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Generation of insulin-producing pancreatic β cells from multiple human stem cell lines

Nathaniel J. Hogrebe[1,†], **Kristina G. Maxwell**[1,2,†], **Punn Augsornworawat**[1,2,†], **Jeffrey R. Millman**[1,2,*]

[1]Division of Endocrinology, Metabolism and Lipid Research, Washington University School of Medicine, St. Louis, MO 63110, USA

[2]Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, MO 63130, USA

Abstract

We detail a 6-stage planar differentiation methodology for generating human pluripotent stem cell (hPSC)-derived pancreatic β cells (SC-β cells) that secrete high amounts of insulin in response to glucose stimulation. This protocol first induces definitive endoderm by treatment with Activin A and CHIR99021, then generates PDX1+/NKX6–1+ pancreatic progenitors through the timed application of keratinocyte growth factor (KGF), SANT1, TPPB, LDN193189, and retinoic acid (RA). Endocrine induction and subsequent SC-β cell specification is achieved with a cocktail consisting of the cytoskeletal depolymerizing compound latrunculin A combined with XXI, T3, ALK5 inhibitor II, SANT1, and RA. The resulting SC-β cells and other endocrine cell types can then be aggregated into islet-like clusters for analysis and transplantation. This differentiation methodology takes approximately 35 days to generate functional SC-β cells, plus an additional 1–2 weeks for initial stem cell expansion and final cell assessment. This protocol builds upon a large body of previous work for generating β-like cells. In this iteration, we have eliminated the need for three-dimensional culture during endocrine induction, allowing for the generation of highly functional SC-β cells to be done entirely on tissue culture polystyrene (TCP). This change simplifies the differentiation methodology, requiring only basic stem cell culture experience as well as familiarity with assessment techniques common in biology labs. In addition to expanding protocol accessibility and simplifying SC-β cell generation, we demonstrate that this planar methodology is amenable for differentiating SC-β cells from a wide variety of cell lines from various sources, broadening its applicability.

EDITORIAL SUMMARY

*Corresponding author. jmillman@wustl.edu.

AUTHOR CONTRIBUTIONS

N.J.H., K.G.M., and P.A. collected data for all experiments. N.J.H., K.G.M., P.A., and J.R.M. designed the experiments, wrote the manuscript, and revised the manuscript.

†These authors contributed equally

PROPOSED TWEET A new protocol from Millman and colleagues describes a planar culture differentiation protocol for generating functional pancreatic beta cells from human pluripotent stem cell lines. #diabetes @JeffreyRMillman @WUSTLmed

COMPETING INTERESTS

N.J.H. and J.R.M. are inventors on patents and patent applications related to the SC-β cell differentiation protocol described in this manuscript.

Millman and colleagues describe a 6-stage monolayer culture differentiation protocol for generating insulin-secreting pancreatic beta cells from a variety of human pluripotent stem cell lines and outline steps for in vitro functional assessment.

Abstract

PROPOSED TEASER Generation of insulin-producing pancreatic β cells

INTRODUCTION

Diabetes results from the loss or dysfunction of insulin-producing β cells within the pancreas, leading to deregulation of glucose homeostasis and the requirement for daily exogenous insulin injections. Successful transplantation of cadaveric islets using the Edmonton protocol has provided a proof-of-principle for a cell-based therapy, demonstrating that inserting mature β cells into a diabetic patient can provide a functional cure for insulin-dependent diabetes.[1,2] Widespread success of this cell therapy depends upon generating a reliable supply of highly functional β cells, however, as cadaveric islets are limited and often have decreased function upon isolation from donors. To this end, substantial progress has been made in the development of stem cell-derived β (SC-β) cells, providing a renewable supply of insulin-producing cells for diabetic disease modeling and cell therapy.

This road to a robust protocol for generating functional SC-β cells has been a gradual process resulting from the excellent work of various research groups developing sequential differentiation stages, each of which utilize the timed application of growth factors and small molecules to mimic developmental cues. The first step of this process specifies definitive endoderm from stem cells, which was first worked out in 2005 by D'Amour et al.[3] and then further refined.[4–10] Subsequent stages of differentiation drive these endodermal cells through a primitive gut tube intermediate to form PDX1+ pancreatic progenitors.[4] While insulin positive cells could originally be generated from these pancreatic progenitors *in vitro*, their insulin secretion was limited, they often also expressed the hormones glucagon or somatostatin, and they lacked other key features of β cells.[5,11] When PDX1-expressing pancreatic progenitors were transplanted into mice or rats, however, they matured over the course of several months into insulin positive cells that were glucose-responsive,[12–15] illustrating the potential of these PDX1+ pancreatic progenitors to produce genuine β cells. An important advance from these transplantation studies was the realization that functional SC-β cells arise from pancreatic progenitors that express the transcription factor NKX6–1.[16,17] Other studies have further illustrated the importance of NKX6–1 in β cell development,[18–20] demonstrating that turning on the transcription factor NEUROG3, which drives all endocrine specification, before NKX6–1 directs cells toward a polyhormonal state that ultimately produces α cells.[21–23]. A breakthrough occurred in 2014 and 2015 when several groups published protocols for specifying functional SC-β cells *in vitro*.[24–26] This was achieved by first introducing a differentiation stage that induced NKX6–1 expression in these PDX1+ pancreatic progenitors and then devising a chemical cocktail to subsequently trigger NEUROG3-induced endocrine formation. The resulting SC-β cells were able to secrete insulin in response to glucose stimulation and protect against moderate diabetes (<300 mg/dL) after transplantation into mice. Nevertheless, the cells generated with these

protocols lacked key features of genuine β cells, including first and second phase dynamic secretion kinetics, and they exhibited lower overall insulin secretion compared with native human islets.

Since then, however, several research groups have further iterated on these protocols to improve SC-β cell generation,[21,27–31] and we refer the reader to more expansive review articles for further details on the development of SC-β cell differentiation strategies.[32–39] Recently, we devised a new maturation stage of the protocol, facilitating acquisition of the first and second phase dynamic insulin release exhibited by primary human β cells.[40] Furthermore, we improved generation of these cells with a new planar version of the protocol so that the cells are now capable of rapidly (1–2 weeks) curing severe preexisting diabetes (>600 mg/dL) in mice,[41,42] demonstrating the utility of these cells for therapy. Here, we describe this efficient differentiation protocol for generating highly functional SC-β cells using traditional planar cell culture techniques. We showcase how to optimize this protocol for different cell lines as well as demonstrate a simple method for aggregating these SC-β cells into functional clusters for use in downstream assays, such as transplantation into diabetic mice. This latest iteration simplifies the differentiation procedure compared to alternative protocols, can be adapted to a wide variety of cell lines, and produces highly functional SC-β cells. Our hope is that this protocol provides an easy-to-follow guide for both experienced researchers and those new to the field to make quality SC-β cells in an effort to accelerate research in β cell development, maturation, and health.

Development of the protocol.

To develop a planar methodology for generating insulin producing SC-β cells from hPSCs, we built upon the previously mentioned protocols that use the timed application of growth factors and small molecules to drive cells through defined stages of development (i.e., definitive endoderm, primitive gut tube, pancreatic progenitors, endocrine, and β cells).[24,25,40] Interestingly, while previous attempts to make SC-β cells in planar culture could robustly generate PDX1+/NKX6–1+ pancreatic progenitors that could mature *in vivo* after transplantation, planar methodologies could only generate immature insulin positive cells from these progenitors *in vitro*.[11,16,20] In contrast, all previous protocols that successfully generated glucose-responsive SC-β cells *in vitro* that secreted high amounts of insulin utilized a three-dimensional arrangement of cells either as suspension clusters[24,40] or as aggregates on transwell culture inserts[25] for the differentiation from pancreatic progenitors to SC-β cells. The reason for this requirement had been unknown, particularly in understanding the effects of the insoluble microenvironment on pancreatic fate choice. These insoluble cues can include substrate stiffness, topography, dimensionality, and the composition of extracellular matrix (ECM) proteins that a cell experiences during culture. These microenvironmental factors have increasingly been shown to influence a range of downstream signaling pathways within cells, such as those involving the actin cytoskeleton, that lead to changes in stem cell fate.[43–45]

In order to explore how such insoluble microenvironmental cues influence SC-β cell specification, we plated suspension-derived PDX1-expressing pancreatic progenitors onto tissue culture polystyrene (TCP) coated with a wide variety of ECM proteins.[41] Surprisingly,

we observed that as long as pancreatic progenitors adhered firmly to any particular ECM-coated TCP plate, NEUROG3-induced endocrine specification was strongly inhibited. We demonstrated that the high stiffness of TCP monolayer culture promoted increased polymerization of the actin cytoskeleton, which prevented premature NEUROG3 induction and facilitated expression of the important β cell transcription factor NKX6–1. Subsequent endocrine specification, however, continued to be blocked. To overcome this inhibition, we demonstrated that depolymerizing the cytoskeleton with latrunculin A allowed for robust NEUROG3 expression in planar culture.[41] Through further optimization, we determined that a 1 μM latrunculin A treatment for the first 24 hours of the endocrine induction stage was sufficient to rescue SC-β cell specification.[41] Thus, monolayer culture on TCP helped facilitate NKX6–1 expression and prevented premature endocrine induction by promoting actin polymerization in pancreatic progenitors (stage 4), while a 24 hour treatment with the actin depolymerizer latrunculin A was required for subsequent endocrine induction (stage 5).

