Differentiation protocol for generating functional pancreatic β cells from human pluripotent stem cells

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Abstract

The efficient generation of pancreatic β cells from human pluripotent stem cells may allow us to study their biological characteristics and use them for the treatment of type I diabetes. The protocol we present in the study provides an efficient method for producing β cells using either human embryonic stem cells or human induced pluripotent stem cells as the starting material.

Introduction

Developments for the differentiation of human pluripotent stem cells (hPSCs) into pancreatic β cells, with the goal of using these cells for cell replacement therapy to cure type I diabetes, are promising [1-9]. However, the application potential of current methodologies remains limited because of several major issues, including moderate rate of efficiency (10%~40% NKX6.1+/INS+), high heterogeneity accompanying with high percentage of unwanted cell types (such as dual-hormonal GCG+/INS+ cells), and highly variable efficiencies depending on the origin of the manipulated cell line. To overcome these issues, we have developed a novel stepwise protocol by creating an efficient method for making pancreatic progenitor 3-D clusters, introducing an extra step to potentiate pancreatic progenitors with a cocktail of 10 chemicals, and identifying new factors or chemicals or their combinations for generating β cells from pancreatic progenitors. Using these improvements, we are able to generate ~60%-80% of β cells from hPSCs, and these β cells can rapidly reverse hyperglycemia in the mouse model of diabetes.

Reagents

- hPSCs (hESCs or hiPSCs)
- mTeSR™1 (STEMCELL Technologies, cat. no. 85850)
- Matrigel (R&D Systems, cat. no. 3433-005-01)
- Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco, cat. no. 11330)
- DMEM (Gibco, cat. no. 11995)
- DMEM-no Glucose (Gibco, cat. no. A14430-01)
- PBS (10X), pH 7.4 (Gibco, cat. no. 70011069)
- Accutase (Stem cell, cat. no. 07920)
- MCDB 131 medium (Gibco, cat. no. 10372)
- KO-DMEM medium (Gibco, cat. no. 10829018)
- Glucose (45%, Sigma-Aldrich, cat. no. D9434)

- Fat acid free BSA (EMD Millipore, cat. no. 126575)
- Sodium Bicarbonate (Gibco, cat. no. 25080-094)
- ITX (Gibco, cat. no. 51500-056)
- Pen/Strep (Gibco, cat. no. 15140)
- Glutamax (Gibco, cat. no. 35050)
- Heparin (Sigma, cat. no. H3149)
- ZnSO4 (Sigma, cat. no. Z0251)
- LDN193189 (LDN, Selleck Chemical, cat. no. S2618)
- T3 (Sigma, cat. no. T6397)
- RA (SIGMA, cat. no. R2625)
- SANT1 (Tocris, cat. no. 1974)
- GABA (Sigma, cat. no. A5835)
- Human EGF (Peprotech, cat. no. AF-100-15)
- NICO (Sigma-Aldrich, cat. no. N0636)
- L-Ascorbic Acid (Vc, SIGMA, cat. no. A4544)
- Forskolin (Tocris, cat. no. 1099)
- B27 (100x, Gibco, cat. no. 17504-044)
- TPB (EMD Millipore, cat. no. 565740)
- Repsox (Tocris, cat. no. 3742)
- KGF (Proteintech, cat. no. HZ-1100)
- SANT1 (Tocris, cat. no. 1974)
- γ-Secretase Inhibitor XXI (GXISS, Millipore, cat. no. 565790)
- IGF1 (PeproTech, cat. no. 100-11)
- FGF inhibitor PD173074 (SELLECK CHEMICALS LLC, cat. no. S1264)

- Betacellulin (BTC, BioLegend, cat. no. 551204)
- ISX-9 (CAYMAN CHEMICAL, cat. no. 16165)
- G-1 (Cayman Chemical, cat. no. 10008933)
- Deazaneplanocin-A (Deza) (BioVision, cat. no. 2060-250)
- ZM447439 (ZM) (Selleck Chemical, cat. no. S1103)
- H-1152 (Medchemexpress, cat. no. HY-15720A)
- CI-1033 (Selleck Chemical, cat. no. S1019)
- RPMI 1640 Vitamins Solution (100×, SIGMA-ALDRICH, cat. no. R7256)
- Sodium pyruvate (200x, Gibco, cat. no. 11360-070)
- NEAA (200x, Gibco, cat. no. 11140-050)
- Lipid (2000x, Thermo Fisher Scientific, cat. no. 11905031)
- Trace Elements A (2000x, Corning, cat. no. MT99182CI)
- Trace Elements B (2000x, Corning, cat. no. MT99175CI)

Antibodies

- Primary antibodies: NKX6.1 (DSHB, F55A12-c), PDX1(R&D, AF2419), INS-APC (Cell signaling, C27C9), NKX6.1-PE (BD, #563023), Human C-peptide (Abcam, Ab14181), Glucagon (Cell signaling, 2760S), Glucagon (Abcam, ab82270), Insulin (Abcam, ab7842), GFP (Abcam, ab6673), NEUROD1 (R&D, AF2746), NEUROD1-APC (BD, 563000).

