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Efficient Generation of Pancreas/Duodenum Homeobox Protein 1+ Posterior Foregut/Pancreatic Progenitors from hPSCs in Adhesion Cultures

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January 8th, 2019

Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Dear Dr. Bajaj,

We would like to express our sincere gratitude for considering our manuscript, "Efficient generation of pancreas/duodenum homeobox protein 1^+ posterior foregut/pancreatic progenitors from human pluripotent stem cells in adhesion cultures." (JoVE57641), for publication in JoVE. In response to the comments raised by the editor and reviewers, we performed additional experiments and revised our manuscript.

Briefly, we examined the differentiation potential of the generated $PDX1^+$ cells by further differentiation and confirmed that they are pancreatic progenitors that can differentiate into $PDX1^+NKX6.1^+$ pancreatic endoderm, $insulin^+$ and $glucagon^+$ endocrine cells. We performed not only immunostaining, but also qRT-PCR analysis to confirm the results. In addition, we confirmed the protocol on two other pluripotent stem cell lines (one hESC and one hiPSC). We also addressed the other concerns raised by the reviewers and revised our manuscript accordingly.

We believe that each of the points raised by the reviewers has been addressed and answered properly. We hope that you and the reviewers now find the manuscript suitable for publication. We thank you again for your time in considering this manuscript for publication in your highly acclaimed journal.

Respectfully yours,

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TITLE:

Efficient Generation of Pancreas/Duodenum Homeobox Protein 1$^+$ Posterior Foregut/Pancreatic Progenitors from hPSCs in Adhesion Cultures

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KEYWORDS:

Pancreas; human embryonic stem cell; human induced pluripotent stem cell; human pluripotent stem cell; differentiation; development; pancreatic progenitor; posterior foregut; regenerative medicine; diabetes

SUMMARY:

Here, we present a detailed protocol to differentiate human pluripotent stem cells (hPSCs) into pancreas/duodenum homeobox protein 1$^+$ (PDX1$^+$) cells for the generation of pancreatic lineages based on the non-colony type monolayer growth of dissociated single cells. This method is suitable for producing homogenous hPSC-derived cells, genetic manipulation and screening.

ABSTRACT:

Human pluripotent stem cell (hPSC)-derived pancreatic cells are a promising cell source for regenerative medicine and a platform to study human developmental processes. Stepwise directed differentiation that recapitulates developmental processes is one of the major ways to generate pancreatic cells including pancreas/duodenum homeobox protein 1$^+$ (PDX1$^+$) pancreatic progenitor cells. Conventional protocols initiate the differentiation with small colonies shortly after the passage. However, in the state of colonies or aggregates, cells are prone to heterogeneities, which might hamper the differentiation to PDX1$^+$ cells. Here, we present a detailed protocol to differentiate hPSCs into PDX1$^+$ cells. The protocol consists of four steps and initiates the differentiation by seeding dissociated single cells. The induction of SOX17$^+$ definitive endoderm cells was followed by the expression of two primitive gut tube markers, HNF1β and HNF4α, and eventual differentiation into PDX1$^+$ cells. The present protocol provides easy handling and may improve and stabilize the differentiation efficiency of some hPSC lines that were previously found to differentiate inefficiently into endodermal lineages or PDX1$^+$ cells.

INTRODUCTION:

The pancreas mainly consists of exocrine and endocrine cells, and its dysfunction or overload causes several diseases, such as pancreatitis, diabetes and pancreatic cancer. To elucidate the pathogeny of pancreatopathy, it is necessary to analyze the developmental process and function of pancreatic cells. In addition, a stable cell supply with robust quality is required to establish cell/tissue supplementation therapy. Human pluripotent stem cell (hPSC)-derived pancreatic cells are a promising cell source for these purposes, and the differentiation protocol toward pancreatic cells has been intensively studied[1-4]. Recent advances in the in vitro generation of pancreatic β cells mimic the generation of β cells in adult human, and these cells show therapeutic efficacy upon implantation into diabetic model mice[2,3]. In addition, the analysis of β cells generated from the induced pluripotent stem cells (iPSCs) of healthy and type 1 diabetes patient donors revealed no functional differences including when under stress[5]. Moreover, disease phenotypes have been partially reproduced in induced pancreatic cells with patient-derived iPSCs or hPSCs harboring genetic mutations in the same site as the patients[6,7].

To generate pancreatic cells from hPSCs, stepwise directed differentiation that recapitulates developmental processes is used. The pancreas is derived from the endoderm layer of the early embryo, which expresses sex determining region Y-box 17 (SOX17) and forkhead box A2 (FOXA2)[8]. Based on the mouse studies, the endodermal layer forms the primitive gut tube, which is marked by the expression of hepatocyte nuclear factor 1-beta (Hnf1β) and hepatocyte nuclear factor 4-alpha (Hnf4α). The primitive gut tube elongates and develops into the respiratory apparatus, digestive tract, and organs. After elongation, the posterior foregut area becomes the presumptive pancreatic region, as marked by the expression of the transcriptional factor pancreas/duodenum homeobox protein 1 (PDX1)[8-10]. The dorsal and ventral parts of the $PDX1^+$ gut tube thicken to form pancreatic buds, which are marked by the co-expression of pancreas transcription factor 1 subunit alpha (PTF1A) and NK6 homeobox 1 (NKX6.1)[8,11]. This expression marks the morphological start of pancreatic organogenesis. Pancreatic endoderm cells, which are components of the pancreatic buds, form a branched tubular network of epithelial structures[12] and eventually differentiate into exocrine and endocrine cells, including insulin-secreting β-cells and glucagon-secreting α-cells. Expression of PDX1 is detected first at the presumptive pancreatic region, which is then observed throughout the entire pancreatic development, and shows localization to β- and δ-cells[9,13,14]. Although the $Pdx1^+$ cell area that does not express Ptf1a or Nkx6.1 differentiates into the gastric antrum, duodenum, extrahepatic bile duct and some intestinal cells at the middle to late stage of development in mice[9], $PDX1^+$ cells are considered the progenitors of the pancreas at the early developmental stage in humans.

Here, we present a detailed protocol to differentiate hPSCs into $PDX1^+$ cells for the generation of pancreatic lineages. The protocol initiates differentiation by seeding dissociated single cells[15-17]. Generally, undifferentiated hPSCs are maintained as colonies or cell aggregates in suspension or in adhesion. As a result, most protocols initiate the differentiation shortly after passaging. However, in the state of colonies or aggregates, cells are prone to spatial and transcriptional heterogeneities[18-22], which might hamper the first differentiation step toward definitive endoderm followed by inefficient differentiation to $PDX1^+$ cells. The present protocol may offer easy handling to improve and stabilize the differentiation efficiency of some hPSC lines that were

previously found to differentiate inefficiently to endodermal lineages and PDX1^{+} cells[23-25].

PROTOCOL:

Experiments using hPSCs were approved by the ethics committee of the Department of Medicine and Graduate School of Medicine, Kyoto University.

1. Preparation of materials

NOTE: Prepare all media and reagents for cell culture in a sterile environment. Warm up base culture media to room temperature before use. Medium for differentiation is used within 6 h at room temperature. Details of the reagents are listed in **Table of Materials**.

1.1 Differentiation (**Figure 1A**)

1.1.1. Stage 1A medium: Transfer RPMI 1640 medium into a tube using a pipette. Add the serum-free supplement, activin A, CHIR99021, and Y-27632 to a concentration of 1x, 100 ng/mL, 3 µM and 10 µM, respectively.

1.1.2. Stage 1B medium: Transfer RPMI 1640 medium into a tube using a pipette. Add the serum-free supplement, activin A and CHIR99021 to a concentration of 1x, 100 ng/mL, ≤1 µM, respectively.

NOTE: The concentration of CHIR99021 should be lower than in Stage 1A medium, and addition is not necessary, but it increases the cell number.

1.1.3. Stage 2 medium: Transfer Improved MEM (iMEM) medium into a tube using a pipette. Add the serum-free supplement and keratinocyte growth factor (KGF) to a concentration of 0.5x and 50 ng/mL, respectively.

1.1.4 Stage 3 medium: Transfer iMEM medium into a tube using a pipette. Add the serum-free supplement, KGF, NOGGIN, 3-Keto-N-aminoethyl-N'-aminocaproyldihydrocinnamoyl cyclopamine (CYC) and 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid (TTNPB) by pipette to concentrations of 0.5x, 50 ng/mL, 100 ng/mL, 0.5 µM and 10 nM, respectively.

1.2. Flow cytometry (FCM)

1.2.1 1x Permeabilization/Wash buffer: Transfer water into a tube by a pipette. Add Permeabilization/Wash buffer by a pipette to a concentration of 1x.

1.2.2 FCM blocking solution: Transfer 1x Permeabilization/Wash buffer into a tube by a pipette. Add donkey serum by a pipette to a concentration of 2% (vol/vol).

1.3. Immunostaining

1.3.1 Blocking solution: Transfer Dulbecco's Phosphate-Buffered Saline (DPBS) into a tube by a pipette. Add donkey serum and Triton X by pipette to concentrations of 5% (vol/vol) and 0.4% (vol/vol), respectively.

2. hPSC differentiation to posterior foregut cells/pancreatic progenitors (PDX1$^+$ cells)

NOTE: Conduct all procedures using sterile techniques. hPSCs are maintained on 6-well plates coated by a synthetic surface material for hPSCs with hPSC maintenance medium according to the manufacturer's instructions[17,26]. When cells reach 50-80% confluency (Stage 0) use them for differentiation.

2.1. Prepare basement membrane matrix-coated plates.

2.1.1 Transfer 6 mL of RPMI 1640 (4 °C) into a tube cooled to 4 °C on ice. Add 2 mg of basement membrane matrix (4 °C) with cooled 1 mL-pipette tips and mix well by gentle pipetting to make 0.33 mg/mL basement membrane matrix.

2.1.2 Transfer 2 mL of diluted basement membrane matrix to each well of 6-well cell culture plates by a pipette. Place the plates at 37 °C for 60-90 min in an incubator. Afterward, keep the plates at room temperature until use (within 3 h).