This new understanding of the importance that the microenvironment and cytoskeletal signaling have on endocrine induction allowed us to adapt cluster-based protocols for generating highly functional SC-β cells to a planar format, simplifying the differentiation methodology while also improving outcomes for multiple cell lines.[41,42] Our results are in agreement with the excellent recent work of the Semb group[46] and others[47,48] that have demonstrated the importance of YAP signaling in pancreatic progenitors, as the state of the cytoskeleton is well-known to control the nuclear localization of YAP.[49] The influence of the cytoskeleton on additional signaling pathways during pancreatic differentiation cannot be ruled out, however, and can be the subject of further study.

Applications of the method.

The methodology described here allows for the production of highly glucose-responsive, insulin-producing SC-β cells. Transplantation of these cells into severely diabetic mice rapidly restores normoglycemia,[41,42] providing hope that these cells will eventually be utilized to functionally cure insulin-dependent diabetes. In addition to such a cell-based therapy, SC-β cell generation can also be implemented to study diabetes pathogenesis and diabetic cell phenotypes. Because this protocol can be adapted to generate SC-β cells from a wide variety of cell lines with different genetic backgrounds, it is amenable to both autologous cell therapy as well as applications that extensively study diabetic disease states, such as defects in SC-β cells generated from patients with monogeneic diabetes.[42] Furthermore, due to the flexibility in culture vessel size, this methodology is conducive to small drug screening and protocol optimization experiments as well as generating batches large enough for cell therapy. Importantly, this protocol simplifies the differentiation procedure and eliminates more complicated and expensive 3D culture systems, removing barriers for many researchers to generate and study these cells in an effort to accelerate research in this field.

Comparison with other methods.

In contrast to previous protocols that required a three-dimensional arrangement of cells either as suspension clusters or as aggregates on transwells for the differentiation from

pancreatic progenitors to SC-β cells, the key feature of our protocol is that it can generate highly functional SC-β cells entirely in traditional monolayer culture. This technique is enabled through the discovery that the cytoskeleton is critical to pancreatic cell fate, and the use of the cytoskeletal depolymerizer latrunculin A at the beginning of endocrine induction allows for robust differentiation of SC-β cells from pancreatic progenitors in planar culture.[41] Our findings solved a longstanding mystery in the field of why three-dimensional cell arrangements were required for the generation of SC-β cells, as prior studies were inadvertently modulating the cytoskeleton to be favorable for SC-β cell generation by use of these three-dimensional cell arrangements.

Several methods of 3D aggregate culture have been successfully used to generate SC-β cells, including magnetic spinner flasks,[24] 6-well plates on an orbital shaker,[26] and manually pipetting cells during endocrine induction onto transwell culture inserts to form clusters.[25] While such cluster-based methods are certainly capable of producing functional SC-β cells, there are several unique advantages to the planar methodology described herein. First, this protocol can be done entirely with traditional cell culture techniques, eliminating the need for these more complicated and expensive 3D culture requirements. Specifically, this protocol does not require additional training and expertise on stem cell suspension culture, expensive spinner flasks and magnetic stirrers, or a time-consuming mid-differentiation manual clustering step. Rather, cells are plated onto standard Matrigel-coated plasticware (e.g., 6-well plate or T-75 flask), and the differentiation media are simply added in the proper order. Because cells are in a monolayer rather than clusters that are hundreds of micrometers in diameter, diffusion gradients of the differentiation factors are minimized, allowing the differentiation to produce more cells per volume of media and further reduce differentiation costs. Specifically, this planar methodology typically produces between 0.5–0.75 million cells per cm^2 and approximately 2–3 times more cells per total media volume used throughout the differentiation than our previous suspension protocol. Furthermore, this planar protocol can be more cost effective for small scale laboratory experiments to test a wide range of conditions due to the ability to perform these differentiations in small formats, such as 6 or 24 well plates, in contrast to larger suspension bioreactors.

While SC-β cells are generated completely in planar culture, our methodology does allow for the generation of islet-sized clusters so that the SC-β cells can be used for additional assays, such as dynamic GSIS and transplantation. A single-cell suspension can be generated during the final stage of differentiation and then distributed into 6-well plates, and the rotational movement of an orbital shaker induces clustering of these cells. We have found that mostly endocrine cells aggregate together at this point in the differentiation after cell fate has already been specified in planar culture, while non-endocrine cell types generated during this protocol[21,42] tend to die off. Therefore, this optional aggregation step can facilitate endocrine purification without the need for a more expensive and time-consuming sorting procedure.

Importantly, this new planar methodology is more reproducible across multiple hPSC lines, as many hPSC lines are often difficult or impossible to adapt to suspension culture. Furthermore, hPSC differentiation protocols for many cell types, not just SC-β cells, often work well with only a single cell line, less well for other cell lines, and not at all for others.

In contrast, we have been able to generate functional SC-β cells from a wide variety of hPSC lines with unique genetic backgrounds using this planar methodology. Here, we provide data for robustly generating SC-β cells from 10 cell lines, including 5 additional lines that were not presented in our previous two publications.[41,42] Importantly, the flexibility in cell line choice that this protocol provides is potentially conducive to an autologous cell therapy to treat diabetes as well as facilitate the study of a wide variety of diabetic phenotypes, as we have recently demonstrated.[42]

Finally, not only does this methodology simplify SC-β cell generation and provide flexibility in terms of cell line and culture size, it can also improve the *in vitro* and *in vivo* performance of these SC-β cells. The cells that we generated with this protocol in our most recent two publications[41,42] are some of the most functional yet presented in the literature. We were able to achieve both first and second phase dynamic insulin secretion across 5 cell lines, with some lines approaching the functional capabilities of human islets. Furthermore, these SC-β cells were capable of reversing severe pre-existing diabetes in mice within 2 weeks at a rate similar to primary human islets, outperforming cells generated with a suspension-based protocol. Importantly, these transplanted SC-β cells were able to maintain normoglycemia indefinitely.

Experimental design.

Here, we detail a step-by-step directed differentiation protocol for generating highly functional SC-β cells (Figure 1). This methodology consists of 6 stages that attempt to recreate phases of pancreatic organogenesis by activating and repressing specific developmental pathways with growth factors and small molecules in serum-free media. We detail methods to assess the cells throughout differentiation, including quality assurance steps at critical points of the protocol as well as *in vitro* functional assessment of the SC-β cells. This methodology can be adapted to differentiate SC-β cells from a range of stem cell lines, and we describe guidelines to optimize the protocol when using a new cell source based on our experience with the lines described here. This protocol takes about 5 weeks to complete, and therefore we recommend that differentiations be started every week or so if a continuous supply of cells is needed in order to avoid lag time between experiments.

HPSC culture (Stage 0, Steps 1–9).—To propagate and expand cells for SC-β cell differentiation, hPSCs are seeded onto Matrigel-coated TCP plates at a density of 0.8×10^5 cells/cm^2 and cultured in mTeSR1. Once cells are 90% confluent after about four days, they are dispersed into single cells using TrypLE and seeded onto new Matrigel-coated plates with mTeSR1 supplemented with the Rho-kinase inhibitor Y-27632. A propagation flask can be thawed and maintained for several weeks using this procedure. When a differentiation is needed, separate flasks (e.g., T-75) or plates (e.g., 6-well plate) can be seeded during a passage of the propagation flask. Unlike the propagation flask, differentiation plates should be seeded near confluency, though the exact cell density must be optimized for each cell line.

