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TITLE:

Differentiation of human pluripotent stem cells into pancreatic beta-cell precursors in a 2D culture system

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SUMMARY:

The present protocol describes an enhanced method to increase the co-expression of PDX1 and NKX6.1 transcription factors in pancreatic progenitors derived from human pluripotent stem cells (hPSCs) in planar monolayers. This is achieved by replenishing the fresh matrix, manipulating cell density, and dissociating the endodermal cells.

ABSTRACT:

Human pluripotent stem cells (hPSCs) are an excellent tool for studying early pancreatic development and investigating the genetic contributors to diabetes. However, hPSC-derived insulin-secreting cells can be generated for cell therapy and disease modeling with limited efficiency and functional properties. hPSC-derived pancreatic progenitors that are precursors to beta cells and other endocrine cells, when co-express the two transcription factors PDX1 and NKX6.1, specify the progenitors to functional, insulin-secreting beta cells both *in vitro* and *in vivo*. hPSC-derived pancreatic progenitors are currently used for cell therapy in type 1 diabetes patients as part of clinical trials. However, current procedures do not generate a high proportion of NKX6.1 and pancreatic progenitors, leading to co-generation of non-functional endocrine cells and few glucose-responsive, insulin-secreting cells. This work thus developed an enhanced protocol for generating hPSC-derived pancreatic progenitors that maximize the co-expression of PDX1 and NKX6.1 in a 2D monolayer. The factors such as cell density, availability of fresh matrix, and dissociation of hPSC-derived endodermal cells are modulated that augmented PDX1 and NKX6.1 levels in the generated pancreatic progenitors and minimized commitment to alternate hepatic lineage. The study highlights that manipulating the cell's physical environment during *in vitro* differentiation can impact lineage specification and gene expression. Therefore, the current optimized protocol facilitates the scalable generation of PDX1 and NKX6.1 co-expressing progenitors for cell therapy and disease modeling.

INTRODUCTION:

Diabetes is a complex metabolic disorder affecting millions of people globally. Supplementation of insulin is considered the only treatment option for diabetes. More advanced cases are treated with beta cell replacement therapy, achieved through transplantation of either whole cadaveric pancreas or islets[1,2]. Several issues surround transplantation therapy, such as limitation with the availability and quality of the tissue, invasiveness of transplantation procedures in addition to the continuous need for immunosuppressants. This necessitates the need for discovering novel and alternative options for beta cell replacement therapy[2,3]. Human pluripotent stem cells (hPSCs) have recently emerged as a promising tool for understanding human pancreas biology and as a non-exhaustive and potentially a more personalized source for transplantation therapy[4-7]. hPSCs, including human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs), have a high self-renewal capacity and give rise to any tissue type of the human body. hESCs are derived from the embryo's inner cell mass, and hiPSCs are reprogrammed from any somatic cell[4,8].

Directed differentiation protocols are optimized to generate pancreatic beta cells from hPSCs that sequentially direct hPSCs through pancreatic developmental stages *in vitro*. These protocols generate hPSC-derived islet organoids. While they have greatly improved at increasing the proportion of pancreatic beta cells therein, the efficiency of protocols is highly variable. It does not increase to more than ~40% of NKX6.1+/INSULIN+ or C-PEPTIDE + cells[5,9-13]. However, the generated beta cells are not entirely identical to the adult human beta cells in terms of their transcriptional and metabolic profiles and their response to glucose[4,5,14]. The hPSC-derived beta cells lack gene expression of key beta cell markers such as PCSK2, PAX6, UCN3, MAFA, G6PC2, and KCNK3 compared to adult humans islets[5]. Additionally, the hPSC-derived beta cells have diminished calcium signaling in response to glucose. They are contaminated with the co-generated polyhormonal cells that do not secrete appropriate amounts of insulin in response to increasing glucose levels[5]. On the other hand, hPSC-derived pancreatic progenitors, which are islet precursors, could be generated more efficiently *in vitro* compared to beta cells and, when transplanted *in vivo*, could mature into functional, insulin-secreting beta cells[15,16]. Clinical trials are currently focused on demonstrating their safety and efficacy upon transplantation in T1D subjects.

Notably, expression of the transcription factors PDX1 (Pancreatic and Duodenal Homeobox 1) and NKX6.1 (NKX6 Homeobox 1) within the same pancreatic progenitor cell is crucial for commitment towards a beta cell lineage[5]. Pancreatic progenitors that fail to express NKX6.1 give rise to polyhormonal endocrine cells or non-functional beta cells[17,18]. Therefore, a high co-expression of PDX1 and NKX6.1 in the pancreatic progenitor stage is essential for ultimately generating a large number of functional beta cells. Studies have demonstrated that an embryoid body or 3D culture enhances PDX1 and NKX6.1 in pancreatic progenitors where the differentiating cells are aggregated, varying between 40%-80% of the PDX1+/NKX6.1+ population[12,19]. However, compared to suspension cultures, 2D differentiation cultures are more cost-effective, feasible, and convenient for application on multiple cell lines[5]. We recently showed that monolayer differentiation cultures yield more than up to 90% of PDX1+/NKX6.1+

co-expressing hPSC-derived pancreatic progenitors[20-22]. The reported method conferred a high replicating capacity to the generated pancreatic progenitors and prevented alternate fate specifications such as hepatic lineage[21]. Therefore, herein, this protocol demonstrates a highly efficient method for the differentiation of hPSCs to pancreatic beta-cell precursors co-expressing PDX1 and NKX6.1. This method utilizes the technique of dissociating hPSC-derived endoderm and manipulating the cell density, followed by an extended FGF and Retinoid signaling as well as Hedgehog inhibition to promote PDX1 and NKX6.1 co-expression (**Figure 1**). This method can facilitate a scalable generation of hPSC-derived pancreatic beta-cell precursors for transplantation therapy and disease modeling.

PROTOCOL:

The study has been approved by the appropriate institutional research ethics committee and performed following the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The protocol was approved by the Institutional Review Board (IRB) of HMC (no. 16260/16) and Qatar Biomedical Research Institute (QBRI) (no. 2016-003). This work is optimized for hESCs such as H1, H9, and HUES8. Blood samples were obtained from healthy individuals from Hamad Medical Corporation (HMC) hospital with full informed consent. The iPSCs are generated from peripheral blood mononuclear cells (PBMCs) of control, healthy individual[23].

1. Preparation of the culture media

1.1. Prepare human pluripotent stem cells (hPSC) culture media

1.1.1. Prepare hPSC culture media from the commercially available medium for maintaining and expanding human embryonic stem cells by supplementing 100 units/mL of Penicillin and 100 ug/mL of Streptomycin (see **Table of Materials**). Aliquot and store the complete media at -20 °C for the long term or 4 °C for immediate use.

1.2. Prepare Stage 1 differentiation media (for Definitive Endoderm (DE)).

1.2.1. Prepare Basal media from MCDB 131 media by supplementing with 0.5% of fatty acid-free Bovine serum albumin (FFA-BSA), 1.5 g/L of Sodium bicarbonate ($NaHCO_3$), 10 mM of glucose, 2 mM of Glutamax, 100 units/mL of Penicillin, and 100 μg/mL of Streptomycin (see **Table of Materials**). Filter the prepared media using a 0.2 μm filter and store at 4 °C.

1.2.2. Prepare Stage 1 media containing CHIR99021 from the Basal media (prepared in step 1.2.1) by warming the basal media at 37 °C and then supplementing with 2 μM of CHIR99021, 100 ng/mL of Activin A, 10 μM of Rock inhibitor (Y-27632), and 0.25 mM of Vitamin C (see **Table of Materials**). Mix the supplemented media well and cover it with aluminum foil to protect it from light.