2.2. Seed the hPSCs and initiate differentiation to definitive endoderm (Stage 1A).

2.2.1 Aspirate the used medium and add 2 mL of 0.5 mM ethylenediaminetetraacetic acid (EDTA) (room temperature) per well by pipette to wash the hPSCs cultured in the 6-well plates.

2.2.2 Aspirate 0.5 mM EDTA and add 2 mL of 0.5 mM EDTA per well by pipette. Incubate the plates at 37 °C for 5 min.

2.2.3 Pipette gently but quickly to blow off attached cells on the plates and to dissociate clumped cells into single cells. Do not bubble the cell suspension during pipetting.

2.2.4 Add 8 mL of hPSC maintenance medium at room temperature (RT) supplemented with 10 µM Y-27632 per well and mix the cell suspension by a pipette.

2.2.5 Count the cell number in the cell suspension using the Trypan Blue stain exclusion procedure.

2.2.5.1 Mix 15 µL of cell suspension and 15 µL of Trypan Blue in a tube.

2.2.5.2 Transfer 10 µL of the cell suspension diluted with Trypan Blue onto cell counting slides in duplicate by a 10-µL pipette.

2.2.5.3 Count the cell number with an automatic cell counter and calculate the cell density in the cell suspension. Viability is usually >98%.

2.2.6 Aliquot the cell suspension in a 50 mL centrifuge tube at 1-1.5 x 10^6 cells per well of 6-well plates (1-1.5 x 10^5 cells/cm^2).

2.2.7 Centrifuge the 50 mL tube at 200 x *g* at RT for 5 min.

2.2.8 Aspirate the supernatant using a pipette and resuspend the cells with 1 mL of Stage 1A medium RT per well. Gently pipette the cell suspension and add another 1 mL of Stage 1A medium (total, 2 mL per well).

2.2.9 Aspirate the diluted basement membrane matrix in the wells of the cell culture plates prepared in step 2.1.2 by a 1000-µL pipette. Proceed to the next step immediately.

2.2.10 Gently pipette the cell suspension in step 2.2.8 again. Immediately after mixing, transfer the cell suspension (2 mL) into each well of a 6-well plate. Cover the plate with an aluminum foil to protect the plate from light. Place the plate in the clean bench at room temperature for 10-15 min.

NOTE: Do not move the plate after seeding.

2.2.11 Gently place the plate into a 37 °C, 5% CO_2 incubator (humidified atmosphere) and culture for 24 h.

NOTE: Do not shake the plate after seeding.

2.3. Induce the differentiation into definitive endoderm (Stage 1B).

2.3.1 Aspirate the used medium and add 2 mL of DPBS to the wells by pipette. Repeat the aspiration and addition of DPBS one time.

NOTE: To remove any dead cells from the monolayer, gently shake the plate before each aspiration.

2.3.2 Aspirate the used DPBS by a pipette and add 4 mL of Stage 1B medium (37 °C) per well. Gently place the plate into the 337 °C incubator (humidified atmosphere of 5% CO_2) and culture for 48 h.

2.3.3 Aspirate the used medium and add 2 mL of DPBS to the well by pipette. Repeat the aspiration and addition of DPBS one time.

NOTE: Remove the dead cells from the monolayer by gentle shaking before each aspiration.

2.3.4 Aspirate the used DPBS by pipette and add 4 mL of Stage 1B medium (37 °C) per well. Gently place the plate into the incubator (37 °C, humidified atmosphere of 5% CO_2) and culture for 24 h.

2.4. Induce the differentiation into the primitive gut tube (Stage 2).

2.4.1 Aspirate the used medium and add 2 mL of DPBS to the well by pipette.

NOTE: Remove the dead cells from the monolayer by gentle shaking before each aspiration.

2.4.2 Aspirate the used DPBS by pipette and add 4 mL of Stage 2 medium (37 °C) per well. Place the plate into the incubator (37 °C, humidified atmosphere of 5% CO_2) and culture for 4 days.

2.5. Induce the differentiation into $PDX1^+$ cells (Stage 3).

2.5.1 Aspirate the used medium and add 2 mL of DPBS to the well by pipette. Repeat the aspiration and addition of DPBS one time.

2.5.2 Aspirate the used DPBS by pipette and add 4 mL of Stage 3 medium (37 °C) per well. Place the plate into the incubator (37 °C, humidified atmosphere of 5% CO_2) and culture for 3 days.

2.6. Induce the differentiation into pancreatic endocrine cells.

2.7. Perform $NKX6.1^+$ cell induction (Stage 4, for 8 days) followed by endocrine cell induction (Stage 5, for 12 days) with previously described protocols[3,16,26]. Characterize and validate the endocrine cell differentiation by immunostaining and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis.

NOTE: Refer to Toyoda et al.[16] and Kimura et al.[26] for detailed experimental procedures. Refer **Table 1** for primer sequences used for qRT-PCR analysis.

3. Flow cytometry (FCM)

3.1 Aspirate the used medium and add 2 mL of 0.5 mM EDTA to the well by pipette. Repeat the aspiration and addition of 0.5 mM EDTA one time.

NOTE: Shake the plate gently to remove any dead cells from the monolayer cells before each aspiration.

3.2 To dissociate the cells (approximately 3-6 × 10^6 cells per well), add 2 mL of 0.25% Trypsin containing 0.5 mM EDTA per well. Incubate the plates at 37 °C for 5-10 min.

3.3 Pipette gently but quickly to dissociate clumped cells into single cells. Do not bubble the cell suspension during pipetting.

3.4 Add 8-10 mL of base medium (RPMI 1640 or iMEM, room temperature) containing 0.5x serum-free supplement and 10 µM Y-27632 per well and mix the cell suspension. Transfer the cell suspension into a centrifuge tube.

3.5 Centrifuge the tube at 300 x *g* at room temperature for 5 min.

3.6 Aspirate the supernatant and resuspend the cells with 2 mL of 0.5 mM EDTA (room temperature). Gently pipette the cell suspension.

3.7 Centrifuge the tube at 300 x *g* at room temperature for 5 min.

3.8 Aspirate the supernatant and resuspend the cells with 100 µL of fixation and permeabilization buffer per 10^6 cells under a hood. Gently pipette the cell suspension under a hood. Keep the tube at room temperature for 30 min.

3.9 Centrifuge the tube at 400 x *g* at room temperature for 5 min.

3.10 Aspirate the supernatant under a hood and resuspend the cells with 500 µL FCM Blocking solution per 10^6 cells. Gently pipette the cell suspension. Keep the tube at room temperature for 30 min.

3.11 Transfer 50 µL of the cell suspension to a tube (approximately 1 x 10^5 cells). Centrifuge the tube at 400 x *g* at room temperature for 5 min. Remove the supernatant and resuspend the cells with 100 µL of diluted primary antibody (see **Table of Materials**) in FCM blocking solution and incubate at 4 °C for >16 h.

3.12 Centrifuge the tube at 400 x *g* at room temperature for 5 min. Remove the supernatant and resuspend the cells with 180 µL of 1x Perm/Wash buffer.

3.13 Centrifuge the tube at 400 x *g* at room temperature for 5 min. Remove the supernatant and resuspend the cells with 100 µL of diluted secondary antibody (see **Table of Materials**) in FCM blocking solution. Incubate the cells at room temperature for 60 min or at 4 °C for >16 h to protect them from light.

3.14 Centrifuge the tube at 400 x *g* at room temperature for 5 min. Remove the supernatant and resuspend the cells with 180 µL of 1x Perm/Wash buffer.

3.15 Centrifuge the tube at 400 x *g* at room temperature for 5 min. Remove the supernatant and resuspend the cells with 180 µL of 2% donkey serum in DPBS.

3.16 Filter the cell suspension by transferring on a 5 mL round bottom polystyrene tube with a cell strainer (35 µm nylon mesh) and keep at 4 °C to protect the cells from light until the analysis.

3.17 Analyze a proportion of positively stained cells by a flow cytometer[27].

4. Immunostaining

4.1 Aspirate the used medium and add 2 mL of DPBS to the well by pipette. Repeat the aspiration and addition of DPBS one time.

NOTE: Gently shake the plate to remove any dead cells from the monolayer cells before each aspiration.

4.2 Add 2 mL of 4% paraformaldehyde (PFA) (4 °C) per well by pipette under a hood. Keep the plate at 4 °C for 20 min.

4.3 Remove the PFA by pipette under a hood. Add 2 mL of DPBS at RT to the well by pipette under a hood. Repeat the aspiration and addition of DPBS one time.

4.4 Add 2 mL of blocking solution per well and keep the plate at room temperature for 30 min.

4.5 Aspirate the used solution and add 1 mL of primary antibody (see **Table of Materials**) in blocking solution per well. Incubate at room temperature for 60 min or at 4 °C for >16 h.

4.6 Aspirate the used solution, add 2 mL of DPBS containing 0.4% Triton X-100 and incubate at RT for 10 min. Repeat the aspiration, addition of solution and incubation one time.

4.7 Aspirate the used solution and add 1 mL of secondary antibody (see **Table of Materials**) in blocking solution per well. Incubate at room temperature for 60 min protected from light.

4.8 Aspirate the used solution, add 2 mL of DPBS containing 0.4% Triton X-100 (room temperature) and incubate at room temperature for 5 min with protection from light. Repeat the aspiration, addition of solution and incubation two times.

4.9 Take micrographs to examine the differentiation efficiency under a fluorescence microscope[28].

REPRESENTATIVE RESULTS

Propagating hiPSCs (585A1[29,30]) are condensed and form a homogenous monolayer (**Figure 1B**) that is suitable for differentiation. Undifferentiated hiPSCs (Stage 0) are dissociated and re-seeded as single cells at low cell densities (1-1.5 x 10^5 cells/cm^2). Within 1 h, the cells are attached to the plate and start to show protrusion. On day 1, the cells are proliferated and well distributed to cover 80-90% of the surface area. During Stage 1B, the media appear cloudy due to dead cells. The removal of dead cells is critical for highly efficient differentiation since dead cells likely disturb the survival and differentiation of cells lying underneath. On days 3-4, cells form a homogenous monolayer sheet that can be described as a cobblestone appearance. At this point, most cells stop expressing sex determining region Y-box 2 (SOX2), a marker for undifferentiated

cells, and instead express a definitive endoderm marker, SOX17, at more than 90% (**Figure 2A and B**). Most SOX17$^+$ cells express FOXA2 (**Figure 2A**). Starting the differentiation with an inappropriate cell density compromises the differentiation efficiency at this step (**Figure 3**). At Stages 2 and 3, cell death relaxes, and the used medium is not as cloudy as it is in Stage 1B. Cells express the primitive gut tube markers HNF1β and HNF4α (**Figure 2C**) and eventually express a posterior foregut/pancreatic progenitor marker, PDX1, at more than 90% (**Figure 2D and E**). The PDX1$^+$ cell induction is reproducible in another hiPSC line, 1231A3 [31], and an hESC line, KhES-3 [32] (**Figure 4**). qRT-PCR results of the mRNA expression of stage markers were consistent with immunostaining (**Figure 5A**). The mRNA expression of PDX1 is evident at Stage 3 and substantially increases afterword.

