

TITLE:

Optimized Protocol for Generating Functional Pancreatic Insulin-secreting Cells from Human Pluripotent Stem Cells

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SUMMARY:

This article presents a protocol for directed differentiation and functional analysis of β -cell like cells. We describe optimal culture conditions and passages for human pluripotent stem cells before generating insulin-producing pancreatic cells. The six-stage differentiation progresses from definitive endoderm formation to functional β -cell like cells secreting insulin in response to glucose.

ABSTRACT:

Human pluripotent stem cells (hPSCs) can differentiate into any kind of cell, making them an excellent alternative source of human pancreatic β -cells. hPSCs can either be embryonic stem cells (hESCs) derived from the blastocyst or induced pluripotent cells (hiPSCs) generated directly from somatic cells using a reprogramming process. Here a video-based protocol is presented to outline the optimal culture and passage conditions for hPSCs, prior to their differentiation and subsequent generation of insulin-producing pancreatic cells. This methodology follows the six-stage process for β -cell directed differentiation, wherein hPSCs differentiate into definitive endoderm (DE), primitive gut tube, posterior foregut fate, pancreatic progenitors, pancreatic endocrine progenitors, and ultimately pancreatic β -cells. It is noteworthy that this differentiation methodology takes a period of 27 days to generate human pancreatic β -cells. The potential of

insulin secretion was evaluated through three experiments, which included immunostaining and glucose-stimulated insulin secretion.

INTRODUCTION:

Human pluripotent stem cells (hPSCs) have the unique ability to differentiate into various cell types, making them a viable alternative to human pancreatic β -cells¹. These hPSCs are categorized into two types: embryonic stem cells (hESCs), derived from the blastocyst², and induced pluripotent cells (hiPSCs), generated by reprogramming somatic cells directly³. The development of techniques to differentiate hPSCs into mature cell types, such as β -cells, has important implications for both research and, ultimately, clinical practice^{1,4}. Diabetes mellitus is a chronic disease affecting >400 million people worldwide and results from the inability of the body to regulate glycemia due to malfunction or loss of pancreatic β -cells⁵. The limited availability of pancreatic islet cells for transplantation has hindered the development of cell replacement therapies for diabetes^{2,4,6-7}. The ability to generate glucose-responsive insulin-secreting cells using hPSCs serves as a useful cellular model for studying human islet development and function. It can also be used to test potential therapeutic candidates for diabetes treatment in a controlled environment. Moreover, hPSCs have the potential to produce pancreatic islet cells that are genetically identical to the patient, reducing the risk of immune rejection after transplantation^{2,4,7}.

In recent years, there have been significant advancements in the refinement of hPSC culture and differentiation protocols, resulting in increased efficiency and reproducibility of the differentiation process toward generating pancreatic β -cells^{8,9}.

The following protocol outlines the essential stages of directed differentiation of pancreatic β -cells and incorporates targeted adjustments to specific cell signaling pathways at specific time points. It is based on the protocol developed by Sui L. et al.¹⁰ (2018) for the generation of hPSCs into pancreatic β -cells. The protocol was adjusted to recent updates from Sui L. et al.¹¹ (2021), as the latest research emphasizes the significance of using aphidicolin (APH) treatment to enhance the differentiation of β -cells. The current protocol includes the addition of APH to the medium during the later stages of the process. Furthermore, modifications have been made to the composition of the medium during the early stages of differentiation compared to the initial protocol. A notable change is the addition of Keratinocyte Growth Factor (KGF) on Day 6 and continuing until Day 8. This adjustment differs from the initial protocol¹⁰, where KGF was not included in the Stage 4 Medium, the specific use of KGF promotes the generation of β -cells by stimulating duct cell proliferation¹².

The first and essential step in the generation of β -cell-like cells is the directed differentiation of hPSCs into definitive endoderm (DE), a primitive germ layer that gives rise to the epithelial lining of various organs, including the pancreas. After the formation of DE, the cells undergo differentiation into the primitive gut tube, which is followed by the specification of the posterior foregut fate. The posterior foregut then develops into pancreatic progenitor cells, which have the potential to differentiate into all cell types of the pancreas, including the endocrine and exocrine cells. Pancreatic endocrine progenitors, which give rise to the hormone-secreting cells

of the islets of Langerhans, are the next step in the process. In the end, the differentiation process reaches its final stage by producing fully functional pancreatic β -cell like cells^{9,10}. It is important to note that this process is complex and often requires optimization of the culture conditions, such as specific growth factors and extracellular matrix components, to improve the efficiency and specificity of differentiation^{9,10}. Furthermore, generating functional β -cell like cells from hPSCs *in vitro* is still a major challenge. Ongoing research focuses on improving differentiation protocols and enhancing the maturation and function of the resulting β -cells⁹.

In this protocol, the use of gentle cell dissociation during the culture and passage of hPSCs is essential to maintain cell viability and pluripotency, significantly improving the efficiency of differentiation into pancreatic β -cells. Additionally, each stage-specific medium has been meticulously optimized following the protocol developed by Sui L. et al.¹⁰ to promote a high yield of insulin-secreting cells in clusters that closely resemble the human islet.