- Secondary antibodies: Donkey anti-mouse Alexa Fluor 488 (Jackson Lab, 715-545-151), Donkey anti-Goat Alexa Fluor 488 (Jackson Lab, 705-545-147), Donkey anti-Rabbit Alexa Fluor 488 (Jackson Lab, 711-545-152), Donkey anti- Guinea Pig Alexa Fluor 488 (Jackson Lab, 706-545-148), Donkey anti-mouse Alexa Fluor 550 (Invitrogen, SA5-10167), Donkey anti-rat Alexa Fluor 555 (Abcam, ab150154), Donkey anti-Rabbit Alexa Fluor Cy3 (Jackson Lab, 711-165-152), Donkey anti-rat Alexa Fluor 647 (Abcam, ab150155), Donkey anti-Goat Alexa Fluor 647 (Jackson Lab, 705-605-147), Donkey anti-Guinea Pig Alexa Fluor 647 (Jackson Lab, 706-605-148), Donkey anti-mouse Alexa Fluor 647 (Jackson Lab, 715-605-151), Donkey anti-Guinea Pig FITC (Jackson Lab, 706-095-148), Donkey anti-Mouse Cy5 (Jackson Lab, 715-175-151), Donkey anti-Mouse Alexa Fluor 647 (AF647) (Jackson Lab, 715-605-151), and Donkey anti-Rabbit Alexa Fluor 647 (Jackson Lab, 711-605-152).

Reagent setup

- MS12 medium (800ml) is made with 744ml MCDB131 medium, 3.2ml 45% glucose, 20ml 20% fat-acid free BSA, and 16ml 7.5% sodium bicarbonate.

- V4b-Medium (800 ml) is made of 340 ml MCDB 131 medium + 170 ml F12 medium + 170 ml KO-DMEM medium + 3.9 ml Glucose (45%, Sigma) + 80 ml 20% FF-BSA + 1 6ml Sodium Bicarbonate (7.5%) + 4 ml ITX + 8 ml Pen/Strep + 8 ml Glutamax.

- 5a-Medium is made of V4b-Medium+ heparin (Sigma, H3149, 10 μg/ml) + ZnSO4 (10 μM, sigma, Z0251) + LDN193189 (LDN, Tocris, 100 nM) + T3 (1 μM, Sigma, T6397) + RA (0.05 μM) + SANT1 (0.25 μM; Tocris) + GABA (1 mM, sigma) + human EGF (100 ng/ml, Peprotech) + NICO (10mM) + Vc (0.25mM).

- Stage-5 medium is made of 5a-Medium + TPB (PKC activator, 100 nM) + Repsox (10 μM, 374210, Thermo Fisher Scientific).

- Stage-6 medium = B26-medium + Forskolin (10 μM) + T3 (1 μM).

- B26-medium = DMEM + B27 (100x), + LDN (500 nM) + TPB (30 nM) + Repsox (1 μM) + KGF (25 ng/ml) + SANT1 (0.25 μM) + RA (0.05 μM) + PS (100x).

- Stage-7 medium = 4#-medium, supplemented with (gamma secretase inhibitor XX (GSIXX, 100 nM, only for day1-6) + RA (0.05 μM, only for day1-6) + HGF (50 ng/ml, only for day1-6) + IGF1 (50 ng/ml, only for day1-6) + FGF inhibitor PD173074 (0.1 μM, only for day3-6)).

- 4#-medium=V4b-medium + $ZnSO_4$ (10 μM) + Heparin (10 μg/ml) + LDN (100 nM) + T3 (1 μM) + Repsox (10μM).