PDX1$^+$ cells at early developmental stages have the potential to differentiate into not only pancreatic cells but also gastric antrum, duodenum, extrahepatic bile duct and a part of the intestine [9]. The differentiation potential of in vitro generated PDX1$^+$ cells to pancreatic cells can be assessed by extended culture with reported protocols for pancreatic endoderm and pancreatic endocrine cells[3,16,26]. The expressions of a pancreatic endoderm marker, *NKX6.1*, and two pancreatic endocrine markers, *INSULIN,* and *GLUCAGON*, were observed on days 19 (Stage 4) and 31 (Stage 5), respectively (**Figure 5**).

FIGURE AND TABLE LEGENDS

Figure 1. Representative appearance of cells under stepwise differentiation. (**A**) A scheme of directed differentiation from hPSCs to pancreatic lineages. Numbers in parentheses indicate concentrations (units are written below). (**B**) Representative bright field micrographs of hiPSCs at key steps of the differentiation culture. 585A1 hiPSCs were dissociated as single cells and induced to differentiate into definitive endoderm, primitive gut tube and posterior foregut/pancreatic progenitor. The lower panels are enlarged views of the upper panels. RPMI, RPMI 1640; AA, activin A (ng/mL); CH, CHIR99021 (μM); KGF (ng/mL); NOG, NOGGIN (ng/mL); CYC, 3-Keto-N-aminoethyl-N'-aminocaproyldihydrocinnamoyl cyclopamine (μM); TTNPB, 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid (nM). Scale bars, 300 μm.

Figure 2. Representative induction toward pancreatic lineages from hiPSCs. (**A**) The proportion of SOX2$^-$SOX17$^+$ cells analyzed by flow cytometry before differentiation (Stage 0) and on day 4 (Stage 1B). Undifferentiated hiPSCs (SOX2$^+$SOX17$^-$) were differentiated into definitive endoderm (SOX2$^-$SOX17$^+$). Most SOX17$^+$ cells co-expressed FOXA2. (**B**) Representative immunofluorescent micrographs on day 4 (Stage 1B). The fixed cells were stained for SOX17 (green), SOX2 (red), and nuclei (blue). (**C**) Representative immunofluorescent micrographs on days 4 (Stage 1B) and 8 (Stage 2). The fixed cells were stained for HNF1β (green), HNF4α (red) and nuclei (blue). (**D**) The proportion of cells positive for PDX1, as analyzed by flow cytometry on days 8 (Stage 1B) and 11 (Stage 3). (**E**) Representative immunofluorescent micrographs of PDX1 (green) and nuclei (blue) on day 11 (Stage 3). Scale bars, 100 μm.

Figure 3. Representative induction toward definitive endoderm initiated from different cell densities. (**A**) Representative bright field micrographs of cells 1 h after differentiation. hiPSCs

were dissociated as single cells and induced to differentiate into definitive endoderm at different cell densities (1-50 × 10^4/cm^2). The lower panels are enlarged views of the upper panels. (**B**) The proportion of $SOX2^-SOX17^+$ cells analyzed by flow cytometry before differentiation (Stage 0) and on day 4 (Stage 1B). (**C**) The proportion of $PDX1^+$ cells analyzed by flow cytometry on days 8 (Stage 2) and 11 (Stage 3). Scale bars, 300 μm.

Figure 4 Representative induction toward $PDX1^+$ cells in hiPCSs and hESCs. An hiPSC line, 1231A3 (**A**), and an hESC line, KhES-3 (**B**), were differentiated to definitive endoderm and $PDX1^+$ cells. The cell composition was analyzed by flow cytometry. The proportion of $SOX2^-SOX17^+$ cells was analyzed before differentiation (Stage 0) and on day 4 (Stage 1B). The proportion of $PDX1^+$ cells was analyzed on days 8 (Stage 2) and 11 (Stage 3).

Figure 5. Representative induction toward pancreatic endoderm and endocrine cells. hiPSCs (585A1) were differentiated into $PDX1^+$ cells. The cells were further differentiated into pancreatic endoderm and pancreatic endocrine cells with reported protocols[3,16,26]. (**A**) mRNA expressions of stage markers were measured by quantitative real-time polymerase chain reaction (qRT-PCR). The data were normalized to *GAPDH* expression and presented as the fold-change in gene expression relative to the peak value. Note that *PDX1* expression was increased at >20-fold from days 8 (Stage 2) to 11 (Stage 3) and at >100-fold from days 11 (Stage 3) to 19 (Stage 4). The expression in the adult human pancreas is shown as Panc. *SOX2*, black; *SOX17*, purple; *HNF1* β, brown; *HNF4* α, orange; *PDX1*, light green; *NKX6.1*, green; *INSULIN*, blue; *GLUCAGON*, red. (**B**) Representative immunofluorescent micrographs of a pancreatic endoderm marker, NKX6.1 (red), on days 11 (Stage 3) and 19 (Stage 4). PDX1 (green) and nuclei (blue) were co-stained. (**C**) Representative immunofluorescent micrographs of two pancreatic endocrine makers, insulin (INS, green) and glucagon (GCG, red), on days 19 (Stage 4) and 31 (Stage 5). Nuclei (blue) were co-stained.

Table 1: Primers for qPCR.

DISCUSSION:

The generation of $PDX1^+$ cells is comprised of multiple steps; therefore, it is critical to treat cells at the appropriate time. Among the steps, the induction efficiency of definitive endoderm largely affects the final induction efficiency, possibly by interference from other contaminating lineage cells (i.e., mesoderm and ectoderm), which may proliferate and/or secrete factors that disrupt specific differentiation. If the proportion of $SOX17^+$ cells is lower than 80% on day 4 (Stage 1B), an efficient induction to $PDX1^+$ cells is likely to be compromised.

Undifferentiated states of hPSCs are maintained as colonies or aggregates of compacted cells. However, methods that start the differentiation from colonies or aggregates may suffer from heterogeneity, because of different cell adhesion and density in the colony, such as in the center and periphery[22], and because cells are at different stages in the cell cycle[25]. On the other hand, our method starts with dissociated single cells, which enables a relatively homogenous state of cell adhesion of single cells or cell density in every single cell. In terms of homogenous handling for each cell, our methods might be easier than others that start with colony or aggregation

cultures.

Although our protocol can be used to induce PDX1$^+$ cells from multiple hPSC lines, the differentiation could still be inefficient. In such cases, differences in adhesion state right after seeding among the hPSC lines could be the cause, and modification of the seeding density could be a solution[33]. Indeed, **Figure 3** shows that inappropriate seeding cell density compromises differentiation into definitive endoderm and PDX1$^+$ cells. Interestingly, the optimal cell density for PDX1$^+$ cell induction was different among the cell populations that achieved >90% definitive endoderm. Another possibility for inefficient differentiation is the poor maintenance condition of the undifferentiated hPSCs, which compromises the quality of pluripotency despite the expression of markers for the undifferentiated state. In this case, the hPSC expansion culture should be recommenced from early passage frozen stocks or sub cloning should be performed to obtain hPSCs in suitable conditions. In the case of inefficient differentiation on days 8 (Stage 2) or 11 (Stage 3), the duration of these steps should be optimized. Supporting this idea, the duration of Stage 3 has been shown critical for acquiring later stage cell characteristics and is cell line-dependent in pancreatic lineage[34].

The generation of PDX1$^+$ cells is crucial for the in vitro generation of pancreatic cells. *PDX1* is functionally essential for pancreatic development based on knowledge from *Pdx1* null mice, which are apancreatic[35]. Consistently, in vitro and in vivo implantation studies showed hPSC-derived PDX1$^+$ cells have the potential to develop into all pancreatic components, including exocrine and endocrine cells such as pancreatic β cells[3,16,36,37]. Thus, the efficient generation of PDX1$^+$ cells from hPSCs leads to a stable pancreatic cell supply for the establishment of β cell therapy against diabetes and the understanding of human pancreas development and pancreatic diseases.

The limitations of this method are related to the two-dimensional (2D) monolayer culture format, which is not suitable for some cell types and cell processing. In recent years, three-dimensional (3D) cultures have been shown to promote the generation of mature cells and tissues, possibly due to mimicking the in vivo microenvironment. For example, β cells generated in 3D cultures but not 2D monolayer cultures could attain the ability to secrete insulin in response to extracellular glucose levels[38].

To use PDX1$^+$ cells for the generation of developmentally later cell types, it is important to shift to 3D cultures, such as suspension cultures of aggregates embedded in an extracellular matrix and aggregate cultures on an air-liquid interface[3,16,37]. In addition, 2D monolayer cultures require more surface area for culturing than suspension cultures, limiting scalability. The processing of large amounts of cells for commercial use requires modifications such as the use of microbeads. At the same time, the present method is suitable for the screening of differentiation-inducing factors and the exploration of molecular mechanisms by gene transfer.

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DISCLOSURES

The authors have nothing to disclose.