PROTOCOL:

Prior to initiating differentiation, it is recommended to determine the required number of islet-like organoids for experimental purposes. In a 6 well plate, a single well with over 80% confluency typically consists of 2-2.3 million hPSCs. While an accurate prediction is challenging due to variations in hPSC lines and differentiation efficiency, a rough estimate is 1.5 times the number of initial wells. An effectively directed differentiation usually yields 1.6 to 2 million cells per well in six-well plates, encompassing all cells within the clusters rather than exclusively insulin-producing cells. For a 50 μ m cluster, it can be approximated to contain around 10,000 cells. **Table 1** provides a summary of the media composition used for each day/stage of directed differentiation on top of stem cell matrix and medium, along with the glucose-stimulated insulin secretion buffer.

1. Passaging human pluripotent stem cells prior differentiation in 6 well plates

NOTE: The appropriate passaging of human stem cells prior to differentiating into β -cell-like cells is a crucial step in establishing the experimental process. Incorrect passage dilution or attachment cell number can compromise differentiation efficiency and fidelity.

1.1. Prepare stem cell culture medium, coating, and dissociation solutions (as specified in **Table 1**).

1.2. 1-2 h before passaging, coat a six-well plate with cold coating solution (1 mL per well) and incubate at 37 °C, 5% CO₂.

1.3. Warm an aliquot of stem cell culture medium at room temperature (15 -25 °C) for 20 min.

1.4. Aspirate the stem cell medium and add 1 mL of calcium/magnesium free D-PBS.

1.5. Repeat step 1.4. twice.

NOTE: D-PBS is added to wash the cells and remove the previous medium and compounds. It is important to note that no specific time frame is required for the washing steps, as exposure to D-PBS does not harm the hPSCs, preventing cell rupture or shrinkage caused by osmosis.

1.6. Aspirate D-PBS, add 500 μ L of dissociation solution for each well, and let it sit for 2–5 min at room temperature (15 – 25 °C).

1.7. While cells are dissociating, aspirate the coating solution of the new 6 well plate and add 1-2 mL of calcium/magnesium-free D-PBS to each well.

1.8. Regularly check the dissociation process under the inverted microscope.

1.9. Aspirate the dissociation solution when 80 - 90% of the cells have rounded but are still adherent.

1.10. Add 1 mL of the stem cell medium containing 10 μ M ROCK inhibitor.

1.11. Collect the dissociated cells into a 15 mL conical tube using 1,000 μ L sterile filtered pipette tips and a P1000 pipette.

1.12. Slowly triturate the cell suspension up and down in the pipette with a 1,000 μ L sterile pipette filtered tip to break the colonies, but do not exceed 10 times.

1.13. Add 1 mL of the stem cell medium containing ROCK inhibitor to help detach the remaining hPSCs and transfer the cell suspension to the same 15 mL conical tube (1.10).

1.14. Aspirate the D-PBS from the newly coated plate and immediately add the cell suspension from the 15 mL conical tube. To ensure optimal cell growth, seed a range of 2×10^5 – 1×10^6 viable cells per well when using a 6 well plate (1 in 10 to 1 in 50 splits).

NOTE: For the volume calculation, each well of a 6-well plate must contain 2 mL of stem cell medium with ROCK inhibitor.

1.15. Move the plate quickly back and forth, and side to side 5 - 10 times.

1.16. Place the plate at 37 °C, 5% CO₂ incubator for 24 h.

1.17. Replace with fresh stem cell medium without ROCK inhibitor the next day, and then every 2 days until the confluency of hPSCs has reached 80 - 95%.

2. Human stem cell-derived β -cells directed differentiation

NOTE: The hPSCs can be used for the direct differentiation process into pancreatic β -cells when 80-95% confluence is achieved.

2.1. Day -1: Passaging of the cells Day -1 of differentiation:

2.1.1. 1 - 2 h before passage, coat a 6 well plate with cold coating solution in a 37 °C, 5% CO₂ incubator for 1 h.

2.1.2. Follow steps 1.1 to 1.10 and proceed to counting cells.

2.1.3. Load 10 μ L of the cell mixture, add an equal volume of Trypan Blue, and gently mix.

2.1.4. Calculate the cell concentration. Use 0.8×10^6 cells/mL – 1.0×10^6 cells/mL of stem cell medium containing 10 μ M ROCK inhibitor to plate the coated 6 well plate with 2 mL of media per well.

2.1.5. Move the plate quickly back and forth, side to side three times.

2.1.6. Place the plate in a 37 °C, 5% CO₂ incubator. Quickly move the plate back and forth, and side to side again 10 times. Then incubate the plate for 4 h.

NOTE: Ensure that the maximum attachment and the even distribution of cells do not disturb the cells by moving the plate once placed in the incubator. Open and close the incubator very carefully.

2.1.7. Place the bottle of basal medium for Days 0 to 4 at 4 °C to thaw it overnight.

2.2. Day 0

NOTE: The hPSCs must be 80 - 95% confluent, do not start the differentiation process to definitive endoderm under that confluency.