Definitive endoderm (Stage 1, Steps 10–12).—24 hours after seeding a differentiation flask, the stem cells should be confluent. To initiate differentiation, mTeSR1

is replaced with differentiation media containing Activin A (TGF-β superfamily member) and CHIR99021 (Wnt agonist). After the first 24 hours in this media, only Activin A is added during the subsequent three days of endoderm induction. At the end of this stage, >90% of the cells should express the endoderm markers FOXA2 and SOX17. Achieving high expression of these markers is critical to the success of the protocol, and so this stage should be optimized for any given cell line before proceeding with the remainder of the differentiation. These markers can be checked visually with immunocytochemistry or quantitatively with flow cytometry.

Primitive gut tube (Stage 2, Steps 13–15).—After endoderm induction, these cells are converted to primitive gut tube with two days of keratinocyte growth factor (KGF) treatment. We have not found a reliable marker to distinguish the ability of these cells to subsequently make SC-β cells, and thus the effects of any stage 2 modulations are assessed in subsequent stages.

Pancreatic progenitors (Stages 3 and 4, Steps 16–20).—The first pancreatic progenitor stage drives cells towards a pancreatic lineage by turning on the transcription factor PDX1 with a high concentration of RA accompanied by KGF, SANT1 (hedgehog signaling inhibitor), TPPB (PKC activator), and LDN193189 (BMP inhibitor). This media should induce >80% of the cells to express PDX1 after two days. The second pancreatic progenitor stage continues culture of the cells for the next four days in this media with the exception of drastically reduced RA concentration. This stage is designed to allow these PDX1+ cells to turn on the important β cell transcription factor NKX6–1. An important quality control step at the end of this stage is measuring the percentage of cells co-expressing NKX6–1+ and PDX1+ with flow cytometry, as these will be the cells that ultimately produce functional, monohormonal SC-β cells. This stage should generate >40% NKX6–1+/PDX1+ cells, but further increasing this percentage can be a major target of protocol optimization.

Endocrine (Stage 5, Steps 21–24).—To induce endocrine formation from these pancreatic progenitors, Notch signaling must be downregulated with XXI (γ-secretase inhibitor) in combination with T3 (thyroid hormone), ALK5 inhibitor II, SANT1, and RA for one week of culture. However, the increased cytoskeletal polymerization induced by monolayer culture on stiff TCP blocks NEUROG3 expression even in the presence of these factors, preventing initiation of the endocrine program. In order to overcome this inhibition, the actin cytoskeleton must be chemically depolymerized with latrunculin A at the start of endocrine induction. Once NEUROG3 has turned on, further cytoskeletal depolymerization is not needed. We have found that a 1 μM treatment for the first 24 hours of stage 5 is sufficient to initiate endocrine differentiation for most cell lines, though this dosing may need to be optimized when adapting the protocol to other lines. Also worthy to note is that while our original suspension protocol included the EGF family member betacellulin, we have found it to not be necessary in the planar differentiations and have since omitted it from the protocol.

SC-β cells (Stage 6, Steps 25–27).—Once the endocrine cells have been specified, the SC-β cells need time to mature before they become glucose-responsive. We developed an enriched serum-free media (ESFM) that facilitates this process, allowing cells to develop a robust insulin secretion response in 10–14 days.[40] These cells can remain on the plate for the remainder of differentiation and characterization. Alternatively, after one week into stage 6, cells can be aggregated into islet-like clusters with a simple method that uses an orbital shaker after single-cell dispersion from the plate. There are several advantages to this aggregation step, including the ability to do assays incompatible with plated cells, such as a dynamic glucose stimulated insulin secretion (GSIS) assay or transplantation into diabetic mice. Furthermore, this aggregation step can eliminate inefficiencies in the differentiation and improve SC-β cell function, as mostly endocrine cells seem to form clusters while other non-endocrine cells tend to die off.

SC-β cell assessment and controls (Steps 28–103).—Our standard set of assessments include static and dynamic GSIS assays to measure glucose responsiveness, insulin content and proinsulin/insulin ratio to determine intracellular insulin levels and processing, flow cytometry to measure the percentages of the different hormone-producing cells, as well as qRT-PCR and immunostaining to confirm the presence of β cell specific markers. In particular, SC-β cells can be identified by their co-expression of C-peptide and NKX6–1, while chromogranin A (CHGA) marks the general endocrine population. After aggregation, >80% of the cells in these clusters should express CHGA, with about 20–60% of these cells being C-peptide+/NKX6–1+.

This protocol was originally developed and optimized with the HUES8 cell line, and therefore it can serve as a good baseline to determine the success of a particular experimental setup. Other cell lines that we have reported on here or previously[41,42] can also serve as controls when adapting this protocol to new cell lines. When first attempting this differentiation protocol, we suggest starting with one of the cell lines we provide data for, particularly HUES8, in order to validate a new experimental setup. Once this setup has been verified, investigators can use the guidelines we provide for adapting and optimizing this protocol to new hPSC lines. We have had good success using these guidelines, though we cannot guarantee that all new cell lines will be capable of generating functional SC-β cells. Once the protocol is running efficiently, primary human islets serve as the best comparison for the functional *in vitro* performance of SC-β cells generated with this protocol. The primary human islets used as controls here were purchased from Prodo Laboratories, Inc (Aliso Viejo, CA). To assess *in vivo* performance, SC-β cell clusters can be injected into the kidney capsule of immunodeficient mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) with streptozotocin-induced diabetes, though this procedure is not detailed here.

Limitations.

This differentiation protocol is a multi-stage process that takes 5 weeks to complete, and therefore there are a number of possible failure points that can prevent successful generation of SC-β cells. We provide quality control steps to try to mitigate unsuccessful differentiations, but due to the length of the protocol, some issues can be time-consuming to troubleshoot. These problems can be exacerbated when attempting to adapt this

methodology to a new cell line, as certain stages may need to be optimized for different cell lines. Although we outline points of optimization that have led to good success with the 10 cell lines presented here, we cannot rule out that some cell lines may need additional optimizations or that other lines may not even be capable of differentiating to an SC-β cell lineage, particularly those derived from patients with various forms of diabetes. Nevertheless, this methodology has allowed for the generation of SC-β cells from cell lines that have not previously worked with other protocols. For all 10 cell lines presented here, we demonstrate co-expression of NKX6–1+/C-peptide+ and the secretion of high levels of insulin in a static GSIS assay, which are the critical assays to show successful generation of SC-β cells. For more in-depth characterization assays, we have chosen to collect data for HUES8 and 2 iPSC lines compared with primary human islets. While data from 3 human cell lines provides a good representation of how SC-β cells produced with this protocol perform in these assays, we have not performed every assay with every cell line presented here. Thus, some lines may require further study to provide more in-depth characterization of cell line variation. Additional characterization and application data for HUES8, 1013–4FA, 1016SeVA, WS4^{corr}, and WS4^{unedit} using this protocol can be found in our recent publications.[41,42]

While we demonstrate generation of highly functional SC-β cells using this protocol, they are less mature compared to primary β cells from adults. The insulin secretion per cell for many cell lines remains lower than primary human islets, and they still lack expression of important maturation genes, such as MAFA. While this protocol generates clusters that consist of more than 80% endocrine cells, it does not produce pure SC-β cells.[42] We have not extensively studied the other endocrine cell types generated in these differentiations, and it remains unclear if these other endocrine cells are necessary for SC-β cell maturation and function. The increased accessibility that this protocol provides, however, will hopefully help accelerate research into maximizing maturation of SC-β cells.

Another potential concern with this planar methodology is its scalability when compared with a suspension-based system. Though suspension systems may theoretically be capable of higher throughput, we have scaled this planar protocol up to T-150 flasks with high differentiation efficiency and function, and there does not seem to be any indication that it could not be scaled further with hyperflask setups. Notably, our T-150 differentiations have been able to generate cell yields comparable to the highest yet reported in literature.[24] Furthermore, while a suspension-based system may ultimately allow for greater cell production on a commercial scale, this planar methodology is more amenable to autologous cell therapy applications as well as small scale laboratory exploration of SC-β cell biology and diabetes pathogenesis.

MATERIALS.

Biological Materials.