NOTE: This media will be used only on the first day of differentiation.

1.2.3. Prepare Stage 1 media without CHIR99021 from the Basal media by warming it at 37 °C and supplementing it with 100 ng/mL of Activin A and 0.25 mM of Vitamin C and mix well.

NOTE: 5 ng/mL of basic FGF or FGF2 can be optionally added to this media. This media will be used for the remaining days of Stage 1.

1.3. Prepare Stage 2 differentiation media (for Primitive Gut Tube; PGT).

1.3.1. Prepare Stage 2 differentiation media containing Rock inhibitor from the Basal media by warming it at 37 °C and supplementing with 50 ng/mL of FGF10, 50 ng/mL of NOGGIN, 0.25 µM of CHIR99021, 10 µM of Y-27632 (Rock inhibitor), and 0.25 mM of Vitamin C.

NOTE: This media is used on the day of dissociation of endodermal cells.

1.3.2. Prepare Stage 2 differentiation media without Rock inhibitor following the same procedure for the Media prepared in step 1.3.1, excluding the Rock inhibitor.

NOTE: This media is used on the second day of Stage 2.

1.4. Prepare Stage 3 differentiation media (for Posterior Foregut).

1.4.1. Prepare DMEM media containing 4.5 g/L of Glucose, then supplement with 1% of Penicillin-streptomycin and 2 mM of Glutamax and use as basal media for Stages 3 and 4.

1.4.2. Warm the DMEM media (prepared in step 1.4.1) and supplement with 2 µM of Retinoic acid, 0.25 µM of SANT-1, 50 ng/mL of FGF10, 50 ng/mL of NOGGIN, 0.25 mM of Vitamin C, and 1% B27 supplement without vitamin A (see **Table of Materials**).

NOTE: This media is used for 4 days of Stage 3 for P2-D (Protocol 2, optimized, dissociated) and 2 days of Stage 3 for P1-ND (Protocol 1, non-optimized, non-dissociated).

1.5. Prepare Stage 4 differentiation media (for Pancreatic Progenitors).

1.5.1. Add DMEM containing 4.5 g/L of Glucose with 100 ng/mL of EGF, 10 mM of Nicotinamide, 50 ng/mL of NOGGIN, 0.25 mM of Vitamin C, and 1% of B27 supplement without Vitamin A (see **Table of Materials**). Use this media for all days of Stage 4.

NOTE: Maintain all reagents at appropriate temperatures, and all cytokine and sensitive reagents need to be aliquoted. Thaw the aliquoted reagents and add to the basal media at the time of changing the differentiation media. The compositions of the different differentiation media are provided in **Table 1**.

2. Preparation of basement membrane matrix coated dishes

2.1. Thaw commercially available basement matrix (see **Table of Materials**) and aliquot on ice; freeze the aliquots at -20 °C.

2.2. Before coating the tissue culture dishes, thaw the frozen aliquots on ice. Add appropriate volume of the membrane matrix solution to the chilled KO-DMEM/F-12 media (KnockOut DMEM/F-12) (see **Table of Materials**) to achieve the desired diluted concentration and mix well. Store the diluted matrix solution at 4 °C for immediate use.

2.3. Cover the surface of the tissue culture-treated plates (see **Table of Materials**) with diluted membrane matrix solution and place the plates at 37 °C for at least 60 min before plating the cells. Use a dilution of 1:50 of the membrane matrix solution in KO-DMEM/F-12 for plating cells for pancreatic differentiation experiment and 1:80 for expansion of undifferentiated hPSCs.

3. Culture of undifferentiated hPSCs

3.1. Passage the hPSCs when the colonies reach a 70%-80% confluency.

3.2. To passage, wash the hPSCs once with warm PBS, aspirate using a portable vacuum aspirator inside the tissue culture hood, and add 0.5 mM of EDTA solution in PBS to cover the colony surface. Incubate at 37 °C, 5% CO_2 for 1 min or until the colonies' borders detach from the plate surface.

3.3. Remove EDTA solution and collect the detaching colonies with hPSC culture medium using a P1000 micropipette. Centrifuge the collected cells at 128 x *g* for 4 min at room temperature. Discard the supernatant and supplement the cells with hPSC culture media containing 10 µM of Y-27632 (Rock inhibitor)[23,24].

NOTE: Passage the hPSCs in at least a 1:3 ratio on 1:80 membrane matrix-coated dishes. However, for differentiation experiments, plate the hPSCs on 1:50 coated dishes.

4. Induction of Definitive Endoderm (DE) differentiation in hPSCs (Stage 1)

4.1. When the hPSC colonies reach a confluency of 70%-80%, wash them twice with warm PBS and aspirate using a portable vacuum aspirator inside the tissue culture hood to begin Stage 1 differentiation.

4.2. Add Stage 1 differentiation medium containing CHIR99021 (from step 1.2.2) to the colonies, 2 mL per 6-well plate, and incubate at 37 °C for 24 h.

4.3. The next day, replace the spent media with Stage 1 differentiation medium without CHIR99021 (step 1.2.3).

4.4. Every 24 h, aspirate the spent media using a portable vacuum aspirator inside the tissue culture hood and replace it with a fresh Stage 1 differentiation medium without CHIR99021.

NOTE: Stage 1 can be extended up to 4 days. The length of Stage 1 is dependent on the hPSC line being used and should be optimized accordingly.

5. Immunofluorescence analysis of hPSC-derived DE (Stage 1)

5.1. For immunofluorescence, aspirate the spent media using a portable vacuum aspirator inside the tissue culture hood from the wells and wash twice with warm PBS. Swirl the plate to get rid of any cell debris.

5.2. Cover the surface of the wells with 4% of paraformaldehyde (PFA) to fix the DE cells; for example, add 250 uL of PFA per well of a 24-well plate. Place the plate on a 2D shaker at 20 x *g* for 20 min.

5.3. After fixation, wash the DE cells with tris-buffered saline with 0.5% of Tween (TBST) (see **Table of Materials**) and place the plate on the shaker at 20 x *g* for 10 min. Repeat this step once more.

5.4. Permeabilize the fixed cells by adding a generous volume of phosphate-buffered saline with 0.5% of Triton X-100 (PBST); for example, add 1 mL of PBST per well of a 24-well plate and place the plate back on the shaker at 20 x *g* for 20 min.

5.5. Freshly prepare 5%-6% of BSA in PBST as blocking buffer and add it to the permeabilized cells. Incubate the plate for at least 1 h in the blocking solution on the shaker.

5.6. Dilute the primary antibodies against SOX17 and FOXA2 together (see **Table of Materials**) in 2%-3% BSA in PBST solution. Add the combined antibodies to blocked cells and place the plate on the shaker at 4 °C overnight at a low speed with gentle shaking.

NOTE: SOX17 and FOXA2 are well-established DE markers[25,26].

5.7. The next day, aspirate the primary antibodies using a portable vacuum filter and wash the wells with TBST three times, each wash for 10 mins on the shaker.

5.8. Prepare 1:500 dilution of Alexa fluor 488- and 568- conjugated secondary antibodies (see **Table of Materials**) against the species the primary antibodies were raised in.

5.9. Add the secondary antibody combination to the stained well and cover the plate with aluminum foil to protect from light. Place the plate on the shaker for 1 h at room temperature.

5.10. Aspirate the secondary antibody solution using a portable vacuum filter and wash the stained wells with TBST on the shaker for 10 min, covering the plate with foil. Repeat the wash step a total of three times.

5.11. Prepare a 1 µg/mL of Hoechst 33342 dilution in PBS to stain the nuclei. Add the Hoechst solution to the wells and place the plate on the shaker for 2-3 min.