2.2.1. On ice, thaw both the basal medium for Days 0 to 4 and supplements (see **Table of Materials**).

2.2.2. Prepare in two conical tubes only the necessary volume to be used on Day 1 (2 mL per well of a 6-well plate), and in a separate tube, double the volume for Day 2.5 to 4 (2 x 2 mL per well of a 6-well plate). Store the Day 2.5 to Day 4 medium at 4 °C.

2.2.3. Warm an aliquot of the necessary volume of the Day 1 medium and washing medium 1 in a 37 °C water bath for 5 min.

2.2.4. Aspirate the stem cell medium and add 2 mL of washing medium 1.

NOTE: The washing medium used in the protocol consists of the basal medium specific to each day/stage, supplemented with 1% antibiotics. The washing steps of the direct differentiation protocol only involve the addition of the designated washing medium (referred to as "washing medium 1 or 2", **Table of Materials**) and subsequent aspiration, following the described procedure 2.2.4. It is important to mention that there is no specific time frame indicated for these washing steps.

2.2.5. Aspirate washing medium 1 and immediately add 2 mL of the Day 1 medium to the side of the well.

2.2.6. Incubate the cells at 37 °C and 5% CO₂ for 24 h.

2.3. Day 1

2.3.1. Pre-warm an aliquot of the necessary volume of Days 2.5 to 4 medium in a 37 °C water bath for 5 min.

2.3.2. Aspirate the Day 1 medium and immediately add 2 mL of the Day 2.5 to 4 medium to the side of the well. Do not wash the cells.

2.3.3. Place the plate back at 37 °C and 5% CO₂ for 36 h.

2.4. Days 2.5 to 4

2.4.1. Pre-warm an aliquot of the remaining volume of the Day 2.5 to 4 medium in 37 °C water bath for 5 min.

2.4.2. Aspirate the medium and immediately add 2 mL of freshly prepared Days 2.5 to 4 medium to the side of the well.

2.4.3. Place the plate back at 37 °C and 5% CO₂ for 36 h.

2.5. Day 4

NOTE: At this stage, the expression of the definitive endoderm markers is at its maximum, and cells can initiate primitive gut tube differentiation.

2.5.1. Prepare an aliquot of the necessary volume of the Day 4 primitive gut stage medium (2 mL per well of a six-well plate) and warm it at room temperature (15 °C -25 °C) for 20 min.

2.5.2. Aspirate Days 2.5 to 4 medium and add 2 mL of washing medium 1.

2.5.3. Aspirate the washing medium 1, and immediately add 2 mL of Day 4 primitive gut stage to the side of the well.

263
264 2.5.4. Place the plate back at 37 °C and 5% CO₂ for 48 h.

265
266 2.6. Days 6 to 8

267
268 NOTE: At this stage, the cells initiate further differentiation onto posterior foregut fate.

269
270 2.6.1. Prepare the necessary volume of Days 6 to 8 posterior foregut medium (2 mL per well of
271 a 6 well plate) and warm the medium at room temperature (15 °C -25 °C) for 20 min.

272
273 2.6.2. Aspirate the Day 4 medium and immediately add 2 mL of Days 6 to 8 posterior foregut
274 medium to the side of the well.

275
276 2.6.3. Place the plate at 37 °C incubator containing 5% CO₂ for 48 h.

277
278 2.7. Days 8 to 12

279
280 NOTE: The cells are ready to undergo pancreatic progenitors' differentiation.

281
282 2.7.1. Prepare the necessary volume of Days 8 to 12 pancreatic progenitors stage medium (2 mL
283 per well of a 6 well plate) and warm the medium at room temperature (15 °C -25 °C) for 20 min.

284
285 2.7.2. Aspirate Days 6 to 8 medium and immediately add 2 mL of Days 6 to 8 posterior foregut
286 medium to the side of the well.

287
288 2.7.3. Place the plate in an incubator at 37 °C containing 5% CO₂ for 48 h.

289
290 2.8. Day 12: Performing the clustering step

291
292 NOTE: At this stage, the cells form a dense monolayer, and will be transitioned to 3D cell culture
293 to form clusters. This step is crucial for the differentiation to enhance the structural resemblance
294 of β -like cells to native human islets but also improves their functional resemblance at both the
295 cellular and cluster levels¹¹ (**Figure 1**).

296
297 2.8.1. Treat a 6 well plate containing microwells that are 400 μ m (as specified in the **Table of**
298 **materials**: Human Stem Cell-Derived β -cells Directed Differentiation, Day 12) with 2 mL of anti-
299 adherence rinsing solution at room temperature (15 °C – 25 °C).

300
301 NOTE: The microwell prevents cells from attaching to the well bottom and facilitates the
302 mechanical formation of 3D clusters.

303
304 2.8.2. Centrifuge plate at 1,300 x g for 5 min.

305

2.8.3. Check for the presence of any bubbles under an inverted microscope. If no bubbles are observed, aspirate anti-adherence rinsing solution and immediately add 2 mL of washing medium 2 (**Table of materials**).