- Stage-8 medium = M18#-medium, supplemented with 7 chemicals/factors (termed as Fβ-7C, including betacellulin (BTC, 10ng/ml, only used for day 7-12), ISX-9 (a NEUROD1 inducer, 10 μM), G-1 (a GPER agonist, 1 μM), Deazaneplanocin-A (Deza, a histone methyltransferase inhibitor, 1 μM), ZM447439 (ZM, an aurora kinase Inhibitor, 2.5 μM), H1152 (a ROCK inhibitor, 10 μM; only used for day1-6), CI-1033 (a pan-ErbB inhibitor, 1 μM, only used for day7-12).

- M18 medium (5.58 mM glucose) = MCD131 (Thermofisher, 340 ml for each 800 ml total medium) + F-12 (Thermofisher, 170 ml for each 800 ml total medium) + DMEM-no Glucose (Thermofisher, 170 ml for each 800 ml total medium) + fatty acid free BSA (final 2%, EMD Millipore, 126575) + 7.5% Sodium Bicarbonate (12.8 ml for total 800 ml medium) +RPMI 1640 Vitamins Solution (100×, SIGMA-ALDRICH, R7256) + ITX (200x) + sodium pyruvate (200x) + NEAA (200x) +PS (100x)+ Glutamax (100x) + 45% glucose (Sigma, 2500x) + Heparin (10 μg/ml) + Lipid (2000x, Thermo Fisher Scientific, 11905031) + Trace Elements A (2000x, Corning, MT99182CI) + Trace Elements B (2000x, Corning, MT99175CI).

Equipment

- 6-well air-liquid interface trans-well (Corning, 07200170)
- Laminar flow tissue culture hood (Thermo, 1300 Series A2)
- 6 well-tissue-culture-grade plates (Sarstedt cat. no. 83-3920)
- Incubator at 37°C with 5% CO2 (Thermo, Heracell 150i)
- Sterile 15-50 ml conical tubes (BioPioneer, Precision CNT-50)
- V-bottom 96-well plate (sigma, P7241-1CS)
- Confocal microscope (Zeiss LSM 710)
- Inverted microscope (Olympus IX51)
- FACS machine (BD, LSRII)
- Frozen section machine (Leica, CM1900)

Procedure

Cell preparation:

1. HESCs are maintained as feeder-free in mTeSR™1 (STEMCELL Technologies Inc.) according to the manufacturer's instructions.

2. Before differentiation, adherent hESCs were rinsed with PBS and then incubated with Accutase for 6-8 min at 37°C.

3. Dissociated single cells were rinsed twice with DMEM/F12 and spun at 300g for 3 min. The resulting cells were re-suspended in mTeSR™1 which was supplied with 10μM Y27632 (Sigma-Aldrich), and seeded on 1:30 diluted Matrigel-coated dishes at a density of ~55,000 cells/cm^2.

4. The next day, the medium was exchanged for mTeSR™1 and maintained for one more day prior to differentiation initiation.

Stage 1: Definitive Endoderm (3 days).

5. After PBS rinse, undifferentiated hESCs (at ~90% confluence) were exposed to differentiation medium as following.

6. Day1: MS12 medium supplied with, 3 μM chir99021, and 115 ng/ml Activin-A (ActA);

7. Day2: MS12 medium with 0.3 μM chir99021, and 110 ng/ml ActA;

8. Day3: MS12 medium with 100 ng/ml ActA.

Tip: (1) 90% confluence (at the moment for starting differentiation) is important as the cell density too low or too high can greatly reduce the final production of the β cells. (2) Some cell line may grow a little slowly, thus increasing the seeding cell quantity or extending the cell growth time will facilitate these cells to reach the appropriate confluence. (3) Handle the cells very gently at this stage as cells are sensitive and are prone to detach from the dish bottom especially at day2 and day3.

Stage 2: Primitive gut tube (3 days):

9. After PBS rinse, cells are exposed to MS12 medium with KGF (50ng/ml), B27 (100x), Vitamin C (Vc, 0.25mM) and dorsomorphin (0.75μM). Cells were fed with fresh medium daily.

Tip: Handle the cells very gently at the beginning of this stage, as cells are prone to detach from the dish bottom.

Stage 3: Posterior foregut (3 days):

10. After PBS rinse, cells are exposed to DMEM with B27 (100x), retinoic acid (RA, 2μM), Noggin (100ng/ml,), SANT1 (0.25μM), Vitamin C (Vc, 0.25mM), Pen/Strep (100x) and Glutamax (100x). Cells are fed with fresh medium every other day.

Tip: Note that RA is light sensitive.