5.12. Aspirate the Hoechst solution using a portable vacuum filter and rinse the wells with PBS twice.

5.13. Finally, add PBS to the stained cells and image them using an inverted fluorescence microscope (see **Table of Materials**) in the dark. Keep the plate covered with foil when not imaging to minimize the fluorophore bleaching.

NOTE: Alternatively, flow-cytometry can be used to assess DE efficiency as described in step 9.2.

6. Generation of the primitive gut tube (PGT) from hPSCs (Stage 2)

NOTE: If the immunofluorescence analysis in step 5.13 is determined to be a SOX17-FOXA2 co-expression of 80% and above, the experiment proceeds to Stage 2. If the efficiency is <80%, extend the duration of Stage 1 to 4 days.

6.1. On day 1 of Stage 2, dissociate the hPSC-derived endodermal cells using TrypLE or Accutase (see **Table of Materials**) for the optimized P2-D protocol. Wash the adherent cells with warm PBS and add warm 1 mL of TrypLE or Accutase solution per well of a 6-well plate for 3-5 min at 37 °C, 5% CO_2, or until the cells begin to detach from one another.

6.2. Dissociate the detached sheets or monolayer of cells in the wells and then collect them together in a 15 mL polypropylene tube using basal Stage 1/2 media without cytokines containing at least 0.5% of either fetal bovine serum (FBS) or KnockOut serum (KOSR) (see **Table of Materials**).

6.3. Spin down the cells at 800 x *g* for 5 min at 4 °C and discard the supernatant. Add 1 mL of sterile PBS and resuspend the pellet into single cells.

6.4. Count the cells using an automated counter (see **Table of Materials**) by loading the recommended volume of cells in the chamber slide. Spin the resuspended cells at 800 x *g* for 5 min at 4 °C and discard the supernatant.

6.5. Resuspend the pellet in the appropriate volume of Stage 2 differentiation medium containing Rock inhibitor at a density of 2.5-3.5 x 10^5 cells/cm^2.

NOTE: This count may come down to a 1:2 splitting ratio for most cell lines, dependent on the proliferation rate. The total volume will be 2 mL of media with resuspended cells in 1 a well of 6-well plate.

6.6. Plate the resuspended cells on 1:50 membrane matrix-coated plates (prepared in step 2) and incubate them at 37 °C, 5% CO_2 in the incubator.

6.7. 24 h later, replace the media with Stage 2 differentiation medium without Rock inhibitor (prepared in step 1.3.2).

7. Generation of posterior foregut from hPSCs (Stage 3)

7.1. Aspirate the spent Stage 2 media using a portable vacuum aspirator inside the tissue culture hood and wash the cells with warm PBS.

7.2. Add Stage 3 differentiation media from step 1.4 to the cells and incubate at 37 °C, 5% CO_2.

7.3. After 24 h, replace the spent media with freshly prepared Stage 3 differentiation media. Repeat this for a total of 4 days for P2-D protocol (optimized) and only 2 days for the non-dissociated P1-ND.

8. Generation of Pancreatic Progenitors from hPSCs (Stage 4)

8.1. After 4 days of Stage 3 treatment, wash the cells with warm PBS, gently swirl the plate, and aspirate using a portable vacuum aspirator. Then, add Stage 4 differentiation media from step 1.5 to the cells.

8.2. After 24 h, replace the spent media with freshly prepared Stage 4 media. Repeat this for a total of 4 days.

9. Assessment of differentiation efficiency of generating pancreatic progenitors from hPSCs

9.1. Perform the immunofluorescence analysis of the hPSC-derived pancreatic progenitors (Stage 4) for expression of PDX1 and NKX6.1.

NOTE: PDX1 and NKX6.1 are well-established pancreatic progenitor markers[17,18].

9.1.1. Perform fixation, permeabilization, blocking, and antibody incubation and washes according to step 5.

9.1.2. Stain the hPSC-derived pancreatic progenitors with a combination of PDX1 and NKX6.1 antibodies (see **Table of Materials**) diluted in 2%-3% BSA in PBST.

9.1.3. Use 1:500 dilutions of appropriate Alexa fluor 488- and 568- conjugated secondary antibodies (see **Table of Materials**).

9.2. Perform flow-cytometry analysis of hPSC-derived pancreatic progenitors for expression of PDX1 and NKX6.1.

NOTE: Flow-cytometry analysis of pancreatic markers in the generated hPSC-derived pancreatic progenitors provides a way to quantify the PDX1 and NKX6.1 co-expressing cells.

9.2.1. At the end of Stage 4, wash the cells twice with warm PBS and add enough TrypLE or Accutase to cover the surface of the wells, for example, 1 mL of TrypLE or Accutase per well of 6-well plate. Place the plate in the incubator for 5-7 min or until the cells detach from the surface.

9.2.2. Dissociate the adherent sheets of cells within the well using a P1000 micropipette before collecting them in a 15 mL polypropylene tube.

9.2.3. Spin down the cells in hPSC-derived pancreatic progenitors at 800 x *g* for 5 min at 4 °C, then discard the supernatant. Wash the cells with PBS by dissociating them into single cells. Count the cells using an automated counter (see **Table of Materials**) and note the concentration in the number of cells per mL.

9.2.4. Spin at 800 x *g* for 5 min at 4 °C, and discard the supernatant. Add 200 µL of chilled PBS to the pellet and dissociate.

9.2.5. Add 2 mL of chilled 80% ethanol dropwise, with the tube on a vortex at low-medium speed (400 x *g* at room temperature). Close the caps tightly and place the tubes slightly tilted on the shaker at 4 °C overnight.

9.2.6. Spin down the cells at 800 x *g* for 5 min at 4 °C, and wash with PBS to dissociate any clumps of fixed cells.

9.2.7. Block the fixed cells with 5%-6% BSA solution in PBST for at least 1 h at room temperature or 4 °C overnight on the shaker.

9.2.8. Stain for the pancreatic progenitor markers following the steps below.

9.2.8.1. Distribute 2,00,000 cells per condition, including appropriate isotype controls dependent on the IgG subclass of the host species of the primary antibody, unstained and secondary antibody controls in a 96-well V bottom plate or 1.5 mL centrifuge tubes.

9.2.8.2. Spin down the plate at 800 x *g* for 5 min at 4 °C and flip the plate with a swift motion to discard the supernatant without losing the pellets. Prepare primary antibody dilutions in 3% BSA solution (see **Table of Materials**).

NOTE: The concentration of primary antibodies can be between 1:50 to 1:200.

9.2.8.3. Incubate the stained cells for at least 2 h at room temperature or overnight at 4 °C on a shaker with gentle shaking at low speed.

9.2.8.4. Wash the stained cells with TBST thrice by pipetting the cells up and down in the

wells. Spin and discard the supernatant as in step 9.2.8.2.

9.2.8.5. Add 1:500 dilution of secondary antibodies (Alexa fluor 488- and 647-conjugated antibodies) prepared in PBS (see **Table of Materials**). Incubate for 30 min at room temperature.

9.2.8.6. Wash the stained cells with TBST at least twice by pipetting the cells up and down. Spin the plate and discard the supernatant as in step 9.2.8.2.

9.2.8.7. Collect the stained cells in at least 100 µL of PBS and transfer them to light-protected FACS tubes (see **Table of Materials**). Run the samples on a flow-cytometry machine.