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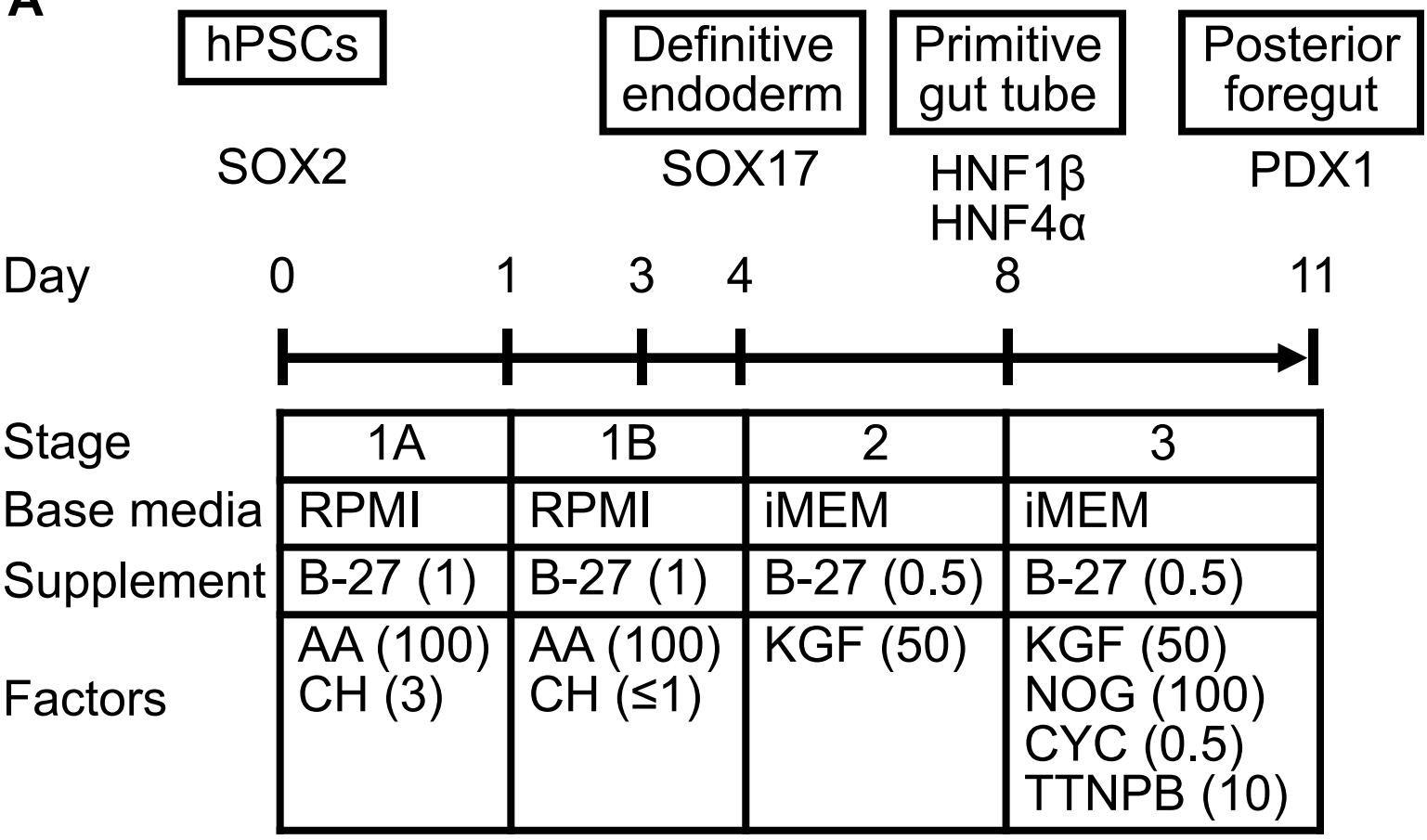
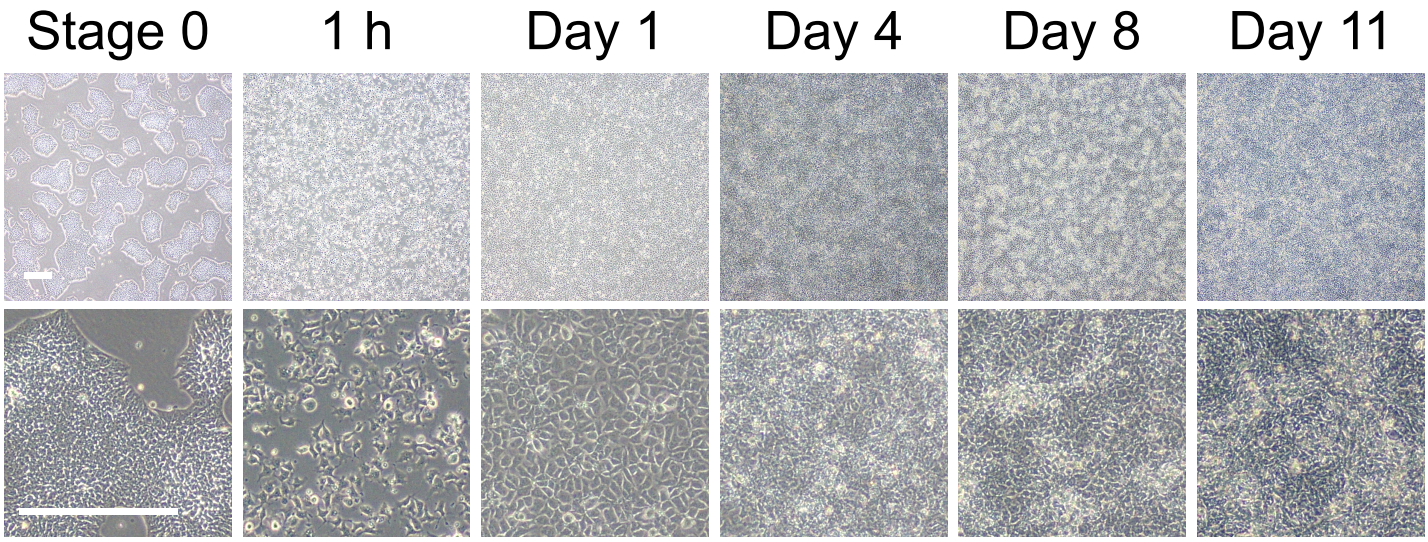
599