Stage 4: Pancreatic progenitor (3-4 days):

11. After PBS rinse, cells are exposed to DMEM with B27 (100x), EGF (100 ng/ml) + Nicotinamide (NICO, 10 mM) + Noggin (100ng/ml) + Vc (0.25mM). Cells are fed with fresh medium every other day.

V-bottom plate based aggregation:

12. Stage-4 cells were rinsed with PBS and then incubated with Accutase for 10-15 min at 37°C.

13. Dissociated single cells were rinsed twice with DMEM/F12 and spun at 300g for 3 min.

14. The resulting cells were re-suspended in aggregation medium (made of 5a-Medium supplied with 10μM Y27632).

15. Cell solution with 0.1-0.4 (according to experimental design) million cells was added into each well of V-bottom 96-well plate, followed by a spun at 300g for 3 min.

16. The plate was put into 37°C incubator for 8-12 hours for cluster formation.

Tip: If cells do not settle to the bottom of plate, centrifuge again by 500g for 3min. This step does not affect the cell viability.

Stage 5: Posing pancreatic progenitor for generation of β cells (4 days):

17. Pancreatic progenitor clusters assembled in V-bottom 96-well plate were collected and rinsed twice using DF12 medium, and loaded on 6-well air-liquid interface trans-well.

18. About 1.3ml stage-5 medium (which will enhance the propensity of pancreatic progenitor for generation of β cells) was added for each well.

19. Cells are fed with fresh medium every other day.

Tip: For transferring the clusters to the air-liquid interface, we use the wide bore 200ul pipette tips.

Stage 6: Endocrine progenitor (6 days).

20. Clusters were rinsed in DF12 and transferred to new trans-well. About 1.3ml Stage-6 medium was added for each well.

21. Cells are fed with fresh medium every other day.

Stage 7: Immature β cells (12days):

22. Clusters were rinsed in DF12 and transferred to new trans-well.

23. About 1.3ml Stage-7 medium was added for each well.

24. Cells are fed with fresh medium every other day.

Stage 8: Functional β cells (12 days):

25. Clusters were rinsed in DF12 and transferred to a new trans-well.

26. About 1.3ml Stage-8 medium was added for each well.

27. Cells were fed with fresh medium every other day.

Troubleshooting

1. The hPSCs are heterogeneous and show early sign of differentiation. As this issue will greatly affect the final efficiency of β cell differentiation, so it must be confirmed before determining whether to proceed or not. If only a few of colonies are differentiated (flattened, enlarged in cell size, or having neurites-like branches), the culture can still be rescued by removing those “bad colonies” using pipette tips before splitting. Repeat this procedure until the whole culture is homogeneous. If too many hPSCs colonies show the propensity of differentiation, start again with a new vial of cells and re-establish the culture.

2. Some hPSC lines may grow a little slowly than others and cannot reach 90% confluence two days after passage (before using for differentiation). Two solutions: (1) Increasing the initial seeding density; (2) Lowering the Activin-A concentrations. Instead of the typical 115-110-100 ng/ml gradient for stage-1, use a gradient of 110-105-95 ng/ml.

3. The final efficiency of generating β cell is not high. There are many possible reasons. (1) The hPSCs are not in high quality. To fix this problem, use a new culture or a new cell line with high quality. (2) The starting point cell confluence (right before stage 1) is not ideal. We typically use ~90% cell confluence to start differentiation. If the confluence is lower than that, the efficiency of NKX6.1+/PDX1+ cells at stage 4 will be low, and the production of β cells at the final stage will be greatly reduced. If the starting point cell confluence is too high, cell differentiation at stage 1 may not be complete. The percentage of definitive endoderm cells at stage 1 will be lower than expected, and thus the β cell efficiency at the final stage will be lower. (3) Reagent related issues, such as quality issues, preparation issues and storage issues, could cause inconsistent experimental results across different experimental batches. Here are some examples: Activin-A (and possibly other growth factors used in the protocol) from different vendors or produced by different approaches (bacteria or eukaryotic cells) or from different batches may have different potency. Therefore, it is better to order a large batch of Activin-A from a certain vendor, aliquot it after dissolving it, and store it at -80°C for long-time usage. (4) Taking the cell culture out of the incubator too often or for too long. Avoid taking the culture dish (with cells) out of the incubator too often (for example to check the cell status), as it would negatively affect the final result. Especially, such practice will significantly increase the cell detachment at stage 1. One possible solution is to prepare two culture replicates with one for checking cell status and the other one for the actual experiment.