REPRESENTATIVE RESULTS:

The results show that optimized protocol P2-D (**Figures 1A**) enhanced pancreatic progenitor differentiation efficiency by upregulating PDX1 and NKX6.1 co-expression (**Figure 2A,B,** and **Figure 3A**). In particular, the results showed that dissociation of endodermal cells and their replating on fresh membrane matrix along with a longer duration of Stage 3 enhanced NKX6.1 expression in hPSC-derived pancreatic progenitors (optimized protocol, P2-D) (**Figure 2** and **Figure 3A**), in comparison to the non-dissociated protocol (P1-ND) that was modified from a previously published study[27]. The present enhanced protocol also generated the highest proportion of PDX1+/NKX6.1+ progenitors compared to "P1-D" (Protocol 1, S3 = 2 days, dissociated) and "P2-ND" (Protocol 2, S3 = 4 days, non-dissociated), and the detailed results have been previously published[21]. Pancreatic progenitors generated using P2-D also have increased numbers of SOX9+ cells compared to the non-dissociated P1-ND. The optimized method also generated a higher proportion of proliferative NKX6.1+ cells that co-express the proliferation marker Ki67 (**Figure 3C**).

The representative results presented here are from iPSCs generated from peripheral blood mononuclear cells (PBMCs) of control, healthy individual. However, we have reproducibly applied the current enhanced protocol on multiple hPSC lines such as H1-hESCs, H9-hESCs, HUES8-hESCs, and several other control iPSC lines to yield ~90% PDX1 and NKX6.1 co-expressing pancreatic progenitors[20,21,28].

Following DE induction in hPSCs, mild levels of cell death are expected. However, if a high cell death rate was observed, the experiment was stopped and started again with a fresh batch of cells. The efficiency of DE induction should be higher than 70%-80% to ensure a high proportion of DE cells in the culture that will serve as an ideal starting source for pancreatic progenitor induction (**Figure 1C**).

The density of replating endodermal cells following dissociation can be manipulated based on the growth rate of that particular cell. For example, slow-growing cells may be replated at a higher density than recommended. This will minimize the chances of obtaining irrelevant pancreatic populations at Stage 4. A high co-expression of PDX1 and NKX6.1 can be obtained following dissociation after Stage 1 and replating at half density (**Figure 1A, Figure 2A,B,** and

Figure 3A). If a high expression of PDX1 is observed but only a moderate expression of NKX6.1, Stage 4 can be extended by 2 days to enhance NKX6.1 expression in PDX1-expressing cells.

FIGURE AND TABLE LEGENDS:

Figure 1: Timeline for the addition of cytokines and growth factors during pancreatic progenitor differentiation. (**A**) Schematic representation of the optimized protocol (P1-D) for generation of PDX1 and NKX6.1 from hPSCs. hPSC-derived definitive endoderm (DE) is dissociated and replated at half density and then sequentially directed towards a pancreatic progenitor fate. (**B**) Images of hPSCs in brightfield before starting differentiation at Day 0. (**C**) Immunostaining analysis for expression of endodermal markers SOX17 and FOXA2 in hPSC-derived endoderm (Stage 1) cells. SOX17, green; FOXA2, red. Scale bars = 100 µm.

Figure 2: Generation of hPSC-derived pancreatic progenitors, co-expressing PDX1 and NKX6.1. Immunostaining analysis for comparison of PDX1 and NKX6.1 expression in hPSC-derived pancreatic progenitors using the enhanced protocol (P2-D) (**A**) and a non-optimized, previously published protocol (P1-ND) (**B**). The enhanced protocol achieved the highest co-expression of PDX1 and NKX6.1. PDX1, green; NKX6.1, red. Magnified images are provided in the second panel for each protocol. Scale bars = 100 µm.

Figure 3: Quantification of pancreatic markers in pancreatic progenitors generated using the enhanced protocol. Flow-cytometry analysis in hPSC-derived pancreatic progenitors yielded using P2-D in comparison to the non-dissociated P1-ND. (**A**) Histograms for PDX1 and NKX6.1 expression and double-positive staining graphs. (**B**) The histograms for SOX9 expression and (**C**) co-expression of NKX6.1 with the proliferation marker Ki67.

Table 1: The compositions of the different differentiation media used in the study.

DISCUSSION:

This work describes an enhanced protocol for generating pancreatic progenitors from hPSCs with a high co-expression of PDX1 and NKX6.1. Dissociation and replating of the hPSC-derived endoderm at half density on fresh matrix resulted in higher PDX1 and NKX6.1 in hPSC-derived pancreatic progenitors.

Although the growth factor cocktail for each stage is highly similar to P1-ND[27], it has been shown that a more extended Stage 3 treatment including FGF and retinoid signaling and BMP and hedgehog inhibition increases NKX6.1 expression, which is in contrast to Nostro et al.'s findings[27]. While PDX1 expression in the pancreas during embryonic development is positively regulated by FGF and retinoid signaling and BMP and hedgehog inhibition[27,29,30], the present results demonstrated the extension of this signaling cocktail also upregulates NKX6.1. Nevertheless, the effect of an extended Stage 3 treatment was aided by dissociation and replating of endodermal cells that led to increased PDX1 and NKX6.1 expression. Furthermore, this method (P2-D) also increased SOX9-expressing cells in hPSC-derived pancreatic progenitors, in addition to the proportion of proliferative NKX6.1+ cells that co-express the proliferation marker Ki67.

Another crucial factor affecting the efficiency of differentiation was the availability of fresh membrane matrix to the dissociated cells. The extracellular matrix components have been previously demonstrated to regulate stem cell fate specification[31,32]. Overall, the favorable effects of extracellular matrix components on pancreatic development have been recorded[33-35], particularly for the membrane matrix, which, along with laminin, was shown to have a pro-endocrine effect on pancreatic lineage cells[36]. Therefore, replating endodermal cells on fresh membrane matrix may have enhanced NKX6.1 expression in hPSC-derived pancreatic progenitors.

The cell density of differentiating cells also controls gene expression. Multiple studies have demonstrated the significance of cell-cell contact in regulating pancreatic development[37-39]. Cellular aggregation or embryoid body formation has been shown to induce higher pancreatic endocrine gene expression than 2D cultures[19]. However, the results demonstrate that higher pancreatic expression, especially NKX6.1, can be obtained by culturing cells in a 2D monolayer at half the endodermal density using our optimized protocol[21]. Interestingly, this method also inhibited hepatic cell fate (the alternative fate of hPSC-derived DE) as noticed by the decreased expression of hepatic genes AFP (Alpha-fetoprotein) and ALB (Albumin)[21], indicating that physical factors play a role in lineage specification of hPSCs.

Overall, the results demonstrate that modulating the physical environment of the differentiating cells can enhance pancreatic gene expression[21]. Specifically, the dissociation of hPSC-derived endoderm and replating on fresh membrane matrix with a longer FGF and retinoid signaling and hedgehog and BMP inhibition can enhance the PDX1 and NKX6.1 co-expression in hPSC-derived pancreatic progenitors. However, the optimized protocol is at least 2 days longer than previously published protocols. Also, the length of Stage 4 may be extended beyond the minimum recommended, i.e., 4 days, based on the cell line being used. Multiple recombinant human growth factors are employed in the optimized protocol that can be substituted by their less expensive, small molecule compounds that perform the same action. Nevertheless, this optimized protocol can facilitate the scalable generation of pancreatic progenitors from hPSCs for cell therapy and disease modeling.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