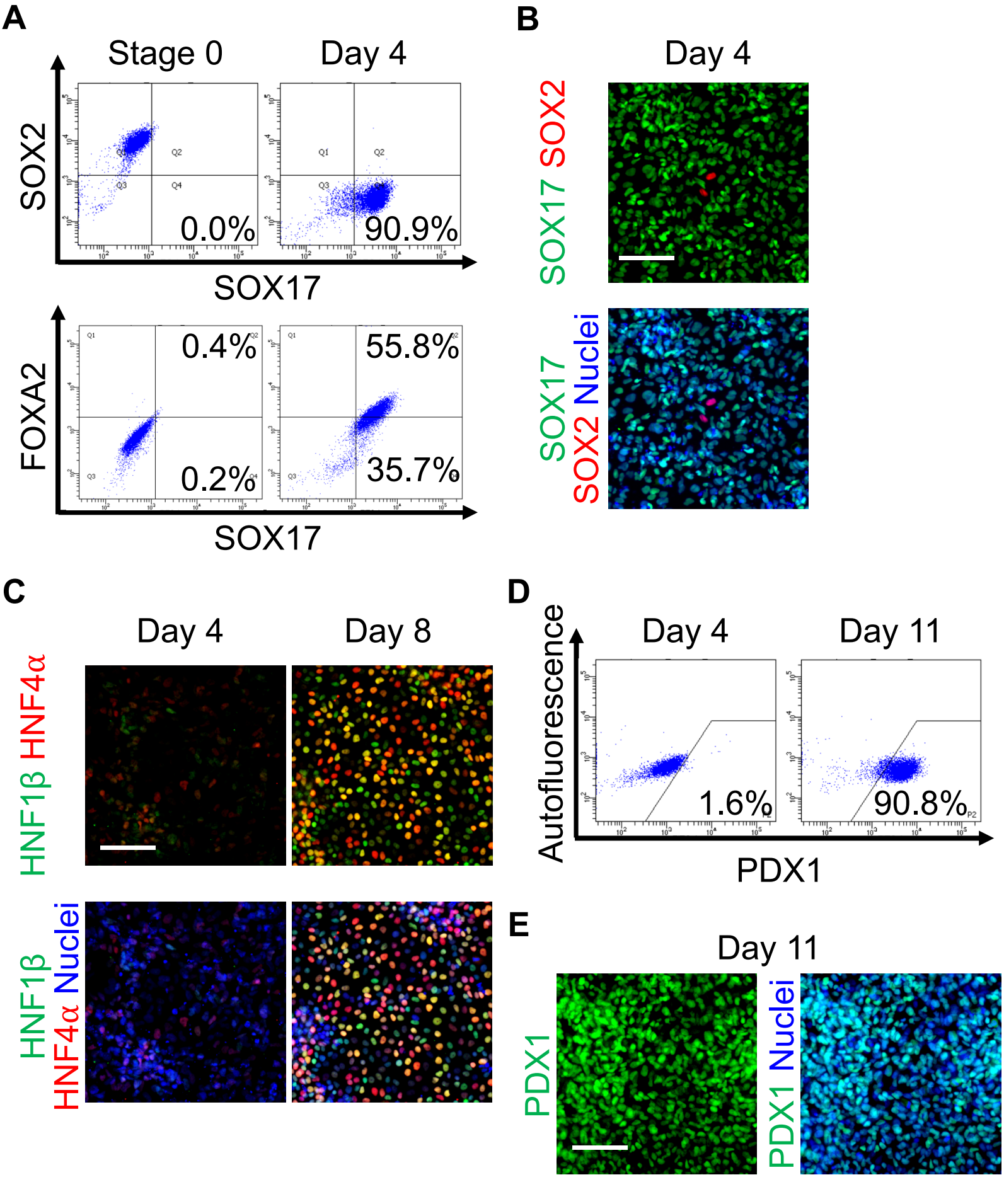
A

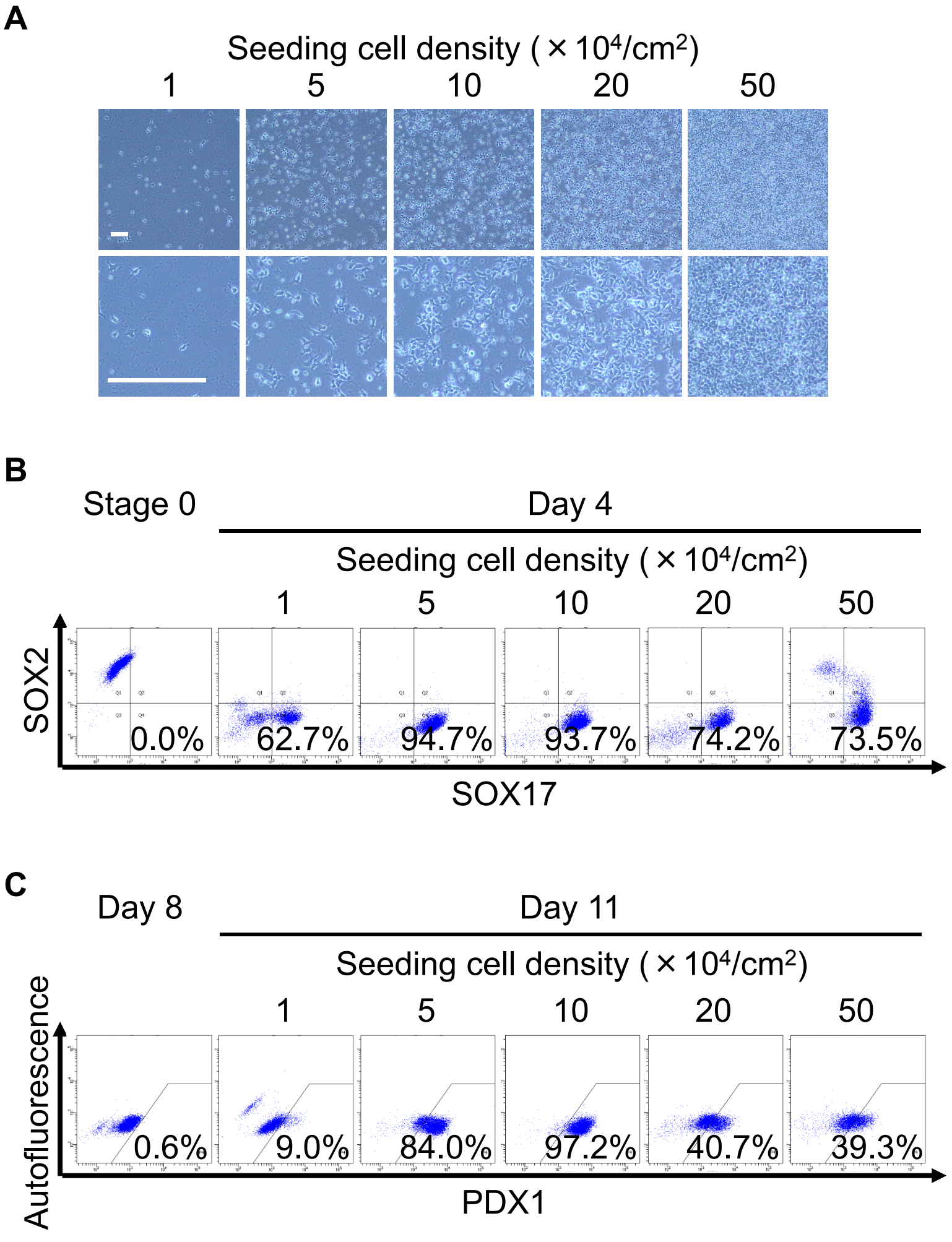


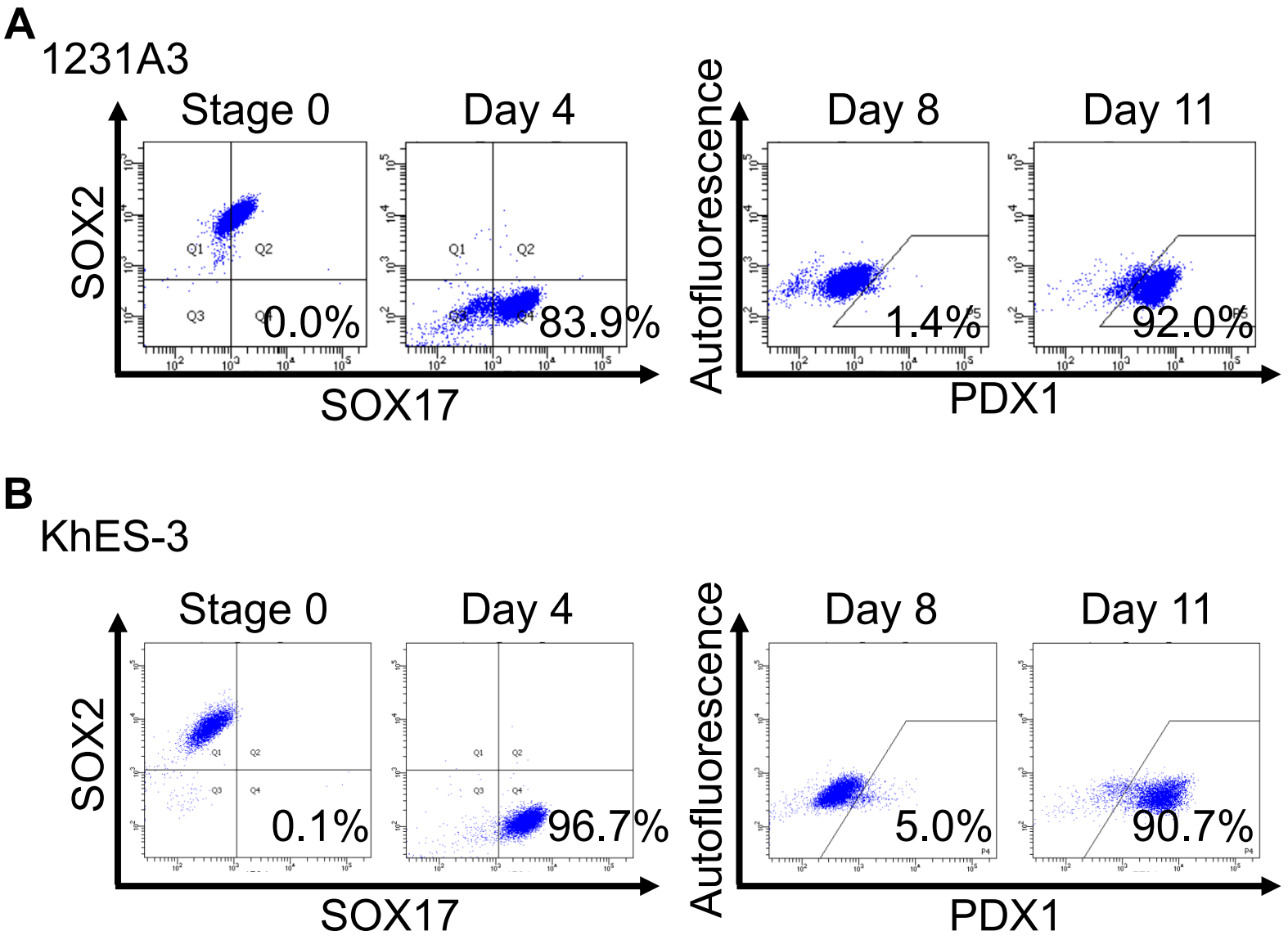
Stage	1A	1B	2	3
Base media	RPMI	RPMI	iMEM	iMEM
Supplement	B-27 (1)	B-27 (1)	B-27 (0.5)	B-27 (0.5)
Factors	AA (100) CH (3)	AA (100) CH (≤1)	KGF (50)	KGF (50) NOG (100) CYC (0.5) TTNPB (10)