2.8.4. Repeat step 1.8.3. two times.

2.8.5. Prepare the necessary volume of the cluster medium and warm it at room temperature (15 -25 °C) for 20 min. Ensure that two wells of a 6 well plate go into one well of the 400 µm microwells 6 well plate with 4 mL of the cluster medium.

2.8.6. Aspirate pancreatic progenitor medium and add dissociation buffer (0.5 mL per well).

2.8.7. Incubate at room temperature (15 °C – 25 °C) for 2-5 min. Regularly check the dissociation process under an inverted microscope and aspirate dissociation buffer when most of the cells have rounded but are still adherent.

2.8.8. Aspirate the dissociation buffer and immediately add 1 mL cluster medium to each well.

2.8.9. Dissociate cells by gently pipetting up and down six times using a P1000 pipette and 1,000 µL filter tips.

2.8.10. Transfer the cells and the cluster medium mixture into a 50 mL conical tube.

2.8.11. Add 1 mL of the cluster medium to collect all remaining cells.

2.8.12. Add the cluster medium to the total volume needed so each well of the microwells plate has 4 mL of mixture cluster medium/cells.

2.8.13. Aspirate the washing medium 2 from microwells 6-well plate and transfer the cell suspension. Incubate the plate at 37 °C for 24 h.

2.9. Day 13: Handling the cells post-clustering and changing their media from Day 13 onward.

NOTE: Clusters are highly sensitive and require careful handling to ensure their integrity and viability in the following days. Therefore, it is crucial to closely monitor the clusters during the post-clustering period after Day 12. Regular evaluation and adjustments are essential to promote successful outcomes in the experimental process.

2.9.1. Prepare the necessary volume of Days 13 medium (2 mL per well of a 6 well plate), and in a separate conical tube of 50 mL for Day 15 to 20 pancreatic endocrine progenitor medium.

2.9.2. Slowly collect clusters from the microwells 6 well plate into a 50 mL conical tube using a p1000 pipette and 1,000 µL filtered tips.

2.9.3. Add 1 mL of Day 13 medium to collect the remaining clusters and transfer the clusters into the tube.

2.9.4. Let the cells set naturally under gravity to the bottom of the conical tube for 5 min.

2.9.5. Aspirate as much supernatant as possible from the tube without disturbing the cell clusters. The pipette tips should not touch nor aspirate the clusters but the supernatant only.

2.9.6. Add the necessary volume of Day 13 medium according to the following: 1 well of microwells 6 well plate goes into three wells of a low-attachment 6 well plate (2 mL of media per well).

2.9.7. Gently pipette up and down, avoiding aspirating clusters.

2.9.8. Transfer the suspension to a very low-adherent 6 well plate.

2.9.9. Move the plate quickly back and forth, side to side six times.

2.9.10. Incubate the plate at 37 °C for 48 h.

2.9.11. Perform the medium changes in all the next steps until the end of the differentiation as described in this section 2.9.

2.10. Days 15 to 20

2.10.1. Change to pancreatic endocrine progenitor stage medium every 48 h until day 21 of differentiation by collecting the cell suspension into a 50 mL conical tube and following steps 2.9.4 to 2.9.10 for Day 13.

2.11. Days 21 to 27

NOTE: At this stage, clusters differentiate onto pancreatic β -cells.

2.11.1. Prepare pancreatic β -cell stage medium.

2.11.2. Change the medium every other day as described for days 15 to 21.

NOTE: After Day 25, the islet-like organoids can undergo analysis to confirm they are fully functional.

3. Staining of pancreatic β -cell clusters

NOTE: Perform this step to study the functional assessment of clusters post-differentiation

- 3.1. Collect five to ten clusters in a 1.5 mL microcentrifuge tube at the end of differentiation.
 - 3.2. Fix the cell clusters with 200 μ L of 4% PFA for 15 min at room temperature.
 - 3.3. Add 1 mL of PBS to the clusters and remove the PBS with a 1,000 μ L pipette tip without disturbing the clusters.
 - 3.4. Add 200 μ L of 30% sucrose to the clusters and incubate overnight at 4 $^{\circ}$ C.
 - 3.5. Transfer the clusters to a cryomold, remove extra sucrose, add a drop of O.C.T. medium, and mix the clusters with O.C.T. medium.
 - 3.6. Freeze the cryomold on dry ice and store at -80 $^{\circ}$ C.
 - 3.7. Cut 5 μ m sections from the frozen block on microscope slides using a microtome/or cryostat.
 - 3.8. Store slides at -80 $^{\circ}$ C freezer until staining.
 - 3.9. On the day of staining, take the slides out of the -80 $^{\circ}$ C freezer.
 - 3.10. Carefully remove the ice around the cryomold's sections.
 - 3.11. Circle cell clusters on slides with a hydrophobic pen.
 - 3.12. Rehydrate slides in a slide staining jar containing PBS for 15 min.
 - 3.13. Add cold methanol to the slide staining jar and place the jar with slides at -20 $^{\circ}$ C for 10 min.
 - 3.14. Immerse slides in a PBS jar.
 - 3.15. Repeat step 3.14 three times.
- NOTE: Use this method for all the following washing steps.
- 3.16. Remove PBS from the slides and cover immediately with 100 μ L of blocking solution with 2-5% BSA in PBS-T.
 - 3.17. Add blocking solution to each slide and cover with paraffin film.
 - 3.18. Incubate for 1 h at room temperature (15 -25 $^{\circ}$ C).