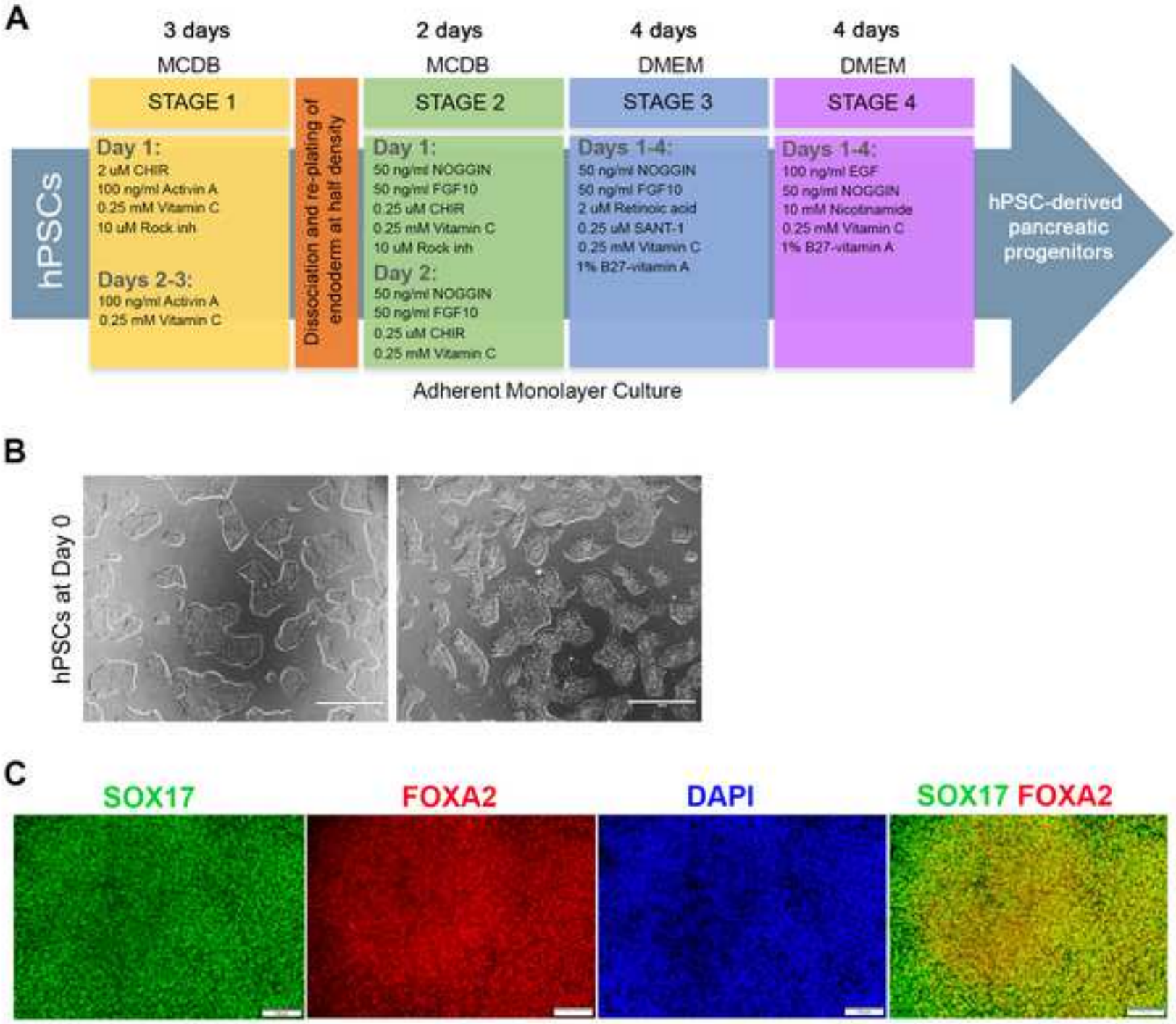

Figure 2

Click here to access/download;Figure;Figure 2_rev.jpg

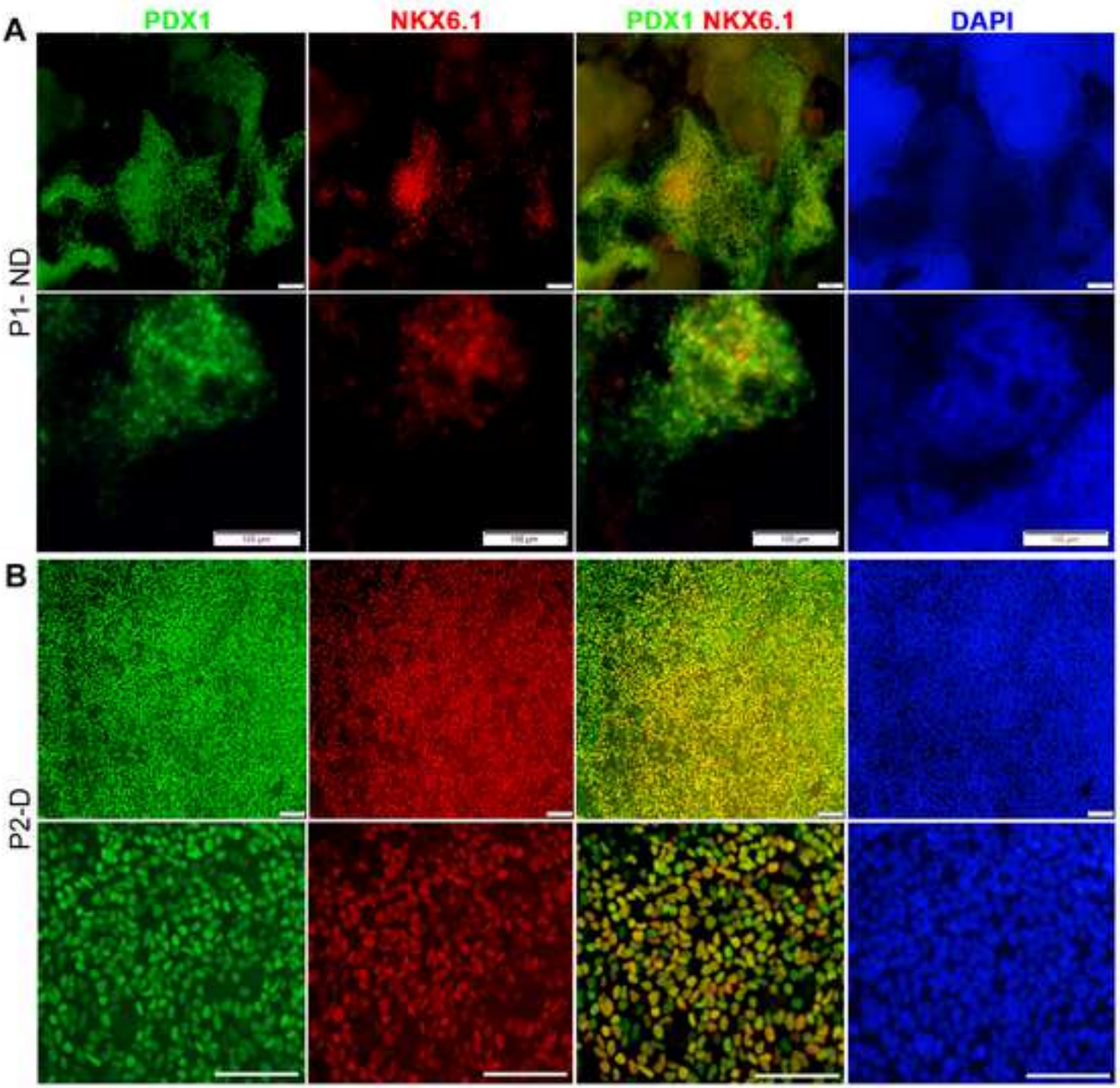

Figure 3

Click here to access/download;Figure;Figure 3_Rev.tif

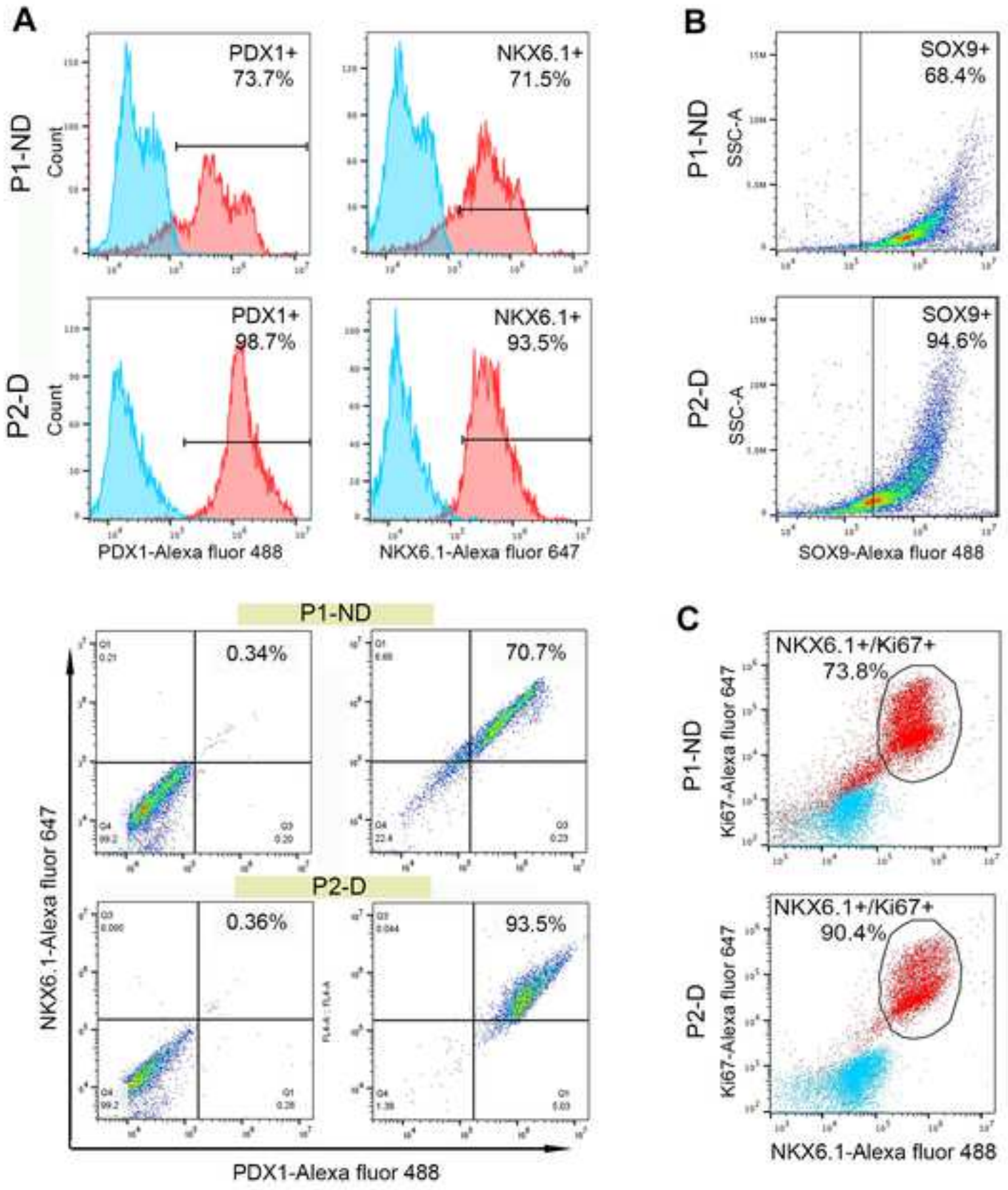

Table 1

Stage	Media	Cytokines	Days
1	MCDB 131 media + 0.5% fatty acid-free bovine serum albumin (FFA-BSA), 1.5 g/L sodium bicarbonate ($NaHCO_3$), 10 mM of glucose	**Day 1**: 2 µM CHIR99021, 100 ng/mL Activin A, 10 µM Rock inhibitor (Y-27632), 0.25 mM Vitamin C. **Day 2 onwards:** 100 ng/mL Activin A, 0.25 mM Vitamin C	3 or 4
2	MCDB 131 media + 0.5% fatty acid-free bovine serum albumin (FFA-BSA), 1.5 g/L sodium bicarbonate ($NaHCO_3$), 10 mM glucose	**Day 1 (Dissociation):** 50 ng/mL FGF10, 50 ng/mL NOGGIN, 0.25 µM CHIR99021, 10 µM Y-27632 (Rock inhibitor) , 0.25 mM Vitamin C. **Day 2:** 50 ng/mL FGF10, 50 ng/mL NOGGIN, 0.25 µM CHIR99021, 0.25 mM Vitamin C.	2
3	DMEM + 4.5 g/L glucose	2 µM Retinoic acid, 0.25 µM SANT-1, 50 ng/mL FGF10, 50 ng/mL NOGGIN, 0.25 mM Vitamin C, 1% B27 supplement without vitamin A	4
4	DMEM + 4.5 g/L glucose	100 ng/mL EGF, 10 mM Nicotinamide, 50 ng/mL NOGGIN, 0.25 mM Vitamin C, 1% B27 supplement without vitamin A.	4

Responses to editor's and reviewer's comments

Editorial comments:

1) ***The editor has formatted the manuscript to match the Journal's style. Please retain it and use the attached version for revision.***

   We have used the editor's version for incorporating any changes we made to the manuscript.

2) ***Please address the specific comments marked in the manuscript. Also, please don't delete the Editor's comments; instead, provide a very brief reply to each comment regarding your actions.***

   We have attempted to address all the comments provided by the editor and replied to them as instructed.

Reviewer's comments:

We thank the reviewer for his comment and acceptance of our revised version of manuscript.

Responses to editor's and reviewer's comments

We thank the editor and reviewers for their constructive comments, which we feel have helped to improve our manuscript. All comments concerning the manuscript were taken into consideration. Below are our point-by-point responses to the reviewer's comments and of the changes that we made in the manuscript. All changes are marked in red *throughout the manuscript.*

Editorial comments:

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

We have corrected the spelling and grammatical errors throughout the manuscript.