Cell lines.—The SC-β cell differentiation protocol detailed here requires a source of hPSCs. We have successfully generated functional SC-β cells using a variety of different genetic backgrounds of both embryonic (ESC) and induced pluripotent stem cell (iPSC) origin. HUES8, 1013–4FA, 1016SeVA, 1026–3FC, and 1031SeVA were provided by

Harvard University. The H1 line was obtained from WiCell. AN1.1 was generated at the Washington University in St. Louis Genome Engineering and iPSC Center, while T2D001A was generated by us from mesenchyme with the Sendai virus kit. We have published previously on $WS4^{corr}$ and $WS4^{unedit}$.[42] Table 1 lists the lines used here, but this protocol can also be adapted to other stem cell lines.

> **!CAUTION** All experiments involving hPSCs and/or mice should be done in accordance with the appropriate institutional approval. Relevant data presented here using human stem cells was approved by the Washington University ESCRO committee (approval #15–002) with appropriate consent and conditions.
>
> **!CAUTION**: The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

Reagents

Stem cell culture

- mTeSR1 complete kit (Stemcell Technologies, 85850)
- Y-27632 (Abcam, ab120129)
- TrypLE Express (Gibco, 12604–039) or Accutase (Stemcell Technologies, 07922)
- Growth Factor Reduced Matrigel (Corning, 356230)
- Dulbecco's Modified Eagle Medium (DMEM) (MilliporeSigma, D5796)

Base media components

- 500 mL MCDB 131 (Corning, 15–100-CV or Gibco, 10372019)
- glucose (MilliporeSigma, G7528)
- $NaHCO_3$ (MilliporeSigma, S5761)
- bovine serum albumin (BSA) (Proliant Biologicals, 68700)

  > ▲**CRITICAL**: It is important that the BSA is fatty-acid free. We strongly recommend using this specific BSA product. We have not observed an effect of different batches of this product on differentiation efficiency.

- ITS-X (Gibco, 51500–056)
- GlutaMAX (Gibco, 35050–079)
- vitamin C (MilliporeSigma, A4544)
- penicillin/streptomycin (P/S) solution (Corning, 30–002-CI)
- heparin (MilliporeSigma, H3149)
- MEM nonessential amino acids (Corning, 20–025-CI)
- $ZnSO_4 \cdot 7H_2O$ (MilliporeSigma, 108883)
- Trace Elements A (Corning, 25–021-CI)

- Trace Elements B (Corning, 25–022-CI)

Differentiation factors.

▲**CRITICAL**: We recommend that the exact differentiation factors be used from the specified vendors listed below. Although some substitutions from other vendors may work, we have validated the listed factors to be compatible with this differentiation protocol. Activin A is particularly sensitive to changes in vendor, and so we strongly recommend using the specified product.

- CHIR99021 (Stemgent, 04–0004)
- Activin A (R&D Systems, 338-AC)
- KGF (Peprotech, AF-100–19)
- LDN193189 (Stemgent, 04–0074)
- TPPB (Tocris, 5343)
- retinoic acid (MilliporeSigma, R2625)
- SANT1 (MilliporeSigma, S4572)
- ALK5i II (Enzo Life Sciences, ALX-270–445-M005)
- L-3,3’,5-Triiodothyronine (T3) (MilliporeSigma, 64245)
- XXI (MilliporeSigma, 565790)
- latrunculin A (Cayman Chemical,10010630)
- bFGF (Stemcell Technologies, 78003)
- 7.5% (wt/vol) BSA for diluting factors (MilliporeSigma, A8412)
- phosphate-buffered saline (PBS) for diluting factors (Corning, 21–040-CV)
- dimethyl sulfoxide (DMSO) for diluting factors (MilliporeSigma, D2650–100ML)
- sterile molecular biology grade water for diluting factors (Corning 46–000-CM)

Antibodies.

▲**CRITICAL**: The following antibodies can be used for both flow cytometry and immunostaining assays. However, note that different glucagon (GCG) and somatostatin (SST) antibodies should be used for immunohistochemistry.

- rat anti-C-peptide (Developmental Studies Hybridoma Bank – University of Iowa, GN-ID4-S, https://scicrunch.org/resolver/AB_2255626)
- mouse anti-NKX6–1 (Developmental Studies Hybridoma Bank – University of Iowa, F55A12-S, https://scicrunch.org/resolver/AB_532379)

- goat anti-PDX1 (R&D Systems, AF2419, https://scicrunch.org/resolver/AB_355257)
- sheep anti-NEUROG3 (R&D Systems, AF3444, https://scicrunch.org/resolver/:AB_2149527)
- rabbit anti-CHGA (ABCAM, ab15160, https://scicrunch.org/resolver/AB_301704)
- rabbit anti-SST (ABCAM, ab64053, https://scicrunch.org/resolver/AB_1143012)
- (histology only) mouse anti-SST (Santa Cruz Biotechnology, sc-55565, https://scicrunch.org/resolver/AB_831726)
- mouse anti-GCG (ABCAM, ab82270, clone IMD-7, https://scicrunch.org/resolver/AB_1658481)
- (histology only) rabbit anti-GCG (Cell Marque, 259A-18, https://scicrunch.org/resolver/AB_1158356)
- mouse anti-SOX17 (R&D Systems, MAB1924, clone # 245013, https://scicrunch.org/resolver/AB_2195646)
- rabbit anti-FOXA2 (MilliporeSigma, 07–633, https://scicrunch.org/resolver/AB_390153)
- mouse anti-OCT-3/4 (Santa Cruz Biotechnology, sc-5279, clone C-10, https://scicrunch.org/resolver/AB_628051)
- goat anti-NANOG (R&D Systems, AF1997, https://scicrunch.org/resolver/AB_355097)
- anti-rat alexa fluor 488 (Invitrogen, A21208, https://scicrunch.org/resolver/AB_141709)
- anti-mouse alexa fluor 488 (Invitrogen, A21202, https://scicrunch.org/resolver/AB_141607)
- anti-mouse alexa fluor 647 (Invitrogen, A31571, https://scicrunch.org/resolver/AB_162542)
- anti-mouse alexa fluor 594 (Invitrogen, A21203, https://scicrunch.org/resolver/AB_141633)
- anti-goat alexa fluor 488 (Invitrogen, A11055, https://scicrunch.org/resolver/AB_2534102)
- anti-goat alexa fluor 647 (Invitrogen, A21447, https://scicrunch.org/resolver/AB_2535864)
- anti-sheep alexa fluor 594 (Invitrogen, A11016, https://scicrunch.org/resolver/AB_10562537)

- anti-rabbit alexa fluor 647 (Invitrogen, A31573, https://scicrunch.org/resolver/AB_2536183)
- anti-rabbit alexa fluor 488 (Invitrogen, A21206, https://scicrunch.org/resolver/AB_2535792)
- anti-rat PE (Jackson ImmunoResearch, 712-116-153, https://scicrunch.org/resolver/AB_2340657)

Krebs Buffer (KrB)

- Milli-Q H_2O
- NaCl (Fisher BioReagents, BP358–1)
- KCl (Fisher BioReagents, BP366–500)
- $CaCl_2 \cdot 2H_2O$ (Acros Organics, 447325000)
- $MgSO_4$ (MilliporeSigma, M7506)
- Na_2HPO_4 (Fisher Scientific, S25563A)
- KH_2PO_4 (MilliporeSigma, PX1562)
- $NaHCO_3$ (MilliporeSigma, S5761)
- HEPES buffer solution (Gibco, 15630080)
- BSA (Proliant Biologicals, 68700)
- NaOH (MilliporeSigma, S8045)

Other assay reagents

- human insulin ELISA (ALPCO, 80-INSHU-E01.1)
- proinsulin ELISA kit (Mercodia, 10-1118-01)
- 4% (wt/vol) paraformaldehyde (PFA) (Electron Microscopy Sciences, 157-4-100)
- Triton X (Acros Organics, 327371000)
- donkey serum (Jackson Immunoresearch, 017-000-121)
- DAPI nuclear stain (Invitrogen, D1306)
- DAPI Fluoromount-G, for histology (SouthernBiotech, 0100–20)
- 200 proof ethanol (Decon, 2716)
- HCl (MilliporeSigma, 258148)
- TRIS buffer (MilliporeSigma, T6066)
- EDTA (Ambion, AM9261)
- Histo-clear II (Electron Microscopy Sciences, 64111–04)

- HistoGel (Thermo Fisher Scientific, HG-4000–012)
- Quant-iT PicoGreen dsDNA kit (Invitrogen, P7589)
- Rneasy Mini Kit (Qiagen, 74104)
- DNAse kit (Qiagen, 79254)
- High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, 4368814)
- PowerUp SYBR Green Master Mix (Applied Biosystems, A25741)
- Custom qRT-PCR primers (MilliporeSigma, see Table 2)

Equipment.