3.19. Dilute the primary antibodies in the blocking solution using the ratios provided in the Reagents and Solutions section.

3.20. Remove the blocking solution from the slides.

3.21. Immediately add diluted antibodies and cover slides with parafilm to prevent the slide from getting dry.

3.22. Incubate overnight at 4 °C.

3.23. The following day wash the slides 3 - 5 times with PBS-T.

3.24. Prepare diluted secondary antibodies and DNA stain.

3.25. Remove excess PBS-T from the slides.

3.26. Add diluted secondary antibodies and DNA stain. Incubate for 45 min at room temperature (15 -25 °C).

3.27. Wash the slides 3 - 5 times with PBS-T.

3.28. Remove any liquid from the slides.

3.29. Add a drop of histology mounting medium to each section.

3.30. Cover the slide with a glass cover.

3.31. Take pictures of stained slides using a fluorescent microscope.

4. GSIS (glucose-stimulated insulin secretion assay)

4.1. Prepare three different solutions: low glucose KREBS solution, high glucose KREBS solution, and low glucose KREBS solution with KCl.

4.2. Warm all the solutions at 37 °C water bath.

4.3. Carefully select around 10 - 15 clusters of similar size using a 1000 µL pipette tip and transfer them into a 1.5 mL tube along with a small amount of differentiated medium to avoid drying out the clusters.

NOTE: The clusters should be visible to the naked eye, but if not, place the plate containing differentiated clusters under an inverted microscope to select the clusters and transfer them to a 1.5 mL tube.

4.4. Place the tube with clusters and medium on a tube rack and wait for the clusters to sink to the bottom of the tube.

4.5. Slowly aspirate the supernatant using a 200 μ L pipette and add 200 μ L of low glucose KREBS solution.

4.6. Incubate the islets in the low glucose solution for 1 h at 37 °C.

NOTE: To ensure the accurate assessment of cluster numbers and consistency in experiments, it is advisable to check for the presence of all clusters in the solution during transfer, as they tend to adhere to the tube.

4.7. Remove the tube from the incubator and slowly aspirate 200 μ L of KREBS solution without aspirating any clusters.

4.8. Add 200 μ L of low glucose KREBS solution and incubate for 30 min at 37 °C.

4.9. Aspirate the volume of 200 μ L of low glucose KREBS solution into a 500 μ L tube and immediately store it at -80 °C for insulin measurements. Repeat for separate treatments.

4.10. To wash the clusters, add 200 μ L of KREBS solution without glucose to the tube containing the clusters. Allow the clusters to settle to the bottom of the tube, and carefully remove the supernatant without disturbing the clusters.

4.11. Add 200 μ L of high glucose KREBS solution and incubate for 30 min at 37 °C.

4.12. Aspirate 200 μ L of high glucose KREBS into a 500 μ L tube and immediately store it at -80 °C for insulin measurement. Repeat for separate treatments.

4.13. To wash the clusters, add 200 μ L of KREBS solution without glucose to the tube containing the clusters. Allow the clusters to settle to the bottom of the tube, and carefully remove the supernatant without disturbing the clusters.

4.14. Add 200 μ L of KCl KREBS solution and repeat for separate treatments.

4.15. Incubate for 30 min at 37 °C.

4.16. Aspirate 200 μ L of KCl KREBS solution into a 500 μ L tube and immediately store it at -80 °C for insulin measurements. Repeat for other treatments.

4.17. Add 50 μ L of ultrapure water and proceed to measure total insulin content.

4.18. Add 150 μ L of acid ethanol solution to the tube with islets and ultrapure water.

4.19. Store samples at 4 °C overnight (12 - 15 h).

4.20. The following day, vortex each sample.

4.21. Perform sonication with an electric sonicator set to 20% power for 1 s to ensure complete lysis of the islet tissue.

NOTE: Repeat step 4.21 until there are no remaining clusters visible.

4.22. Centrifuge all samples at 10,000 × *g* for 10 min and keep the supernatant.

4.23. Measure DNA concentration using a nanodrop spectrophotometer, which can then be used to normalize insulin measurements.

REPRESENTATIVE RESULTS:

The protocol described in this paper offers a highly efficient approach for differentiating β-like cells from hPSCs¹⁰. This process utilizes a 2D culture system that is easily scalable, enabling its use in various experimental settings, such as learning differentiation, smaller projects and laboratories, and pilot tests to assess the potential of an iPSC line for differentiation.

It is essential to characterize the functional properties of differentiated β-cells in islets to gain insight into glucose homeostasis. This is typically achieved through various experiments such as immunostaining for β-cell markers and insulin expression, as well as glucose-stimulated insulin secretion (GSIS) assays, which test islet function in response to low and high glucose concentrations^{13,14}. β-cells possess signature genes, including *Nkx2-2*, *Pdx1*, *Nkx6-1*, and *Neurod1*, which are critical for establishing and maintaining β-cell identity⁹. Immunostaining techniques are increasingly valuable for investigating protein expression and localization within tissue sections. Immunostaining for β-cell markers can assess the expression levels of key pancreatic lineage markers, providing insights into the differentiation process's fidelity and optimization for specific applications^{9,13}.