B









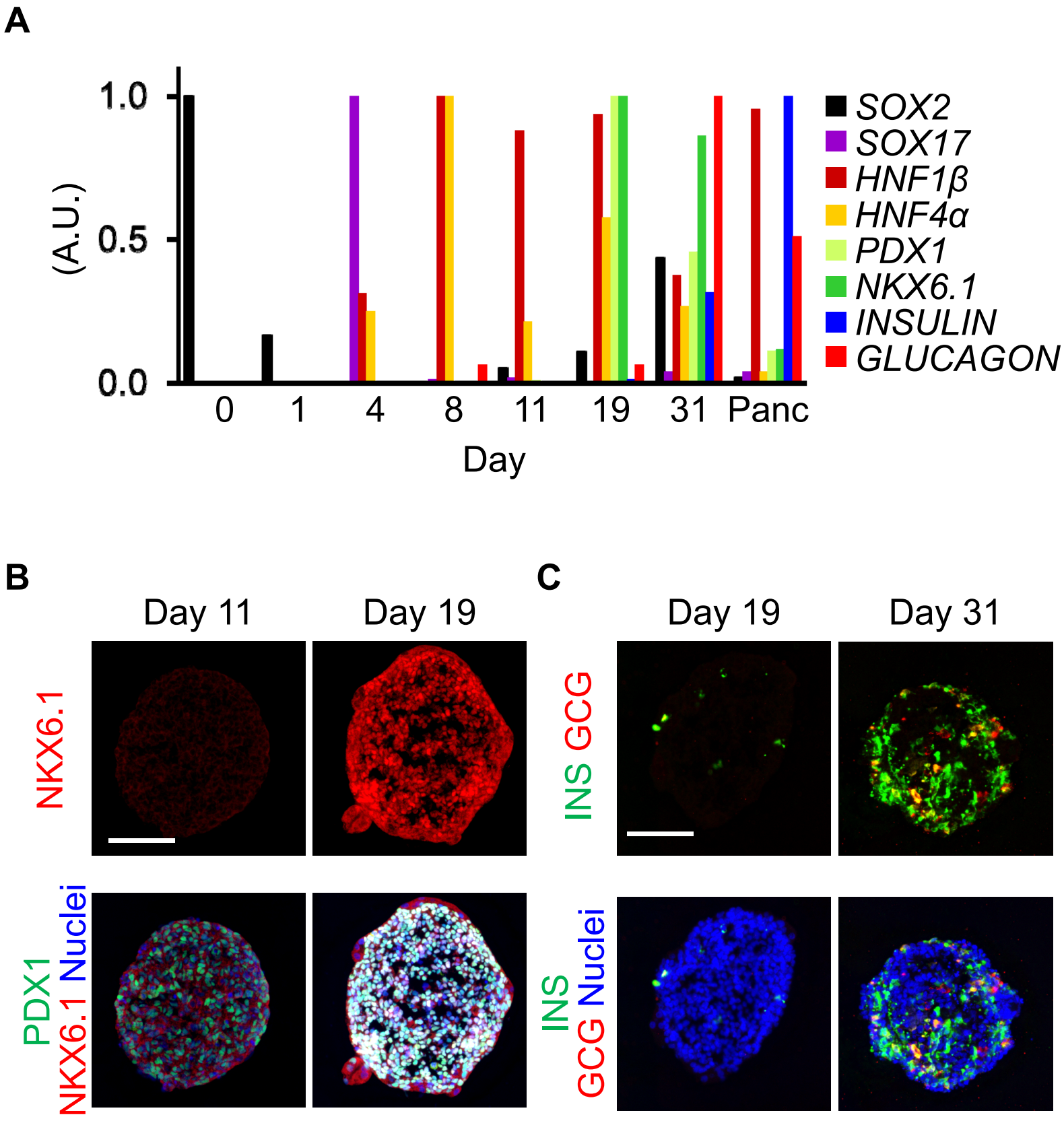


Table 1

Gene name	Gene symbol	Forward primer	Reverse primer
glyceraldehyde-3-phospha te dehydrogenase	*GAPDH*	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
SRY-box 2	*SOX2*	AGTCTCCAAGCGACGAAAAA	TTTCACGTTTGCAACTGTCC
SRY-box 17	*SOX17*	CGCACGGAATTTGAACAGTA	TTAGCTCCTCCAGGAAGTGTG
HNF1 homeobox B	*HNF1β*	CCTCTCCTCCAAACAAGCTG	TGTTGCCATGGTGACTGATT
hepatocyte nuclear factor 4 alpha	*HNF4α*	GAGCTGCAGATCGATGACAA	TACTGGCGGTCGTTGATGTA
pancreatic and duodenal homeobox 1	*PDX1*	AGCAGTGCAAGAGTCCCTGT	CACAGCCTCTACCTCGGAAC
NK6 homeobox 1	*NKX6.1*	ATTCGTTGGGGATGACAGAG	TGGGATCCAGAGGCTTATTG
glucagon	*GCG*	GAATTCATTGCTTGGCTGGT	CGGCCAAGTTCTTCAACAAT
insulin	*INS*	CTACCTAGTGTGCGGGGAAC	GCTGGTAGAGGGAGCAGATG

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3-Keto-N-aminoethyl-N′-aminocaproyldihydrocinnamoyl cyclopamine	Toronto Research Chemicals	K171000	CYC
4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid	Santa Cruz Biotechnology	SC-203303	TTNPB
50 mL Conical Sterile Polypropylene Centrifuge Tubes	Thermo Fisher Scientific	339652	
Anti-CDX2 antibody [EPR2764Y]	Abcam	Ab76541	Anti-CDX2, × 1/1000 dilution
B-27 Supplement (50 ×)	Thermo Fisher Scientific	17504-044	Serum-free supplement
BD FACSAria II Cell Sorter	BD Biosciences		For flow cytometry
Biomedical freezer	SANYO	MDF-U538	For -30 °C storing
Cell Counting Slides for TC10/TC20 Cell Counter, Dual-Chamber	BIO-RAD	1450011	Counting slide glass
CELL CULTURE MULTIWELL PLATE, 6 WELL, PS, CLEAR	Greiner bio-one	657165	For differentiation culture/6-well plate
Centrifuge	TOMY	AX-310	For cell culturing
Centrifuge	TOMY	MX-305	For RT-qPCR
CHIR99021	Axon Medchem	Axon 1386	
CLEAN BENCH	SHOWA KAGAKU	S-1601PRV	Clean bench
Corning CellBIND 6-well plate	Corning	3335	For feeder-free culture of hPSCs/6-well plate
Corning Matrigel Basement Membrane Matrix Growth Factor Reduced	Corning	354230	Basement membrane matrix
Corning Synthemax II-SC Substrate	Corning	3535	For feeder-free culture of hPSCs/synthetic surface material for hPSCs

Cryostat	Leica	Leica CM1510 S	For immunostaining of aggregates.
Cytofix/Cytoperm Kit	Becton Dickinson	554714	Perm/Wash buffer is Permeabilization/Wash buffer. Cytofix/Cytoperm buffer is fixation and permeabilization buffer.
Dako pen	Dako	S2002	For immunostaining of aggregates
dNTP mix (10 mM)	Thermo Fisher Scientific	18427-088	For RT-qPCR
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A11055	Secondary antibody, × 1/500 dilution
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	Thermo Fisher Scientific	A10036	Secondary antibody, × 1/500 dilution
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	Thermo Fisher Scientific	A10040	Secondary antibody, × 1/500 dilution
Donkey Serum	Merck Millipore	S30	Donkey serum
D-PBS(-) without Ca or Mg	Nacalai tesque	14249-95	DPBS
Essential 8 Medium	Thermo Fisher Scientific	A1517001	For feeder-free culture of hPSCs/hPSC maintenance medium
Falcon 5mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap	Corning	352235	5 mL round bottom polystyrene tube with cell strainer
Filter Tip, 1000 µL	Watoson	124-1000S	Use together with pipettes
Filter Tip, 20 µL	Watoson	124-P20S	Use together with pipettes
Filter Tip, 200 µL	Watoson	124-P200S	Use together with pipettes
Fluorescence Microscope	Keyence	BZ-X700	For immunostaining

Forma Steri-Cycle CO_2 incubator	Thermo Fisher Scientific	370A	Incubator
HNF-1β Antibody (C-20)	Santa Cruz Biotechnology	sc-7411	Anti-HNF1β, × 1/200 dilution
HNF-4α Antibody (H-171)	Santa Cruz Biotechnology	sc-8987	Anti-HNF4α, × 1/200 dilution
Hoechst 33342	Thermo Fisher Scientific	H3570	For nucleus staining, × 1/200 dilution
Human Pancreas Total RNA	Ambion	AM7954	For RT-qPCR
Human PDX-1/IPF1 Antibody	R&D Systems	AF2419	Anti-PDX1, goat IgG, × 1/200 dilution
Human SOX17 Antibody	R&D Systems	AF1924	Anti-SOX17, × 1/200 dilution
Improved MEM Zinc Option medium	Thermo Fisher Scientific	10373-017	iMEM
Incubation chamber	Cosmo Bio	10DO	For immunostaining of aggregates
Latex Examination Gloves	Adachi		
MAS coated slide glass	Matsunami Glass	83-1881	For immunostaining of aggregates
MicroAmp Fast 96-well Reaction Plate	Applied Biosystems/Thermo Fisher Scientific	4346907	For RT-qPCR
Microscope	Olympus	CKX41N-31PHP	For cell culturing
Microtube	Watoson	131-515CS	
Monoclonal Anti-α-Fetoprotein	SIGMA	A8452	Anti-AFP, × 1/200 dilution
Nanodeop 8000	Thermo Fisher Scientific		For RT-qPCR
Oligo dT	FASMAC	Custom made Oligo	For RT-qPCR of sequence is "TTTTTTTTTTTTTTTTTT"

Paraformaldehyde, powder	Nacalai tesque	26126-54	PFA, fixative, diluted in DPBS
Pharmaceutical refrigerator	SANYO	MPR-514	For 4 °C storing
PIPETMAN P	GILSON		Pipette
Recombinant Human KGF/FGF-7	R&D Systems	251-KG	KGF
Recombinant Human Noggin	PeproTech	120-10C	NOGGIN
Recombinant Human/Mouse/Rat Activin A	R&D Systems	338-AC	Activin A
ReverTra Ace (100 U/μL)	TOYOBO	TRT-101	For RT-qPCR
Rnase-Free Dnase Set (50)	QIAGEN	79254	For RT-qPCR
Rneasy Mini Kit	QIAGEN	74104	For RT-qPCR
RPMI 1640 with L-Gln	Nacalai tesque	30264-85	RPMI 1640
Sealing Film for Real Time	Takara	NJ500	For RT-qPCR
Serological pipettes 10 mL	Costar/Corning	4488	For cell culturing
Serological pipettes 25 mL	Costar/Corning	4489	For cell culturing
Serological pipettes 5 mL	Costar/Corning	4487	For cell culturing
Sox2 (D6D9) XP Rabbit mAb	Cell signaling	3579S	Anti-SOX2, × 1/200 dilution
StepOnePlus	Applied Biosystems/Thermo Fisher Scientific		For RT-qPCR
Sucrose	Nacalai tesque	30406-25	For immunostaining of aggregates
TB Green Premix Ex Taq II	Takara	RR820B	For RT-qPCR
TC20 Automated Cell Counter	BIO-RAD	1450101J1	Automatic cell counter
Tissue-Tek OCT compound 4583	Sakura Finetechnical	4583	For immunostaining of aggregates
Tissue-Tek Cryomold Molds/Adapters	Sakura Finetechnical	4566	For immunostaining of aggregates
Triton X-100	Nacalai tesque	35501-15	
Trypan Blue	BIO-RAD	1450021	
Ultracold freezer	SANYO	MDF-U33V	For -80 °C storing

UltraPure 0.5M EDTA, pH 8.0	Thermo Fisher Scientific	15575-038	Dilute with DPBS to prepare 0.5 mM EDTA
Veriti Thermal Cycler	Applied Biosystems/Thermo Fisher Scientific		For RT-qPCR
Y-27632	Wako	251-00514	

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We thank the reviewers for their helpful reviews of our manuscript "Efficient generation of pancreas/duodenum homeobox protein 1^{+} posterior foregut/pancreatic progenitors from human pluripotent stem cells in adhesion cultures" (JoVE57641). We are pleased that there is considerable interest in our work in this important area of investigation. In order to address the questions raised in the reviews, we performed additional experiments and revised the manuscript as requested. Our point-by-point responses are outlined below.

Response to Reviews

Editorial comments:

Point 1.

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We revised the entire manuscript and had it read by a native English speaker.

Point 2.

Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines 59-64, 66-68, 69-71, 90-91, 117, 140, 158-159, 168, 174, 182, 197.

We apologize for the improper writing and corrected the sentences in the revised manuscript including lines 59-78, 96-98, 143-144, 169, 194-196, 207-208, 217-218, 228-231 and 254-255.