Time Taken

1. hPSCs preparation: (two-weeks)
2. Stage 1: Definitive Endoderm (3 days).
3. Stage 2: Primitive gut tube (3 days).
4. Stage 3: Posterior Foregut (3 days).
5. Stage 4: Pancreatic progenitor (3-4 days).
6. V-bottom plate based aggregation: (half day)
7. Stage 5: Posing pancreatic progenitor for generation of β cells (4 days).
8. Stage 6: Endocrine progenitor (6 days).
9. Stage 7: Immature β cells (12 days).
10. Stage 8: Functional β cells (12 days):

Anticipated Results

1. HPSCs preparation. HPSCs should show homogeneous morphology (typical stem cell morphology and high nuclei/cytoplasm ratio). There should be very few (< 0.1%) or none differentiated hPSCs. After passaging and further culture, adherent hESCs should reach ~90% confluence within about 2 days (before the first stage differentiation).

2. Stage 1, definitive endoderm. There will be some cells detaching from the dish within these 3 days, and at least ~80-90% of cells will still attach to the dish. At the end of this stage, we should see cells with typical definitive endoderm morphology covering the whole dish bottom. More than 90% (and frequently ~95%) of the cells are expected to be definitive endoderm cells (determined by immunostaining for the expression of definitive endoderm markers FOXA2 and SOX17).

3. Stage 2, primitive gut tube. Detachment of cells from the dish bottom ceases and cells gradually regain the propensity of proliferation. At the end of this stage, ~90% of the cells are expected to be positive for both HNF1B and HNF4A.

4. Stage 3, posterior foregut. Cells proliferate quickly and gradually become dense. At the end of this stage, ~90% of the cells are expected to express PDX1.

5. Stage 4, pancreatic progenitor. Cells gradually reduce proliferation. At the end of this stage, >80% of the cells are expected to be PDX1+/NKX6.1+.

6. Making pancreatic progenitor 3D-clusters in V-bottom plate. After 8-12 hours incubation, each well will form a 3D-cluster. There might be some cells (~1% - 5%) not integrating into the clusters, which will not affect the later stage of cell differentiation. The percentage of PDX1+/NKX6.1+ cells will be comparable to that of stage 4.

7. Stage 5, posing pancreatic progenitor for generation of β cells. After the treatment, the clusters will become a little more bright and compact. The percentage of PDX1+/NKX6.1+ cells will be comparable to that of stage 4. Typically less than 3% NEUROD1+ cells (representing endocrine related cells) should be observed. Few cells might detach from the clusters at this stage.

8. Stage 6, endocrine progenitor. Most NKX6.1+ cells would become NKX6.1+/NEUROD1+ cells. The morphology of the cluster will not change too much. Few cells might detach from the clusters at this stage.

9. Stage 7, immature β cells. At the end of this stage, about ~55%-75% cells would be NKX6.1+/INS+. Cells can weakly respond to glucose stimulation.

10. Stage 8, functional β cells. At the end of this stage, about ~60%-80% cells would be NKX6.1+/INS+. Cells could respond to glucose stimulation. The stimulation index (high glucose over low glucose) ranges from 2 to 4.5 folds.

References

1. Baeyens, L. *et al.* (RE) generating Human B Cells: Status, Pitfalls, and Perspectives. *PHYSIOL REV* **98**, 1143-1167 (2018).

2. Ghazizadeh, Z. *et al.* ROCKII inhibition promotes the maturation of human pancreatic beta-like cells. *NAT COMMUN* **8**, 298 (2017).

3. Jiang, W. *et al.* In vitro derivation of functional insulin-producing cells from human embryonic stem cells. *CELL RES* **17**, 333-344 (2007).

4. Kroon, E. *et al.* Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *NAT BIOTECHNOL* **26**, 443-452 (2008).

5. Kushner, J.A., MacDonald, P.E. & Atkinson, M.A. Stem Cells to Insulin Secreting Cells: Two Steps Forward and Now a Time to Pause? *CELL STEM CELL* **15**, 535-536 (2014).

6. Pagliuca, F.W. *et al.* Generation of Functional Human Pancreatic β Cells In Vitro. *CELL* **159**, 428-439 (2014).

7. Rezania, A. *et al.* Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. *NAT BIOTECHNOL* **32**, 1121-1133 (2014).

8. Russ, H.A. *et al.* Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro. *EMBO J* **34**, 1759-1772 (2015).

9. Vegas, A.J. *et al.* Long-term glycemic control using polymer-encapsulated human stem cell–derived β cells in immune-competent mice. *NAT MED* **22**, 306-311 (2016).

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