In this study, the Mel1 InsGFP/w (Mel1 INS-GFP) (15) hESC reporter line was used to differentiate clusters comprising different cell types, including β-cells resembling those found in human native islets. **Figure 2** in this paper offers significant findings regarding the efficiency and accuracy of the differentiation process. The results demonstrate a high enrichment of insulin-expressing cells within the pancreatic lineage, and these cells exhibit glucose-stimulated insulin secretion. This indicates the successful generation of functional β-like cells through the differentiation process. The differentiated cells were stimulated with low and high glucose concentrations, and GSIS results showed that the clusters derived from Mel1 cells functioned similarly to islets in their insulin secretion response to glucose. The Mel1-derived clusters were found to secrete 100-fold more insulin in response to high glucose concentrations compared to low glucose concentrations. Specifically, the insulin content was 0.003 ± 0.002% at 3.3 mM low glucose and 0.236 ± 0.197% at 16.7 mM high glucose.

The clusters derived from Mel1 INS-GFP hESCs were subjected to further analyses to determine their composition and functionality, in addition to the GSIS assay. Specifically, the expression of β -cell signature genes and the presence of different cell types within the clusters were investigated. To assess the differentiation efficiency, more than 18,000 cells were counted across three separate differentiations from 9 islets. The results showed that the pancreatic lineage obtained from this process is highly enriched in insulin-positive cells, indicating a high level of success in the differentiation process of hESCs into β -cell-like cells. Furthermore, the expression of signature genes, such as Nkx6.1 and Pdx1, important for the establishment and maintenance of β -cell identities, were examined. The analysis revealed that approximately 25% and 40% of cells expressed Nkx6.1 and Pdx1, respectively, providing additional evidence that the clusters contained differentiated β -like cells (mean Nkx6.1+ cells per cluster $24.9\% \pm 6.2\%$, n=9 clusters, Pdx1+ cells $40.2\% \pm 6.2\%$, n=9, SEM, **Figure 2**). Additionally, the clusters contained other cell types, such as glucagon-positive cells, which accounted for around 15% of the total cell population. These cells are typically found in alpha cells of native islets of Langerhans, suggesting that the clusters closely resemble human islets in terms of cell composition.

FIGURE and TABLE LEGENDS:

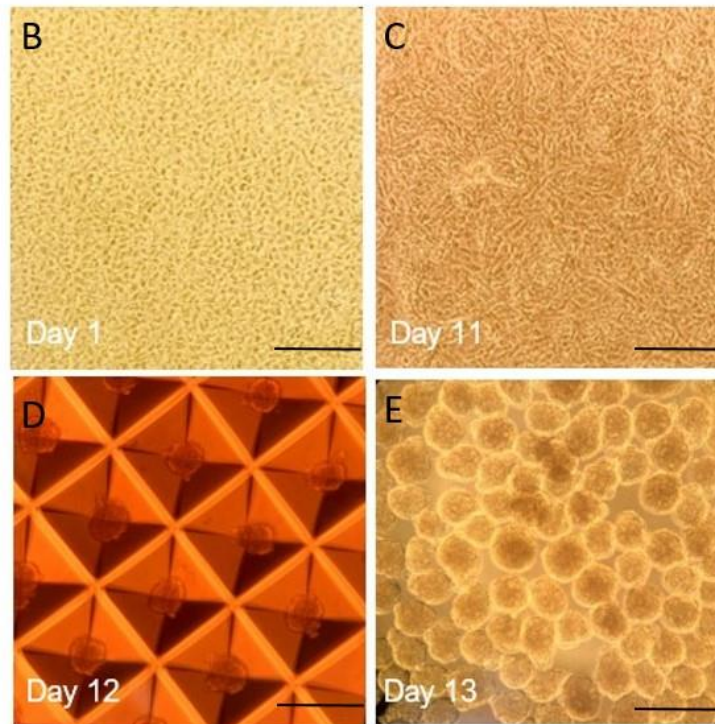
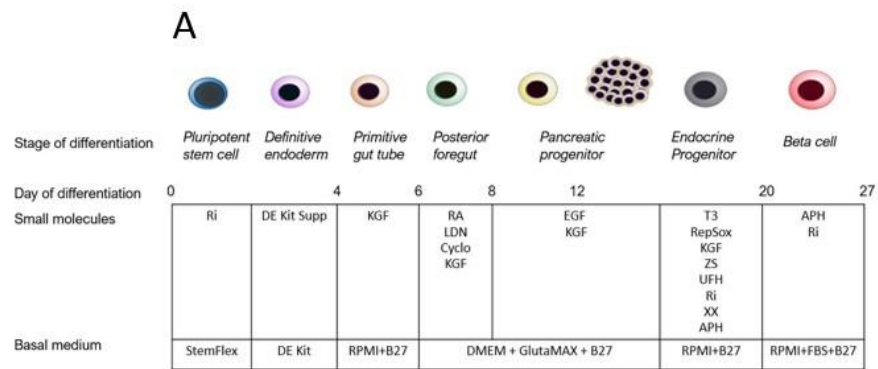