2. Please make the Title concise: Differentiation of human pluripotent stem cells into pancreatic beta cell precursors in 2D culture system.

We have made this change.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the manuscript to make these changes.

4. Please ensure that abbreviations are defined at first usage.

We have added these definitions.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. All action steps should be numbered.

We have edited the numbering of steps throughout the manuscript.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). Any text that cannot be written in the imperative tense (e.g., provide extraneous details, optional steps, or recommendations) may be added as a "Note."

The language has been changed throughout the protocol as per the editor's instructions.

7. The Protocol should be made up almost entirely of discrete steps without large

paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We have shortened any steps that had more than 4 sentences as per the above instructions.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have attempted to add more details to each step as per the editor and reviewer's comments.

9. Please add more details to your protocol steps: Step 2.1/3.1/3.4/4.1/4.7/4.10/4.12/6.1: How was the aspiration done? A pipette was used? Please specify.
Step 2.2/5.4: Please mention the centrifugation temperature.
Step 5: Indicate 'stage 2' within brackets in the heading as done for steps 6 and 7. Similarly, indicate 'stage 1' in the previous step(s).

All the above-mentioned concerns have been addressed and details added in the respective steps.

10. Please ensure you provide composition of all the media, solution, reagents, buffers used in a separate table format as .xlsx file.

We have included another table (Table 2) titled "Table for Differentiation Media Compositions".

11. Please insert single-line spacing between individual steps and sub-steps in the Protocol and then highlight up to 3 pages of protocol sections including headings and spacings. Please ensure that the highlighted steps form a cohesive narrative with a logical flow and is in line with the Title of the mansucript.

We have highlighted 3 pages of our protocol for narrating our protocol in yellow highlight.

12. As we are a methods journal, please ensure that the Discussion cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have made sure the above points are covered in discussion.