Point 3.

Please avoid using any abbreviation in the title.

We revised the manuscript title.

Point 4.

Please provide an email address for each author on the first page

We added the email addresses on the first page.

Point 5.

Please use SI units, e.g. please use "mL" instead of "ml", "h" instead of "hour", "min" instead of "minute" etc. Please leave a white space between the values and the units.

We revised the entire manuscript in accordance with SI units.

Point 6.

Please define all abbreviations before use.

We have defined all abbreviations the first time they are called in the revised manuscript.

Point 7.

Please remove all commercial language from your manuscript and use generic terms instead.

We have removed all commercial language as requested.

Point 8.

Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique…Please include all safety procedures and use of hoods, etc.

We revised the entire protocol section accordingly.

Point 9.

The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step.

We revised the protocol section accordingly.

Point 10.

Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have added several new references in the revised manuscript (lines 150-152, 244-250, 309 and 342).

Point 11.

For steps that involve software or analyzing tools, please make sure to provide all the details such as "click this", "select that", "observe this", etc.

We used software to operate analytical equipment according to the manufacturer's instruction. We did not use any special software for the analyses.

Point 12.

Please leave a blank line between all protocol steps as well as Notes.

We have added blank lines as requested.

Point 13.

Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors.

We have revised the numbering accordingly.

Point 14.

Protocol: 1.1: Please use imperative tense for all the sentences of the protocol steps. Please use sub-steps like 1.1.1, 1.1.2 etc. Please include all the instruments and containers used.

We have revised Protocol: 1.1 as requested.

Point 15.

Protocol: 1.2, 1.3: Please clearly describe the actiosn in the imperative tense.

We have revised Protocol: 1.2, 1.3 as requested.

Point 16.

Protocol: 2, Note: Please avoid using any commercial language, please use a generic term instead.

We removed commercial language in the revised manuscript (lines 144, 148, 150-152, 169 and 191).

Point 17.

Protocol: 2.1.1: "Mix" where? Please include the container and the pipette size.

We include the requested information and provide more detail about the mix in the revised manuscript (lines 150-152).

Point 18.

Protocol: 2.1.2: How is that done?

We explain in the revised manuscript (lines 154-156).

Point 19.

Protocol: 2.1.3: How?

We relocated the step in 2.1.3 to 2.2.9 with the requested information in the revised manuscript (lines 191-192).

Point 20.

Protocol: 2.2.5.2: How is that done?

We provide the requested information in the revised manuscript (lines 176-177).

Point 21.

Protocol: 2.3.1, 2.3.3: "rinse" how? With how much solution?

We provide the requested information in the revised manuscript (lines 207-208 and 217-218).

Point 22.

Protocol: 2.3.2, 2.3.4, 2.5.2: Incubate at which temperature?

We added the conditions of the incubator in the revised manuscript (lines 214, 224 and 242).

Point 23.

Protocol: 2.5.1: How? How much solution is needed to rinse?

We provide the requested information in the revised manuscript (line 238-239).

Point 24.

Protocol: 3.16: What is the filter size?

We added the filter size in the revised manuscript (line 307).

Point 25.

Protocol: 3.17, 4.9: Please include an appropriate reference.

We added the appropriate references in the revised manuscript (lines 317 and 342).

Point 26.

After formatting, please ensure that the highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We revised the highlighted steps in the revised manuscript.

Point 27.

Please avoid numbering Figures inside them, e.g., please remove "Figure 1" from Figure 1.

We removed numbering from the Figures.

Point 28.

Figure 2: Please define the colors.

We apologize for the imprecise writing. We defined the colors in the revised manuscript (lines 391-395).

Point 29.

If you are reusing figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure).

We did not reuse figures.

Point 30.

Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.

We revised the tables and added information about the instruments.

Point 31.

Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

We removed those superscripts.

Reviewer 1:

Point 1.

General comments:

However, this article cannot be recommended for publication as is. The protocol for producing PDX1+ cells has been well understood for over a decade. The changes the authors have made to this protocol appear superficial. The authors claim this protocol reduces cellular heterogeneity and make cell lines difficult to differentiate more efficient, however they provide no evidence of this. These weaknesses make this article not useful and with zero general impact, which are key review criteria for JoVE as per the website.

(1) The protocol for robustly generating PDX1+ cells was first published in 2006 (D'Amour et al Nat Biotech) and has been reproduced with minor variation in the decade since by a large number of groups. Producing PDX1+ cells is very well understood in the field, with this method published several times in the field. It is even available as a kit from Stem Cell Technologies (STEMdiff™ Pancreatic Progenitor Kit).

As the reviewer commented, the protocol for producing $PDX1^+$ cells has been established for over a decade. At the same time, there are reports that failed to reproduce the differentiation at high efficiency (Gage BK, 2013) as the references and the suggested report (Millman JR, 2016). The purpose of this manuscript is to provide a detailed protocol and a couple of troubleshoots. The manuscript is basically in line with the concept that the journal does not require novelty. With regard to the reduced heterogeneity by our protocol, the reviewer is correct that we do not provide evidence. We corrected

the description in the revised manuscript (lines 42, 43, 86, 88, 437 and 479-480). We thank the reviewer for the comment.

Point 2.

(2) The authors claim their protocol reduces heterogeneity and allows efficient differentiation of lines found "resistant" to differentiation. There is no evidence to back up these claims. In particular, does heterogeneity exist that affects pancreatic differentiation? If yes, does their method reduce this? It seems unlikely their method would reduce this since the cells are grown as colonies before the dispersion, so the heterogeneous cellular state would be maintained. Where is the data that cell lines are resistant to different and that this method overcomes that?

We thank the reviewer for the comment. Many research groups reported no difference in the differentiation efficiency of different iPSC lines, including Millman JR (2016), which the reviewer cites in the next comment. At the same time, many reports have found differences in the differentiation efficiency along with the transcriptome and epigenome states among iPSC lines (Kilpinen H, 2017; Carcamo-Orive I, 2017). The reason for the discrepancy is unknown, but differences in protocols for the cell reprograming and maintenance culture might be factors. In addition, single cell analysis revealed transcriptional heterogeneity in mouse and human PSCs (Cahan P, 2013; Nguyen QH, 2018). It was also reported that the outer edges of human ESC colonies have distinct transcriptional profiles (Narsinh KH, 2011), mechanical properties and differentiation potential (Rosowski KA, 2015). In addition, PSCs are highly proliferative and heterogeneous in terms of their cell cycle, with about 40, 35 and 15% being G1, S and G2 phase cells, respectively, and these cells have different differentiation tendency (Sela Y, 2012). Since dissociation into single cells modifies cell-to-cell interactions, the signals derived from these interactions are likely to change. In fact, increasing the number of cells at the edge of colonies by plating small colonies improves epidermal differentiation efficiency (Rosowski KA, 2015). Noting all these studies, we hypothesize that there exists heterogeneity in cell lines and intra-colony cells and that dissociation modifies cell-to-cell interaction signaling. Admittedly though, we do not have data showing that some cell lines are resistant to differentiation. We have therefore revised our description (lines 86-88, 437 and 479-480) and added references (line 439) in the revised manuscript. We thank the reviewer for pointing out this important issue.

References.

Millman JR, Xie C, Van Dervort A, Gürtler M, Pagliuca FW, Melton DA. Generation of stem cell-derived β-cells from patients with type 1 diabetes. Nat Commun. 2016 May 10;7:11463.

Gage BK, Webber TD, Kieffer TJ. Initial cell seeding density influences pancreatic endocrine development during in vitro differentiation of human embryonic stem cells. PLoS One. 2013 Dec 4;8(12):e82076.

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Rosowski KA, Mertz AF, Norcross S, Dufresne ER, Horsley V. Edges of human embryonic stem cell colonies display distinct mechanical properties and differentiation potential. Sci Rep. 2015 Sep 22;5:14218.

Sela Y, Molotski N, Golan S, Itskovitz-Eldor J, Soen Y. Human embryonic stem cells exhibit increased propensity to differentiate during the G1 phase prior to phosphorylation of retinoblastoma protein. Stem Cells. 2012 Jun;30(6):1097-108.

Point 3.

(3) Millman et al. Nature Communications 2016 is an extremely relevant reference that has been left off. It shows differentiation of 6 different human induced pluripotent stem cell (hiPSC) lines to generate between 85%-97% PDX1+ cells with a planar protocol very similar to that described by Toyoda et al. This data contradicts the authors' claims on cell lines resistant to differentiation and issues with cell heterogeneity. Furthermore, this reference is the first publication where actual beta-like cells are generated from patient hiPSC, as opposed to off target polyhormonal cells are known to be fated to the alpha cell fate.

We thank the reviewer for the thoughtful comment. We cite this paper in the revised manuscript (lines 53-55). The paper reported the generation and analysis of patient hiPSC-derived pancreatic beta cells and showed high efficiency and little variation in the $PDX1^+$ cell generation. However, there are many research groups that have difficulty in generating definitive endoderm and $PDX1^+$ cells at high efficiency or little variation. Why Millman has had greater success than other studies may be due to the protocols for reprograming and maintenance culture of iPSCs or the differentiation protocol, as we explain in our response to the previous comment. On the other hand, the main purpose of the submitted manuscript is to provide a solution to readers who have difficulty in generating $PDX1^+$ cells with high efficiency.

Point 4.

(4) How is it known that these PDX1+ cells have pancreatic fate potential and fate potential to become beta cells? PDX1+ cells can be fate restricted to intestinal cells or to pancreatic exocrine.

We thank the reviewer for the comment. This point was also raised by Reviewer 3, Comment 2. We performed additional experiments to further induce the differentiation of $PDX1^+$ cells toward pancreatic endoderm and examined the marker expression by qPCR and immunostaining. We detected the expressions of *NKX6.1*, *INSULIN* and *GLUCAGON* by both analyses. These data suggest the generated $PDX1^+$ cells have potential to differentiate into beta cells. We added the data in Figure 5. Accordingly, we also revised the Methods (lines 244-250) and Results (lines 366-372) sections. In addition, we revised the description of $PDX1^+$ cells to clearly state that $PDX1^+$ cells are progenitors of pancreas, gastric antrum, duodenum, extrahepatic bile duct and a part of the intestine (lines 75-78 and 366-368).