Figure 1: Differentiation of hPSCs towards pancreatic β -cells. (A) Schematic representation of the *in vitro* directed differentiation of hPSCs into pancreatic β -cells, which involves six successive stages: definitive endoderm induction, primitive gut tube formation, posterior foregut fate specification, pancreatic progenitor generation, pancreatic endocrine progenitor formation, and ultimately, pancreatic β -cell differentiation. Pancreatic β -cell differentiation uses key stages of human islet development, with the regulation of specific cell signaling pathways at specific times. B27: B-27 Supplement; Ri: rho-associated protein kinase inhibitor or ROCK inhibitor; T3: thyroid hormone; KGF: human KGF / FGF-7 protein; RepSox: Activin/Nodal/TGF- β pathway inhibitor; Inhibits ALK5; RA: Retinoic acid; ZS: zinc sulfate; UFH: unfractionated heparin; XX: gamma-Secretase Inhibitor XX; APH: aphidicolin; EGF: epidermal growth factor; LDN: BMP Inhibitor III, LDN-212854; Cyclo: Cyclopamine- KAAD. (B) Images of cellular morphology captured at various stages of differentiation from pluripotent stem cells to pancreatic β -cells. The first image shows human pluripotent stem cells on the first day of differentiation (monolayer of HPSCs). (C) On Day 11, cells are in the pancreatic progenitor stage. Scale bar of 100 μ m. (D) On day 12, clusters are formed in the microwells of 6-well plate after the dissociation of cells at the pancreatic progenitor stage. (E) On day 13, clusters are in a low-attachment 6-well plate. Scale bar of 100 μ m.