▲**CRITICAL**: In contrast to many of the cell culture media components, the following equipment and consumables can be substituted with suitable replacements. We provide some suggestions that have worked well in our differentiations.

- Pipet-aid
- pipettors (P10, P20, P200, and P1000)
- multi-channel pipettor, 200 μL
- forceps
- automated (Beckman Coulter, Vi-Cell XR) or manual cell-counting system
- humidified cell culture incubator set at 37°C and 5% (vol/vol) CO_2
- orbital shaker suitable for use in a cell culture incubator (Benchmark Scientific, Orbi-Shaker CO_2)
- 37°C water bath
- biosafety cabinet
- −20°C and −80°C freezers
- light microscope
- confocol microscope
- flow cytometer
- qRT-PCR thermocycler
- plate reader capable of reading at 450 nm and 520 nm
- pH meter
- centrifuges for use with 50 mL conical tubes and 1.7 mL microcentrifuge tubes
- pressure cooker for antigen retrieval (Electron Microscopy Sciences, Retriever 2100)

Consumables

- serological pipettes (5 mL, 10 mL, and 25 mL; USA Scientific, 1075–0110, 1071–0810, and 1072–5410)
- 2 mL aspirating pipettes (Falcon, 13-675-16)
- barrier pipet tips (10 μL, 20 μL, 200 μL, and 1000 μL; Corning, 4135, 4136, 4139, and 4140)
- 1.7 mL microcentrifuge tubes (BioExpress, C-3262–1)
- 50 mL conical tubes (USA Scientific, 5622–7261)
- 96-well tissue culture treated plates (Thermo Fisher Scientific, 167008)
- 24-well tissue culture treated plates (Falcon, 353226)
- 6-well tissue culture treated plates (Corning, 3506)
- T25 tissue culture treated flasks (TPP, 90026)
- T75 tissue culture treated flasks (TPP, 90076)
- T150 tissue culture treated flasks (TPP, 90151)
- disposable sterile filter systems (50 mL, 500 mL, and 1000 mL; MilliporeSigma, SE1M179M6; Corning, 431097 and 431098)
- 96-well qRT-PCR reaction plates and adhesive film (Applied Biosystems, 4346906 and 4311971)
- transwell inserts for static GSIS (MilliporeSigma, PIXP01250)
- reagent reservoirs (MIDSCI, P8010)
- tubes for flow cytometry (Falcon, 352235)
- tissue-loc histoscreen cassettes (Thermo Fisher Scientific, C-1000-BL)
- micro coverslips (VWR, 48393–081)
- glass slides (VWR, 48300–026)

Dynamic GSIS setup

- Bio-Gel P-4 polyacrylamide beads (Bio-Rad, 150–4124)
- high precision 8-channel dispenser pump (Ismatec, ISM931C)
- 0.015” ID inlet/outlet tubing and 0.04” ID connection tubing (BioRep Diabetes, PERI-TUBSET)
- 275-μl cell chambers (BioRep Diabetes, PERI-CHAMBER)
- dispensing nozzles (BioRep Diabetes, PERI-NOZZLE)
- filter paper (BioRep Diabetes, PERI-FILTER)

Reagent Setup

Stem cell culture.—Thaw the mTeSR1 5x Supplement and mix it into the mTeSR1 Basal Medium. Do not, however, add Y-27632 until use during passaging. This media can be stored at 4°C for up to two weeks, or aliquoted and stored at −20°C for up to 6 months.

For easier handling, the Matrigel used for coating plasticware should be aliquoted from the stock bottle into microcentrifuge tubes. Determine the desired aliquot size of concentrated Matrigel stock assuming that at least 1 mL of diluted Matrigel coating solution is needed per 12.5 cm^2 of culture surface area. While the Matrigel product information should be consulted for the appropriate dilution factor due to lot-to-lot variation of protein concentration, a typical dilution is approximately 13 μL of concentrated Matrigel stock per mL DMEM. Thus a 234 μL aliquot of concentrated Matrigel stock would yield 18 mL of diluted Matrigel coating solution, which is enough to coat three T75 flasks. Before aliquoting, place pipette tips and tubes in a −20°C freezer to get them cold, as warming concentrated Matrigel to room temperature will cause it to solidify. Thaw the stock vial overnight at 4°C and keep on ice during aliquoting. Store the aliquots at −80°C until use.

Base media.—All stages of this differentiation protocol use custom media consisting of MCDB131 supplemented with the components detailed in Table 3. Note that BE3 is used for both stages 3 and 4. Make stock solutions of heparin at 10 mg/mL in PBS and $ZnSO_4 \cdot 7H_2O$ at 1 mM in sterile Milli-Q water and store them at 4°C for up to 4 months. The remaining components can be added directly from the purchased stock containers. For each media, mix all components together in the MCDB131 bottle and warm in a 37°C water bath to dissolve the materials, particularly the BSA. Sterile filter and store at 4°C for up to 2 months. Avoid extended light exposure when using all media. Antibiotics have been omitted from BE1-BE3, but we have successfully generated SC-β cells with P/S added during all stages, so it can be added to early stages if desired.

> ▲**CRITICAL**: Do not add differentiation factors to these base media yet, as those factors will be added each day before feeding cells.

Differentiation factors.—The differentiation factors for each stage are added fresh to the base media immediately prior to every feed. In order to maintain optimum activity of these differentiation factors, they should be aliquoted in microcentrifuge tubes to ensure no more than 2 freeze/thaw cycles for each aliquot. Factors reconstituted in a PBS solution containing 0.1% (wt/vol) BSA or in molecular biology grade water can be kept at 4°C for up to one week after a thaw, while factors in DMSO should always be put back in the freezer after a thaw since they will already freeze at 4°C. Frozen factors can be kept at −20°C in the short-term but should be kept at −80°C for extended storage up to a year. Furthermore, avoid extended light exposure for all factors but particularly with retinoic acid.

To reconstitute factors, first create a sterile 0.1% (wt/vol) BSA solution by diluting a 7.5% BSA solution (MilliporeSigma, A8412) in PBS. Then, sterilely open each factor in a biosafety cabinet and add the amount of either DMSO, 0.1% BSA solution, or molecular biology grade water to the lyophilized powders as described in Table 4. Once reconstituted, aliquot into microcentrifuge tubes and store in the −80°C freezer. Determine the appropriate

aliquot size for each factor based on how much media will be needed each feed for the chosen plate or flask, and always make aliquots a little bigger than needed to avoid running out on the day of feeding. The amounts listed in Table 4 are enough to perform 7 differentiations in T-75 flasks. There will be a surplus of many of the relatively inexpensive small molecules, but additional amounts of the more expensive growth factors will need to be purchased for additional differentiations. Note that bFGF is only needed for optimizing the differentiation of some cell lines.

> **!CAUTION**: Take care when reconstituting factors to avoid creating dust from the powders. See the MSDS of each factor for safety information.

Krebs Buffer (KrB).—Properly making KrB is critical to accurately assessing the glucose responsiveness of SC-β cells in a GSIS assay. First, make stock salt solutions by dissolving the salts in Milli-Q water as detailed in Table 5. These stocks can be stored at room temperature (20–25°C) and may be used for several months.

In contrast to the stock salt solutions, fresh KrB should be made the day of performing GSIS. Mix components as detailed in Table 6, adjust the pH to 7.4 with 1M NaOH, and sterile filter before use.

In addition to KrB, make 50 mL of a 1 M glucose solution in Milli-Q water and sterile filter. This glucose stock solution can be stored at 4°C for several months. On the day of the assay, use this stock 1 M glucose solution to make 2 mM (1:500 dilution in KrB) and 20 mM (1:50 dilution in KrB) glucose solutions. When performing GSIS in a 24-well plate, you will need 2 mL of the 2 mM low glucose solution per sample and 1 mL of the 20 mM high glucose solution per sample.

Immunocytochemistry (ICC) solution.—Make a solution of 0.1% (vol/vol) Triton X and 5% (vol/vol) donkey serum in PBS. This solution is used during immunostaining for ICC, flow cytometry, and immunohistochemistry. This solution can be stored at room temperature for up to 6 months.