13. Ensure that all the essential supplies, reagents, and equipment used in the protocol are included in the Table of Materials. Please sort the Table of Materials alphabetically by the name of the material.

We have made these changes to our Table of Materials. In addition, we have provided another table "Table of differentiation media compositions" summarizing the media reagents and cytokine cocktails for each stage.

14. Please do not abbreviate the journal names in the References.

We have made this change.

Reviewers' comments

Reviewer #1:

Major Concerns:

Q1. In stage 4, generation of pancreatic progenitors from hPSCs, authors detected PDX1 and NKX6.1 via immunostaining and FACS, insulin1, insulin2, MafA, MafB, and glucagon are also needed to be tested. RNAseq analysis for hPSCs is highly recommended.

Pancreatic progenitors that are generated at stage 4 from hPSCs are multipotent cells and can generate endocrine lineage cells, including beta cells, upon appropriate cues. PDX1+ and NKX6.1+ co-expressing progenitors will give rise to insulin secreting beta cells in vitro and in vivo upon further differentiation. Therefore, at stage 4, which is the focus of the current protocol, we assessed the expression levels of PDX1 and NKX6.1, but not for beta cell markers, because the islet cells are not obtained at the current stage. However, to obtain beta cells that express INSULIN, MAFA, MAFB, and GLUCAGON, we would be required to differentiate the PDX1+/NKX6.1+ progenitors further to endocrine progenitors and then to endocrine cells (beta cells). Therefore, the abovementioned markers are not assessed at stage 4, because the signaling pathways until stage 4 are designed to generate a high number of pancreatic progenitors.

Also, human islets do not have insulin 1 and insulin 2, but have Insulin, which is different from rodents.

Q2. Percentage of insulin-secreting cells need to statistics.

As described above, our protocol aims to generate pancreatic progenitors at stage 4, which are the precursors of beta cells; therefore, insulin-secreting cells are not quantified at this stage.

Minor Concerns:
Q3. Quality of immunofluorescence images should be improved.

We have improved the images by enhancing brightness/ contrast in the red and green channels.

Reviewer #2:

Major Concerns:

Q1. In its current state the abstract does not clearly convey why there is a need for another differentiation protocol for hPSCs. For example, the sentence below makes me question why we need another system when there are already clinical trials ongoing. "hPSC derived pancreatic progenitors are currently being used for cell therapy in type 1 diabetes patients as part of clinical trials. We, therefore, developed an enhanced protocol for generating hPSC-derived…."
The authors should highlight what is missing or making current systems inefficient and how their system solves those issues.

We added the following sentence in the abstract to point out the gap and significance of our protocol:

"However, current protocols do not generate a high proportion of NKX6.1+ pancreatic progenitors that leads to co-generation of non-functional endocrine cells along with few glucose-responsive, insulin-secreting cells. We, therefore, developed an enhanced protocol for generating hPSC-derived pancreatic progenitors that maximizes the co-expression of PDX1 and NKX6.1 in 2D monolayer."

Q2. It would be helpful if the authors could make the reason why it could be advantageous to generate pancreatic progenitors rather than endocrine cells even clearer. Additionally, alternative uses of their system (e.g., developmentally focused studies of pancreatic progenitors and potentially also endocrine cells) should be added to the discussion.

The sentences addressing the above points have been added in the "Introduction" on pages 2-3:

"These protocols generate hPSC-derived islet organoids and, while they have greatly improved at increasing the proportion of pancreatic beta cells therein, the efficiency of protocols is highly variable that does not increase to more ~40% of NKX6.1+/INSULIN+ or C-PEPTIDE+ cells. However, the generated beta cells are not entirely identical to the adult human beta cells, in terms of their transcriptional and metabolic profiles as well as in their response to glucose [6-8]. The hPSC-derived beta cells lack gene expression of key beta cell markers such as PCSK2, PAX6, UCN3, MAFA, G6PC2 and KCNK3 in comparison to adult human islets [6]. Additionally, the hPSC-derived beta cells have diminished calcium signaling in response to glucose and are contaminated with the co-generated polyhormonal cells that do not secrete appropriate amounts of insulin in response to increase glucose levels [6]."

<u>Q3.</u> Miss highlighting of critical steps in the protocol.

Critical steps have been highlighted in bold font. For example: Line 235, step 5.7: **"Resuspend the pellet in the appropriate volume of stage 2 differentiation medium containing Rock inhibitor at a density of 2.5-3.5 x 10^5 cells/cm^2."**

<u>Q4.</u> A table of the reagents and what temperature they should be stored at and for how long would be helpful. As well as the solvent used for lyophilized growth factors/inhibitors.

This has been provided in 'comments' section of the table of materials.

<u>Q5.</u> A table listing things that can go wrong at the different steps, the potential/ most likely cause and what action to take would be most useful for readers trying to replicate the experiments.

These points are discussed in "Notes" throughout the text.

<u>Q6.</u> Did the authors try different hPSC/iPSC lines, do they see any difference in efficiency between the lines? (Would the useful for the readers to know that if they try another line except the one mentioned here - the results may vary). Please specify which hPSC line(s) were used to generate the representative results.

The representative results shown here have been obtained from hiPSC lines generated in our lab from a healthy individual. However, we have published the efficiencies for other cell lines such as H1-hESCs and other control iPSCs generated by us in previous publications. We have applied the differentiation protocol across different hESC and hiPSC lines in our lab. Differences in efficiencies are inherent in different hPSC cell lines as demonstrated by several groups globally. For example, using the same protocol, NKX6.1 expression levels varied between 37%-85% at stage 4 across for different hPSC lines. In our lab, we also obtained different efficiencies across H1-hESCs, HUES8-hESCs and other in-house generated hiPSCs from normal individuals; however, our protocol still yielded a reasonably high proportion of PDX1+/NKX6.1+ pancreatic progenitors across different cell lines.

We have described this in the 'Representative Results' section. Line 324: *"*The representative results presented here are from iPSCs generated from PBMCs of control, healthy individual. However, we have reproducibly applied our enhanced protocol on multiple hPSC lines such as H1-hESCs, H9-hESCs, HUES8-hESCs and several other control iPSC lines to yield ~90% PDX1 and NKX6.1 co-expressing pancreatic progenitors*".*

Q7. In the text the authors are comparing their method to previously published methods. It would be very helpful to have a side-by-side comparison of the different protocols (not only the more direct comparison to P1-ND). At least a table summarizing the percentages of double positive cells obtained by the different protocols should be included. This would highlight why the readers could benefit from using this method.

We compared our protocol to P1-ND (that is a modified version of Nostro et al. (*Stem Cell Reports 2015; 4 (4), 591-604*), because this protocol, prior to ours, was the most recent and generated the highest percent of NKX6.1+ progenitors, which is crucial to beta cell differentiation. Since our aim was to maximize NKX6.1 expression in pancreatic progenitors, we chose to compare to P1-ND alone. However, we also compared our protocol to different versions of P1-ND which are described in our previous paper [10] and mentioned in the next response below.

Q8. The authors claim that an extended stage 3 treatment and replating of endodermal cells however the representative results lack the single controls (only replating or only extended stage 3 treatment) to show that both these factors are of importance. Also, a comparison (at least a table) comparing the authors' method to that of others should be included to highlight the advantage with this protocol compared to others.

We wanted to focus on the enhanced protocol alone in this paper as using the different single control protocols would confuse the readers. However, we have published a comprehensive analysis of results from these single control protocol, that is, the P1-D (S3 not extended, dissociated) and P2-ND (extended S3, non-dissociated) in our previous paper.