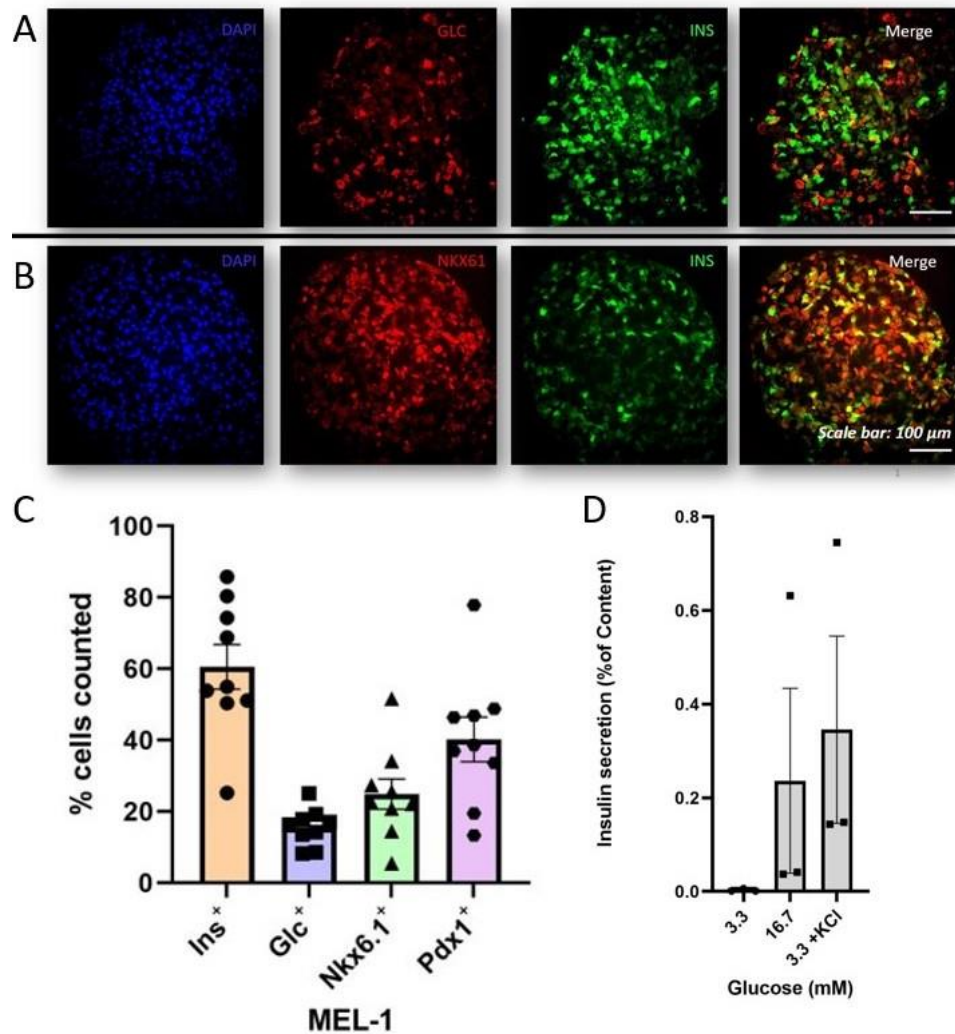


Figure 2: Clusters obtained from differentiated Mel1 InsGFP/w hESC reporter line¹⁵ were evaluated for the presence of insulin-producing cells expressing β -cell maturity markers. (A) Immunofluorescence images of the clusters were captured from cryomold sections (5 μ m) using spinning disk confocal microscopy, which revealed the predominance of insulin-producing cells (approximately 60%) compared to glucagon-producing cells (approximately 15%) (n=9 clusters, approximately 18,000 cells, SEM). **(B)** Immunofluorescence images of the clusters were obtained from cryomold sections (5 μ m) using spinning disk confocal microscopy, which showed the predominance of insulin-producing cells co-expressing the pancreatic β -cell markers Nkx6.1 (n=9 clusters, approximately 18,000 cells). **(C)** ImageJ cell counter macro, specifically designed for the immunostaining of markers, was employed to determine the percentage of insulin-positive, glucagon-positive, and β -cell markers Nkx6.1 positive cells, and β -cell markers Pdx1 positive cells. **(D)** The glucose-stimulated insulin secretion of Mel1 InsGFP/w hESC derived clusters was evaluated, which exhibited an increase of 100-fold in response to high glucose stimulation (16.7mM glucose) in the differentiated clusters (n=9, SEM).

DISCUSSION:

The successful differentiation of hPSCs into pancreatic β -cells depends on optimizing all aspects of routine culturing and passage of the selected hPSCs. This includes ensuring that the cell line has a normal karyotype, is negative for mycoplasma infection, and is free of plasmid or viral vector genomes. Furthermore, when using hiPSCs, it is important to avoid using the earliest passage which are still undergoing reprogramming, for pilot experiments. These experiments should be conducted on a small scale to identify the hPSC line with the best differentiation potential and the optimal number of passages.

Other parameters that can influence differentiation efficiency include the quality of the stem cell medium used, the coating density, and the number of passages^{10,13}. This protocol has been optimized to maximize the efficiency of differentiation by ensuring that all relevant parameters are optimized¹⁰.

Differentiation media with specific formulations are used at each stage to support the differentiation of hPSCs into β -cells. Activin A and a Wnt agonist are used in the differentiation medium to initiate the transition to definitive endoderm cells. During the primitive gut tube stage, KGF is added to the medium to promote further differentiation into β -cells¹², and this inclusion of KGF is maintained from day 6 to 8, differing from the original protocol by Sui, Egli, et al.¹⁰. During the pancreatic progenitor stage, the specific media composition is optimized to enhance the expression of the PDX1 transcription factor. This is achieved by using a high concentration of retinoic acid (RA), KGF, and LDN193189, which inhibits the bone morphogenetic protein (BMP) pathway⁸. As the differentiation progresses to the endocrine stage, the culture medium is modified to downregulate Notch signaling. This is achieved by incorporating XXI, a γ -secretase inhibitor, along with T3 (thyroid hormone), RA, and RepSox, an inhibitor of the Activin/BMP/TGF- β pathway⁸. This specific combination of compounds is sustained to promote the differentiation of endocrine progenitors into pancreatic progenitors. Finally, to optimize the direct differentiation process, aphidicolin (APH) is introduced during the differentiation from endocrine progenitors to pancreatic progenitors. This addition of APH aims to further enhance β -cell differentiation, and it represents a distinct modification from the initial protocol proposed by Sui, Egli, et al.^{10,11}.

During the differentiation process, it is crucial to monitor cell density and prevent over-confluence, as this can hinder proper differentiation. High-density cultures can maintain high Oct4 expression, inhibiting differentiation to definitive endoderm. Removing the ROCK inhibitor during the first washing step is essential for initiating differentiation and allowing the pluripotent state of hPSCs to be altered. Using a fluorescence marker, such as Mel1 INS-GFP with a GFP integrated at the insulin locus, facilitates the assessment of differentiation progress at the pancreatic progenitor and β -cell stages, aiding downstream experiments¹⁵.

The current protocol for differentiating human pluripotent stem cells into pancreatic β -like cells has demonstrated variability in efficiency among different hPSC lines¹⁰. Additionally, the resulting β -like cells exhibit functional immaturity compared to human pancreatic islets, showing lower insulin secretion per cell. To address this limitation further, *in vivo* maturation of β -like cells can

be achieved by transplantation of islet organoids into animal models during the final stages of differentiation^{6,7}.

Despite these limitations, the differentiation of hPSCs into pancreatic β -cells has significant potential with respect to existing methods^{8–10}. This technique allows to produce large numbers of β -cell-like cells that are responsive to glucose and express β -cell markers (*Pdx1* and *Nkx6.1*, see **Figure 2**). This is done without the ethical, technical, and source limitations associated with the use of human pancreatic islets. In addition, this technique has the potential to be applied to personalized medicine, as patient-specific β -cells can be generated for drug testing and disease modeling^{4,6–7}. The technique may also have future applications in treating diabetes that involve the loss or dysfunction of pancreatic β -cells^{4,6–7}.

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