Acid-ethanol extraction solution.—Make an acid-ethanol solution consisting of 1.5% (vol/vol) HCl and 70% (vol/vol) ethanol in Milli-Q water. This solution can be stored at room temperature for up to a year. **!CAUTION**: Use a chemical fume hood and appropriate personal protective equipment when handling concentrated HCl.

DNA content lysis buffer.—For lysing cells for DNA quantification for the dynamic GSIS assay, make a solution consisting of 10 mM Tris, 1 mM EDTA, and 0.2% (vol/vol) Triton X. This solution can be stored at room temperature for up to 6 months.

PROCEDURE

> ▲**CRITICAL**: This SC-β cell differentiation protocol takes approximately 5 weeks to complete, plus 1–2 weeks to expand the stem cells as well as assess the SC-β cells at the end of the differentiation (Figure 1). The cells need to be fed every day for a majority of the protocol, and there are no pause points until the end of

the differentiation when assessing SC-β cell quality and function. Thus if planning consecutive experiments with SC-β cells generated with this protocol, we recommend starting differentiations every week or so to eliminate lag time between experiments by ensuring a continuous supply of SC-β cells. Troubleshooting of earlier stages can be done on shorter time scales, however, as issues with differentiation efficiency can often be checked with flow cytometry, immunocytochemistry, and qRT-PCR at the end of particular stages.

Stage 0: Stem cell culture and seeding differentiations •TIMING ~8 days (1–2 hrs/d hands-on)

1. Prepare tissue culture-treated polystyrene (TCP) for cell culture by coating it for 1 hour at 37°C with at least 1 mL of diluted Matrigel per 12.5 cm^2. See the Matrigel product information for the appropriate dilution as it is lot-dependent, though a typical dilution is approximately 13 μL concentrated Matrigel stock per mL DMEM. After coating, aspirate the DMEM and replace it with mTeSR1 supplemented with 10 μM Y-27632.

2. Thaw a vial of hPSCs. Mix well with 10 mL mTeSR1 supplemented with 10 μM Y-27632 and centrifuge at 300g for 3 minutes at room temperature to pellet cells.

3. Aspirate the media and resuspend the cells in mTeSR1 supplemented with 10 μM Y-27632. Seed the Matrigel-coated flask at a minimum cell density of 0.8×10^5 cells/cm^2 using 0.2 mL /cm^2 of mTeSR1 supplemented with 10 μM Y-27632.

4. Keep cells in a 37°C incubator at 5% (vol/vol) CO_2 for all cell culture. Feed the hPSCs every day with mTeSR1 (without Y-27632) and increase this volume (e.g., day 2 = 0.267 mL/cm^2, day 3 = 0.333 mL/cm^2, day 4 = 0.467 mL/cm^2) as cells become more confluent (Figure 2a,f, Supplementary Figure 1a,k). Culture the cells until approximately 90% confluency, which should take several days but will vary depending on the growth rate of each cell line.

5. Before passaging the hPSCs, prepare a new Matrigel-coated flask as described in Step 1 for continuing this propagation. Multiple and/or larger flasks can be used to further expand the hPSCs and ensure adequate cell numbers for seeding differentiations. If ready to start a new differentiation at this point, also prepare the desired number of plates/flasks.

   ▲**CRITICAL STEP**: We typically aim to generate enough cells to both start differentiations and re-seed a propagation flask, as maintaining a propagation flask minimizes lag time between starting new differentiations.

   ▲**CRITICAL STEP**: As cells need time to recover after thawing from cryofrozen storage, do not start differentiations directly from a thaw, instead allowing for 1–2 passages before seeding a differentiation. Confirm that the proliferation rate is similar to the pre-frozen rate, and check pluripotency markers such as OCT4 and NANOG with immunostaining (steps 46–55) or flow cytometry (steps 30–45).

!CAUTION: We have found that plates with smaller well sizes (24-well plates and smaller) can sometimes experience issues with differentiation efficiency, as some edge effects can arise. Therefore, we recommend starting with 6-well plates or larger when first testing this protocol.

6. Passage the hPSCs by first aspirating the media and adding approximately 0.2 mL TrypLE/cm^2. Incubate for 10 minutes or less at 37°C.

7. Once the cells start releasing from the plate, gently pipette up and down or gently tap the sides of the flask to finish dispersing the cells. Mix the cell suspension with an equal volume of mTeSR1 supplemented with 10 μM Y-27632 and perform a cell count.

   ▲**CRITICAL STEP**: Successful hPSC culture relies on accurate and consistent cell counts. While this can be done manually using a hemocytometer, we have found it helpful to use an automated cell counter to ensure consistent cell counts across multiple users.

8. Centrifuge the cell suspension at 300g for 3 minutes at room temperature to pellet the cells, aspirate the supernatant, and resuspend the cells in mTeSR1 supplemented with 10 μM Y-27632.

   ▲**CRITICAL STEP**: If the user finds it difficult to disperse the cell pellet after centrifuging at 300g for 3 minutes at any step in this protocol, a gentler centrifugation procedure can be used as an alternative in future experiments. While this procedure adds an extra step, it can prevent extensive cell pellet compaction with certain cell lines and thus circumvent the need for excessive mechanical force to break up the cell pellet.

   To perform this alternative procedure, first centrifuge the cell solution at 200g for 2 minutes to pellet most of the cells. Transfer the supernatant to another tube, and immediately disperse the cell pellet with media. Centrifuge the supernatant at 300g for 3 minutes to pellet the remaining cells, aspirate the supernatant, disperse this second pellet with media, and add these cells to the original cell suspension.

9. To continue a propagation flask, seed a Matrigel-coated flask at a cell density of at least 0.8 × 10^5 cells/cm^2. To start differentiations, seed Matrigel-coated flasks/plates at a cell density of between 0.32–0.74 × 10^6 cells/cm^2, with cell density dependent on cell line (see Table 11).

   ▲**CRITICAL STEP**: To avoid differences in proliferation rates and maintain a consistent starting cell density for differentiation, we have found it best to seed near confluency and start the differentiation the next day. Thus, a T-75 propagation flask will usually only be enough to start one T-75 differentiation. Plate format should be determined by the specifics of each experiment. If the user wants to perform characterization assays of the cells while still in planar culture (such as flow cytometry during the differentiation or static GSIS of planar stage 6 cells), it is easiest to start the

differentiation in either a 24-well or 6-well plate. If characterization assays are to be performed after aggregation in stage 6, then larger formats (6-well, T-75, or T-150) are easier to use.

!CAUTION: Single-cell passaging of stem cells over several weeks to months can lead to karyotype abnormalities, and thus undifferentiated stem cells should be checked frequently for proper karyotype if cultured for extended periods. To avoid extended passaging, stem cells can be initially expanded and frozen down to create a cell bank, and each thawed vial can be used for only a few passages when seeding differentiations. Alternatively, a clump passaging method, such as those described in the manufacturer's manual for using mTeSR1, can be used to reduce the risk of karyotype abnormalities.

Stage 1: Definitive endoderm differentiation •TIMING 4 days (30–60 min/d hands-on)

10. 24 hours after seeding the differentiation, cells should be a confluent monolayer (Figure 2b,g, Supplementary Figure 1b,l). Prepare stage 1 media by adding Activin A (final concentration of 100 ng/mL; 1:1,000 dilution from stock aliquot) and CHIR99021 (final concentration of 3 μM; 1:10,000 dilution from stock aliquot) to BE1 base media and warm in a 37°C water bath. The amount of media needed for each feed throughout the differentiation can be calculated from the Table 7, and Table 8 provides a convenient summary of the full differentiation feeding schedule.

▲**CRITICAL STEP**: The differentiation factors throughout the protocol need to be added fresh each day before feeding to ensure maximum efficacy. Adhere to the 2 freeze/thaw limit for the factor aliquots. Furthermore, once the differentiation factors have been added to the base media, do not let the media remain in the water bath for extended periods before feeding.

11. Wash cells once with PBS and add BE1 containing Activin A and CHIR99021.

12. After 24 hours, replace this media with BE1 containing only Activin A. Culture for an additional 3 days with this media, replacing the media every day. Note that it is normal for there to be some amount of cell death throughout stage 1, but the remaining cells should still completely cover the plate (Figure 2c,h, Supplementary Figure 1c,m).

▲**CRITICAL STEP**: The duration of exposure to CHIR99021 is important for endoderm specification, so be sure to wait a full 24 hours before replacing the media containing both Activin A and CHIR99021 with the stage 1 media containing only Activin A.