Point 5.

(5) Minor concern with use of human and mouse gene name conventions. This is a human paper, so the human convention (all caps) should be used, except in specific instances referring mouse genes.

We apologize for the imprecise writing. We correctly describe gene names in the revised manuscript.

Point 6.

(6) Liens 96-97, what determines the concentration of CHIR99021?

We apologize for the imprecise writing. The concentration of CHIR99021 should be decreased at this step to less than 1 μM. We have modified the protocol (line 109-112) and Figure 1 in the revised manuscript accordingly.

Point 7.

(7) Lines 68, 349-350, should say exocrine and endocrine or acinar, endocrine, and duct, not exocrine, endocrine, and duct.

Again, we apologize for the imprecise writing. We corrected the sentence in the revised manuscript (lines 72 and 464).

Reviewer 2:

Point 1.

Is this method more broadly applicable to multiple different human ES cell lines and iPS cell lines? The authors appear to use the 585A1 human iPS cell line in the results described in Fig 1. It would be valuable to see data, or there to be a description, of outcomes with other human iPSC or hESC lines. Additional information with regard to how the 585A1 human iPSC line was derived would also be of value to the reader. There are many different methods published for derivation of pancreatic lineage cells including islet-like clusters from human pluripotent stem cells. This method is different and straightforward, however, it is essential to know whether this method is applicable to cell lines other than the one described in Fig 1.

We performed additional experiments to examine the protocol with another iPSC line (1231A) and ESC line (Kh-ES3). FCM analysis showed that >90% of cells were $PDX1^+$ with both cell lines. We added the data in Figure 4 and a description in the Results section (lines 360-362) in the revised manuscript.

585A1 was generated from T lymphocytes of a healthy donor by the introduction of OCT4, SOX2, KLF4, L-MYC, LIN28, and p53-shRNA at our institute (Okita K, 2011; Kajiwara M, 2012). 1231A3 was generated from the peripheral blood cells of a healthy donor by the introduction of OCT4, SOX2, KLF4, L-MYC, LIN28, and dominant negative form of p53 again at our institute (Kikuchi T, 2017). Kh-ES3 was generated from surplus frozen embryos at Kyoto University (Suemori H, 2011). We cited the following reports on the derivation of the cell lines used in this manuscript (lines 346 and 361) of the revised manuscript.

References.

Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa M, Tanabe K, Tezuka K, Shibata T, Kunisada T, Takahashi M, Takahashi J, Saji H, Yamanaka S. A more efficient method to generate integration-free human iPS cells. Nat Methods 2011;8:409-412.

Kajiwara M, Aoi T, Okita K, Takahashi R, Inoue H, Takayama N, Endo H, Eto K, Toguchida J, Uemoto S, Yamanaka S. Donor-dependent variations in hepatic differentiation from human-induced pluripotent stem cells. Proc Natl Acad Sci U S A 2012;109:12538-12543.

Kikuchi T, Morizane A, Doi D, Magotani H, Onoe H, Hayashi T, Mizuma H, Takara S, Takahashi R, Inoue H, Morita S, Yamamoto M, Okita K, Nakagawa M, Parmar M, Takahashi J. Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. Nature. 2017 548(7669):592-596.

Suemori H, Yasuchika K, Hasegawa K, Fujioka T, Tsuneyoshi N, Nakatsuji N. Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. Biochem Biophys Res Commun 2006;345:926-932.

Point 2.

Definitive endoderm expression is typically characterized by co-expression of FOXA2 and SOX17. The authors show flow cytometry data for SOX17 only. It would be of value to the reader to understand what percentage of the highly enriched $SOX17^+$ population co-expresses FOXA2. One would expect this to be a high frequency of expression, but information regarding such co-expression is not presented but would be of interest.

We performed additional experiments to examine the expression of FOXA2 in the $SOX17^+$ cell population. Although the separation of FOXA2 staining was not clear when double staining with SOX17, the majority of cells co-expressed FOXA2 as expected. The result suggests that the enriched $SOX17^+$ population was definitive endoderm. We added the data in Figure 2A and a description in the Results section (line 356) in the revised manuscript.

Point 3.

The authors nicely demonstrate the correlation of seeding density with SOX17 expression showing that 1 x 10^5 cells/cm^2 is a near ideal seeding density for achieving $SOX17^+$ cells. However, they do not show how the seeding density impacts the $PDX1^+$ population frequency at Day 11. Since this is the ultimate outcome, it would be recommended to demonstrate how seeding density impacts the frequency of PDX1+ cells.

We thank the reviewer for the thoughtful comment. We performed additional experiments to examine whether the seeding density impacts the proportion of $PDX1^+$ cells on day 11 (Stage 3) as well as the proportion of $SOX17^+$ cells on day 4 (Stage 1B). As a result, the initial seeding density impacted the proportion of $PDX1^+$ cells, and the induction of $PDX1^+$ cells indicated an optimal density. We revised the data in Figure 3 and added a description in the Discussion section (lines 449-451) in the revised manuscript.

Point 4.

PDX1 is expressed in the foregut endoderm and also in pancreatic progenitor cells that also express NKX6.1. The ratio of $PDX1^+$ that are also expressing NKX6.1 would be essential to enumerate since this is believed to be the true pancreatic progenitor population. If the cells do not highly express NKX6.1 then they may be considered a mixed population of $PDX1^+NKX6.1^+$ pancreatic progenitors. Methods are widely available to identify this PDX1 and NKX6.1 co-expressing population.

We thank the reviewer for the comment. The $PDX1^+$ cells that we presented were posterior foregut endoderm cells which can differentiate into $NKX6.1^+$ pancreatic endoderm. The cells did not express NKX6.1 on day 11 (Stage 3), but began to express NKX6.1 with further differentiation, suggesting that the $PDX1^+$ cells are progenitors of $PDX1^+NKX6.1^+$ pancreatic endoderm. We added the data in Figures 5A and B. We also added descriptions in the Methods (lines 244-250) and Results (lines 366-372) sections in the revised manuscript.

Reviewer 3:

Point 1.

The authors present flow cytometry and imaging data to support their conclusions. Inclusion of gene expression (e.g. qPCR) data is necessary to further strengthen.

We performed additional experiments to demonstrate the mRNA expression of representative markers at each differentiation step. Consistent with the immunostaining data, the mRNA expression of *SOX2* was decreased by the initiation of differentiation, and *SOX17* expression was elevated on day 4 (Stage 1B). The expressions of *HNF1β* and *HNF4α* were elevated on day 8 (Stage 2). PDX1 expression was scarcely detected on day 8 (Stage 2), but was increased by >20-fold on day 11 (Stage 3). These data support our conclusion. We added the data in Figure 5. We also added descriptions in the Methods (lines 244-250) and Results (lines 362-372) sections in the revised manuscript.

Point 2.

It should be clear in the text that PDX1$^+$ is also a duodenum marker and it is not exclusively expressed in pan-pancreatic progenitors. Addition of downstream differentiation data (e.g. immunostaining and qPCR for pancreatic endocrine markers) is necessary to support the latter statement.

We apologize for the imprecise writing. This point was also raised by Reviewer 1, Comment 4. We revised the description of PDX1$^+$ cells in the Introduction section (lines 75-78). In addition, we further induced the PDX1$^+$ cells to differentiate toward pancreatic endoderm and examined the marker expression by qPCR and immunostaining, as both reviewers suggested. We detected the expressions of *NKX6.1*, *INSULIN* and *GLUCAGON* by both analyses. These data support our statement. We added the data in Figure 5. We also added descriptions in the Methods (lines 244-250) and Results (lines 366-372) sections in the revised manuscript.

Point 3.

It is not clear whether these experiments have been performed only with one line (585A1 hiPSCs) or multiple lines.

We apologize for the imprecise writing. The data in the previous manuscript were obtained from only one line (585A1). Reviewer 2 (Comment 1) also asked to see the protocol applied to more lines. Accordingly, we tested the protocol on an hiPSC line (1231A) and hESC line (Kh-ES3). FCM analysis showed that >90% of cells were PDX1$^+$ with both cell lines. We added the data in Figure 4 and a description in the Results section (lines 360-362) in the revised manuscript.

Point 4.

Although the main differentiation stages will be the same for different hPSC lines, timing may change. I suggest that the authors change the names of the various differentiation media to indicate the stage rather than the days in the differentiation.

We thank the reviewer for the suggestion. We revised the name of the media to indicate the stage (lines 104, 109, 116 and 121). Accordingly, we added description of the differentiation stages in the entire manuscript.

Point 5.

There are no controls for flow cytometry (isotype or FMO controls).

We thank the reviewer for the comment, however, we think the samples before and after differentiation is the best control. As the Reviewer suggested, staining with isotype IgG or fluorescence minus one (FMO) can be used as controls in some cases. On the other hand, while many parameters, such as affinity, concentration and solvent, must be titrated and qualified for each antibody, the appropriate isotype control is not available for some antibodies. For FMO, antibodies bind non-specifically at some concentrations, which shift the entire population even if they are negative. As far as we tested, FMO cannot be used as the control of the antibodies used in the manuscript.

Point 6.

Line 106: Perm/Wash buffer is not defined.

We apologize for the imprecise writing. This buffer is included in “Cytofix/Cytoperm Kit.” We added the description of the buffer in Table 1 of the revised manuscript.

Point 7.

Lines 119-122: Since one may want to use more than 3 Matrigel-coated wells for the experiment, it is better to give a diluted Matrigel concentration rather than a specific media volume in 2.1.1.

We added the description of the final concentration of diluted Matrigel in the revised manuscript (lines 150-152).

Point 8.

3) I suggest that the authors substitute "micrographs" for "images" (e.g. Line 293).

We use “micrographs” instead of “images” in the revised manuscript.

Point 9.

I suggest that the authors use "0 h" instead of "Before" in their figures (e.g. Figure 1B).

We thank the reviewer for the thoughtful comments. The data were obtained from undifferentiated cells before the initiation of differentiation. To avoid misleading readers, we revised the description to “Stage 0” in the revised manuscript.

Point 10.

In the materials table, the name of the company "nacalai tesque" has been misspelled "nacali tesque".

We apologize for the misspelling. We write “nacalai tesque” in Table 1 of the revised manuscript.