This has been cited in the "Representative results" section on page 10 as follows: "*Our enhanced protocol also generated highest proportion of PDX1+/NKX6.1+ progenitors compared to "P1-D" (S3 not extended, dissociated) and "P2-ND" (S3 extended, non-dissociated) and the detailed results have been previously published (10)".*

Minor Concerns:

Q9. These two sentences can be rewritten to make the message clearer. The second sentence is almost a repetition of the first. "hPSC-derived pancreatic progenitors, that are precursors to beta cells and other endocrine cells, when co-expressing the two transcription factors PDX1 and NKX6.1 specify the progenitors to a beta cell fate. PDX1- and NKX6.1 co-expressing pancreatic progenitors derived from hPSCs can give rise to functional insulin-secreting cells both in vitro and in vivo."

In the Abstract, we deleted the first sentence and changed the second sentence to:

"hPSC-derived pancreatic progenitors, that are precursors to beta cells and other endocrine cells, when co-express the two transcription factors PDX1 and NKX6.1, specify the progenitors to functional, insulin-secreting beta cells both in vitro and in vivo."

Q10. 38: the word choice growing is interesting.

We changed the sentence to *"Diabetes is a complex metabolic disorder affecting millions of people globally"* on page 2, line 38.

Q11. 48: self-renewal instead of self-replicative.

We changed the word to self-renewal on page 2, line 48.

Q12. 47-51 before 45-47, and remove the bit about disease models and drug screens. Not relevant here.

We removed the part about drug screening and disease models in line 57.

Q13. "On the other hand, hPSC-derived pancreatic progenitors, that are islet precursors, could be generated rather more efficiently in vitro and when transplanted in vivo, could mature into functional, insulin-secreting beta cells 12,13"
Rather more efficiently compared to what?

We added "*compared to beta cells*" in line 82.

Q15. 70: is this higher than for aggregates? Authors could mention the advantages for 2D over aggregates to highlight why their protocol would be useful.

The percentage of PDX1+/NKX6.1+ pancreatic progenitors generated by our enhanced protocol at day 4 of stage 4 (80-90%) is higher than the two studies cited; 40-50% as aggregates in Toyoda et al, and ~80% in Russ et al.

However, 2D culture is more convenient, cost-effective, and feasible as well as more practical. This is now mentioned on page 3, line 72-78: *"*Studies have demonstrated that an embryoid body or 3D culture enhances PDX1 and NKX6. 1 in pancreatic progenitors where the differentiating cells are aggregated, **varying between 40-80% of the PDX1+/NKX6.1+ population; however, compared to suspension cultures, 2D differentiation cultures are**

more cost-effective, feasible, and more convenient for application on multiple cell lines. We recently showed that monolayer differentiation cultures can yield more than up to 90% of PDX1+/NKX6.1+ co-expressing hPSC-derived pancreatic progenitors ".

Q16. A paragraph summarizing the protocol and the aims of the main different differentiation steps (step1-4) as well as rationale with regards to the chosen growth factors/inhibitors would provide the reader with an overview before going into the detailed protocol steps. The authors can also refer to Fig1A.

We have added the following sentence towards the end of introduction: *"This method utilizes the technique of dissociating hPSC-derived endoderm and manipulating the cell density, followed by an extended FGF and Retinoid signaling as well as Hedgehog inhibition to promote PDX1 and NKX6.1 co-expression (Figure 1A)."*

Q17. Fig1A: indicate that it is B27-vitaminA.

It is now mentioned on the schematic under stages 3 and 4.

Q18. Fig1B: please include inserts with higher magnification and quantification of double positive cells.

We have provided higher magnification images for PDX1 and NKX6.1 co-expression in Figure 2 as second panels for each protocol.

Q19. For step 2: pictures of the desired confluency and what the hESCs should look like prior to starting the differentiation procedure would be useful for readers that are not familiar to hESC cultures.

We added this image in Figure 1B as "hPSCs at Day 0".

Q20. 138: add the different Matrigel ratios already at this point. Have the authors tried BME? Any difference?

We have not tried BME.

Q21. 164: When is it preferred to extend step 1 for up to 4 days? Could the authors please explain based on what parameters this decision should be made and provide representative images?

The extension of stage 1 to 4 days or limiting it to 3 days is dependent on the cell line being used based on their inherent differences. The goal of stage 1 is to generate a high proportion of Endoderm or DE (SOX17+/FOXA2+) cells so that the percentages of subsequent lineages to be differentiated can be maximized. This is now stated in **step 3.4.**

Q22. For step 4: Immunofluorescence analysis of hPSC-derived DE, would FACS be an alternative? If so that could make the process faster as overnight incubation of primary antibodies would not be needed.

The reviewer is right, it is dependent on the researcher's preferred method of choice. However, immunofluorescence can also be performed within a day if the blocking step is shortened, and primary incubation is done for a shorter time (like 3-4 hours). The possibility of this alternative is stated in Step 4.1.

Q23. 199: there is no step 4.13.

Step 4.13 is now step 4.14. and mentioned in line 248.

Q24. 298: modified in what way? Please provide details and the rationale behind these modifications.

Step 5.1: what if less than 80% of the cells are double positive? Please refer the reader to the correct troubleshooting section and discuss what could have gone wrong?

It is now mentioned in step 5.1 that if the efficiency is below 80%, they can extend stage 1 to 4 days.

Q26. Step 5.7: either counting or doing a 1:2 split. When would the authors choose to do which option? This is a bit unclear, why ever count if one can just split 1:2. Please comment. What is the appropriate volume of media? Please be specific.

For most experiments, the count mentioned is roughly equivalent to 1:2 ratio for the cell lines we tested. Therefore, if the experiment is being repeated multiple times on the same cell line, and if it is confirmed that the count suggested comes down to 1:2 splitting for the particular

cell line, a 1:2 ratio splitting can be used instead. However, different cell lines may have different proliferation rates and hence may give different counts of total cells. However, for the simplicity of the protocol and to prevent confusion, we have changed the sentence to:

"*For most cell lines, this count may come down to a 1:2 splitting ratio, dependent on the proliferation rate. Total volume should be 2 ml of media with resuspended cells in 1 well of 6-well plate."*

Q27. Step 5.9: spent media?

We have changed it to be "*replace the media with…."*.

Q28. 233: step 1.6 describes coating of plates with Matrigel

We have changed this to step 1.5. Thank you for the correction.

Q29. 271: give examples of controls.

8.2.8.1: The sentence is edited to *"Distribute 200,000 cells per condition including appropriate isotype controls dependent on the IgG subclass of the host-species of the primary antibody, unstained and secondary antibody controls in a 96-well V bottom plate or 1.5 mL Eppendorf tubes."*

Q30. 7.2.8.2 refer the reader to a table (please include this) with the dilutions for the antibodies used in this study

We have added this detail in Table of Materials under "comments" section.

Q31. 295-296: Show controls where only one variable is changed at a time. First only do the dissociation and then only stage 3 for a longer time period, and re-plating vs no replating. Compare that to dissociation and longer treatment time together - or refer to previously published data. However, images are preferred as it would highlight the usefulness of the authors differentiation protocol in an easily accessible way for the readers.

Detailed results for each individual condition that the reviewer has described in this comment is explained and presented elaboratively in our previously published paper *Stem Cell Res Ther* **9,** 15 (2018)[10]. In this paper, we have only elaborated details for our enhanced protocol only.

Q32. Fig2A: please include inserts with higher magnification.

We have included these in Figure 2A and 2B.

Q33. Fig 2B-D: Please show the double positive PDX1 and NKX6.1 population. Since this is the main aim of the protocol it is strange that they (the double positive cells) are not quantified.

We have provided the double positive PDX1 and NKX6.1 plots in Figure 3A. All FACS plots are now in Figure 3.

Q34. Fig2D: texts in the FACS plots are blurry. Please use higher resolution images.

The font size in FACS plots has been increased.