▲**CRITICAL STEP**: Efficient endoderm induction is crucial for the success of the rest of the differentiation protocol. Thus, it can be beneficial to check the percentage of FOXA2+/SOX17+ cells with flow cytometry (Figure 3a) or immunocytochemistry (Figure 3d) to ensure expression in >90% of cells. While we have empirically found these culture parameters

and markers to work well, there are many published variations for making definitive endoderm that could be used.[3–10]

?TROUBLESHOOTING

Stage 2: Primitive gut tube generation •TIMING 2 days (30–60 min/d hands-on)

13. Prepare stage 2 media by adding KGF (final concentration of 50 ng/mL; 1:1,000 dilution of stock aliquot) to BE2 base media and warm in a 37°C water bath.

14. Aspirate stage 1 media and add BE2 supplemented with KGF. At the beginning of this stage, cells should completely cover the plate (Figure 2c,h, Supplementary Figure 1c,m), though there may be cells floating in the media as a result of stage 1 endoderm induction.

15. Culture the cells for 2 days, replacing the media after the first 24 hours.

▲**CRITICAL STEP**: We have not found a good marker that indicates whether cells at the end of stage 2 will be competent to further differentiate into SC-β cells. Thus, any modulations in stage 2 must be assessed at later stages.

Stage 3: Generating pancreatic progenitor 1 cells •TIMING 2 days (30–60 min/d hands-on)

16. At the beginning of this stage, the cells often adopt a “hill/valley” morphology, with some cells clustering together while others remaining in a monolayer (Figure 2d,i, Supplementary Figure 1d,n). Prepare stage 3 media by adding KGF (final concentration of 50 ng/mL; 1:1,000 dilution of stock aliquot), TPPB (final concentration of 0.2 μM; 1:10,000 dilution of stock aliquot), SANT1 (final concentration of 0.25 μM; 1:4,000 dilution of stock aliquot), RA (final concentration of 2 μM; 1:5,000 dilution of stock aliquot), and LDN193189 (final concentration of 0.2 μM; 1:5,000 dilution of stock aliquot) to BE3 base media and warm in a 37°C water bath.

17. Aspirate stage 2 media and add BE3 supplemented with the differentiation factors.

18. Culture the cells for 2 days, replacing the media after the first 24 hours.

▲**CRITICAL STEP**: Stage 3 is designed to turn on the pancreatic transcription factor PDX1. To determine the quality of the differentiation thus far, PDX1 expression can be measured quantitatively with flow cytometry (Figure 3b) or visually with ICC at the end of stage 3. Differentiations should achieve PDX1 expression in at least 80% of the cells.

?TROUBLESHOOTING

Stage 4: Generating pancreatic progenitor 2 cells •TIMING 4 days (30–60 min/d hands-on)

19. At the beginning of this stage, cells should be a confluent monolayer, although some cell clustering may persist from the previous stage (Figure

2e,j, Supplementary Figure 1e,o). Prepare stage 4 media by adding KGF (final concentration of 50 ng/mL; 1:1,000 dilution of stock aliquot), TPPB (final concentration of 0.2 μM; 1:10,000 dilution of stock aliquot), SANT1 (final concentration of 0.25 μM; 1:4,000 dilution of stock aliquot), RA (final concentration of 0.1 μM; 1:100,000 dilution of stock aliquot), and LDN193189 (final concentration of 0.2 μM; 1:5,000 dilution of stock aliquot) to BE3 base media and warm in a 37°C water bath. Note that this is the same media used in stage 3 with the exception of a significantly lower RA concentration.

20. Aspirate stage 3 media and add BE3 supplemented with stage 4 differentiation factors. Culture the cells for 4 days, replacing the media every day.

▲**CRITICAL STEP**: Stage 4 is designed to turn on the important β cell transcription factor NKX6–1. Checking for the percentage of PDX1+/NKX6–1+ cells with flow cytometry (Figure 3c) and ICC (Figure 3e) at this stage is our most routinely used quality control check during this differentiation protocol. Differentiations should have >40% PDX1+/NKX6–1+ cells at the end of stage 4.

?TROUBLESHOOTING

Stage 5: Endocrine induction •TIMING 7 days (30–60 min/d hands-on)

21. At the beginning of this stage, the cells should continue to be in a monolayer, though often some holes may appear in this monolayer (Figure 2k,p, Supplementary Figure 1f,p). Prepare stage 5 media by adding SANT1 (final concentration of 0.25 μM; 1:4,000 dilution of stock aliquot), RA (final concentration of 0.1 μM; 1:100,000 dilution of stock aliquot), XXI (final concentration of 1 μM; 1:10,000 dilution of stock aliquot), Alk5i II (final concentration of 10 μM; 1:10,000 dilution of stock aliquot), and T3 (final concentration of 1 μM; 1:10,000 dilution of stock aliquot) to S5 base media and warm in a 37°C water bath.

▲**CRITICAL STEP**: For the first 24 hours only, add latrunculin A (final concentration of 1 μM; 1:237.2 dilution of stock aliquot) to this stage 5 media.

?TROUBLESHOOTING

22. Aspirate stage 4 media and add S5 media supplemented with the differentiation factors, including latrunculin A.

23. After the first 24 hours, aspirate the media and replace it with S5 media without latrunculin A. Note that because latrunculin A depolymerizes the cytoskeleton and thus the ability of cells to pull against their substrate, the cells will look spherical after the first 24 hours of stage 5 (Figure 2l,q, Supplementary Figure 1g,q). The cells should begin to flatten out again after removal of latrunculin A.

24. Continue to culture the cells with S5 media (without latrunculin A) for six more days. Replace the media every day through day 4 of stage 5. Starting on day 5,

cells for the remainder of the protocol can be fed every other day instead of every day.

▲**CRITICAL STEP**: Stage 5 is designed to convert the pancreatic progenitor population into endocrine. There can be up to 30% cell death during this stage, the extent of which is often dependent upon the number of progenitors lacking PDX1 expression. The cells tend to recover, however, and most of the plate should still be covered with cells in a successful differentiation by the end of this stage (Figure 2m,r, Supplementary Figure 1h,r). The degree of overall endocrine induction can be checked with flow cytometry by quantifying the percentage of CHGA+ cells, which should be >80% at the end of stage 5. Within this population, the percentage of SC-β cells can be determined by those that co-express NKX6–1+/C-peptide+. Furthermore, qRT-PCR at this point should reveal high expression of the hormones insulin, glucagon, and somatostatin.

Stage 6: SC-β cell maturation •TIMING 14–30+ days (30–60 min/d hands-on)

25. At the beginning of this stage, there will often be some regions of the plate with bare spots and others where cells are beginning to cluster together (Figure 2m,r, Supplementary Figure 1h,r). Warm ESFM in a 37°C water bath. Note that there are no differentiation factors that need to be added to this media.

26. Aspirate stage 5 media and replace with ESFM. Continue to replace the media every other day. After a few days in ESFM, the cells often become larger and more rounded (Figure 2n,s, Supplementary Figure 1i,s). They may also start to have a golden hue to them when observed under a brightfield microscope.

▲**CRITICAL STEP**: We have found that *in vitro* function peaks around 14 days into stage 6. The cells can remain functional for weeks in culture (Supplementary Figure 2a), though their function may deteriorate after extended periods of *in vitro* culture.

27. SC-β cell quality and function can either be assessed in planar culture (Option A) or alternatively, the SC-β cells can be aggregated into clusters (Figure 2o,t, Supplementary Figure 1j,t) for use in assays which cannot be performed on TCP, such as dynamic GSIS and transplantation into diabetic mice (Option B).

A. Assessing cells in planar culture

i. Continue planar culture by replacing the ESFM every other day through day 14, at which point the standard set of assessments (static GSIS, steps 28–29; intracellular flow cytometry, steps 30–45; immunostaining, steps 46–55; qRT-PCR, steps 76–78; insulin content and proinsulin/insulin ratio, steps 79–86) can be performed to evaluate the SC-β cells.

B. Aggregating endocrine cells into islet-like clusters

i. On day 7 of stage 6, aspirate the media and add approximately 0.2 mL TrypLE/cm^2. Incubate for 10 minutes or less at 37°C.

ii. Once the cells start releasing from the plate, gently pipette up and down or gently tap the sides of the flask to finish dispersing the cells. Add an equal volume of ESFM to the cell suspension, mix well, and perform a cell count.

iii. Centrifuge the cell suspension at 300g for 3 minutes at room temperature to pellet the cells, aspirate the supernatant, and resuspend the cells in ESFM at a concentration of 1 million cells per mL.

iv. Pipette 5 mL of this cell solution into one well of a 6-well plate. Put this plate on an orbital shaker at 100 RPM in the cell culture incubator at 37°C and 5% CO_2.

v. Continue to feed the cells every other day with ESFM on the orbital shaker. Cells will form clusters over the next several days, reaching a stable cluster size of approximately 200–250 μm in diameter (Figure 2t, Supplementary Figure 3a).

?TROUBLESHOOTING

vi. After allowing the cells to form clusters and recover over the next week, the islet-like clusters can be assessed around day 14 of stage 6 (static GSIS, steps 28–29; dynamic GSIS, steps 87–103; intracellular flow cytometry, steps 30–45; histology, steps 56–75; qRT-PCR, steps 76–78; insulin content and proinsulin/insulin ratio, steps 79–86).

▲**CRITICAL STEP**: Up to 50% of cells can be lost during aggregation depending on the efficiency of the differentiation. Mostly endocrine cells seem to aggregate into clusters with this method, and thus this simple aggregation step tends to purify the endocrine population (Supplementary Figure 3b) since any remaining progenitor and off-target cell types are prone to dying during this procedure. As a result, we tend to get better performance out of aggregated clusters when compared to cells left on the plate. If functional performance is lacking during static GSIS assays performed in planar culture, this aggregation may help to improve results (Supplementary Figure 3c). After aggregation, clusters should consist of >80% CHGA+ cells and approximately 20–60% NKX6–1+/C-peptide+ cells as assessed by flow cytometry. For any given cell line, the percentage of SC-β cells generated after

aggregation is similar from different culture vessel sizes used for the differentiation (Supplementary Figure 3d).

?TROUBLESHOOTING

Static GSIS •TIMING 6 hours (4 hrs for setup and sample collection, 2 hrs to quantify insulin with ELISA)

28. The SC-β cells attain function typically between days 10–14 of stage 6, at which point they can be assessed by a static glucose-stimulated insulin secretion assay. On the day of the assay, prepare fresh KrB as well as the low (2 mM) and high (20 mM) glucose solutions using this KrB as described in “Reagent Setup.” The remainder of the procedure differs slightly depending on whether it is performed on cells still attached to the plate (Option A) or on aggregated clusters (Option B).

A. Static GSIS for cells attached to TCP

i. Aspirate the media and gently wash each well 3x with KrB.

ii. Add the 2 mM glucose solution into each well and incubate for 1 hour at 37°C and 5% CO_2. For a 24-well plate, use 1 mL of the glucose solutions for all steps. Scale accordingly for other well sizes.

▲**CRITICAL STEP**: During all incubations, return the unused low and high glucose solutions to the incubator with the caps loosened to maintain optimal temperature and pH.

iii. After one hour, aspirate the low glucose solution and wash once with KrB. Add fresh 2 mM low glucose to each well again and incubate for 1 hour.

iv. After one hour, pipette the supernatants into a separate, adequately labeled plate. The insulin within these samples that was secreted at low glucose will be quantified later. The samples can either be placed in the fridge at 4°C if they are to be quantified immediately following the assay, or at −80°C for up to 3 months.

▲**CRITICAL STEP**: View all collected supernatants under a microscope to ensure that no cells have come off the plate. If a few cells are in the collected sample, allow them to settle and transfer the supernatant to a new collection well. Any cell left in the supernatant can lyse and release all of its insulin content, which can lead to incorrect values calculated for the amount of secreted insulin.

v. Wash each well once with KrB and replace with the 20 mM high glucose solution. Incubate for 1 hour.

vi. After one hour, pipette the supernatants into a separate, adequately labeled plate. The insulin within these samples that was secreted at high glucose will be quantified later. The samples can either be placed in the fridge at 4°C if they are to be quantified immediately following the assay, or at −80°C for up to 3 months.

vii. Add TrypLE to each well and place in the incubator for 10 minutes. Use 1 mL TrypLE for a 24-well plate, and scale accordingly for other plate sizes.

viii. Pipette up and down to disperse the cells. Use this cell solution from each well to perform cell counts, which will be used later to calculate the amount of insulin secreted per cell in each well. Any leftover dispersed cells can be discarded or used for other assays, such as flow cytometry (steps 30–45) or qRT-PCR (steps 76–78).

■**PAUSE POINT** The supernatant samples can be either quantified immediately (Step 29) or stored at −80°C for up to 3 months.

B. Static GSIS for aggregated clusters

i. Prepare two 24-well plates by pipetting 1 mL of the 2 mM glucose solution into a well of each plate for every sample to be run. Prepare a third 24-well plate by pipetting 1 mL of the 20 mM glucose solution into one well for every sample to be run.

ii. In a fourth 24-well plate, use forceps to place a transwell insert (MilliporeSigma, PIXP01250) into a well for every sample to be run. Pipette 0.5 mL of KrB into each transwell.

iii. Collect approximately 30 SC-β cell clusters with a P1000 and pipette them into one of the transwell inserts. We recommend running at least three replicates per condition.

iv. Add another 0.5 mL of KrB into each transwell and allow the clusters to settle.

v. Use forceps to remove each transwell, being careful to do so slowly and angling the transwell to allow wicking to drain the liquid. Place each transwell into another well containing fresh KrB to further wash each sample. Repeat this wash step one additional time.

▲**CRITICAL STEP**: During all steps that transfer the transwell inserts to another well, care should be taken to ensure that almost all of the liquid has drained. When performed correctly, this draining should only take about 5 seconds per transwell.

vi. Transfer each transwell into a well of one of the 24-well plates containing the 2 mM glucose solution. Incubate for 1 hour at 37°C and 5% CO_2.

▲**CRITICAL STEP**: During all incubations, also place the 24-well plates containing the unused low and high glucose solutions in the incubator to maintain optimal temperature and pH.

vii. After 1 hour, transfer each transwell into a well of the other 24-well plate containing the fresh 2 mM glucose solution and incubate for another hour. Discard the used plate.

viii. After 1 hour, transfer each transwell into a well of the 24-well plate containing the fresh 20 mM glucose solution and incubate for another hour. Keep the used plate, as the insulin within these samples that was secreted at low glucose will be quantified later (Step 29). The samples can either be placed in the fridge at 4°C if they are to be quantified immediately following the assay, or at −80°C for up to 3 months.

ix. After 1 hour, transfer each transwell into a well of another 24-well plate containing 1 mL of TrypLE and incubate at 37°C for 10 minutes. Keep the used plate, as the insulin within these samples that was secreted at high glucose will be quantified later. The supernatant samples can either be placed in the fridge at 4°C if they are to be quantified immediately following the assay, or at −80°C for up to 3 months.

x. Pipette up and down to disperse the cells. Use this cell solution from each well to perform cell counts, which will be used later to calculate the amount of insulin secreted per cell in each well. Any leftover dispersed cells can be discarded or used for other assays, such as flow cytometry (steps 30–45) or qRT-PCR (steps 76–78).

■**PAUSE POINT** The supernatant samples can be either quantified immediately or stored at −80°C for up to 3 months.

29. After either procedure, quantify the total amount of insulin within each sample from both the low and high glucose challenges on a human insulin ELISA according to the manufacturer's instructions. Here, we present insulin units in

either μIU (international units) or ng, where 1 μIU equals 0.0348 ng insulin. To normalize each sample, divide this calculated insulin value by its respective cell count to determine the total insulin secreted per cell.

▲**CRITICAL STEP**: Due to the high functionality of the SC-β cells generated with this protocol, we often have to dilute our samples in KrB (typically by 3–5x) in order to avoid saturating the ELISA.

▲**CRITICAL STEP**: In order to account for variations in differentiation efficiency between cell lines, insulin secretion can also be normalized to the number of SC-β cells rather than the total cell number (Supplementary Figure 2b). Multiply the cell count for each GSIS sample by the percentage of C-peptide+/NKX6–1+ cells as assessed by flow cytometry to get the total number of SC-β cells per sample, and divide the insulin secreted in each sample by its number of SC-β cells.

▲**CRITICAL STEP**: Because C-peptide is a byproduct of insulin processing and is secreted with insulin, a C-peptide ELISA can alternatively be used to quantify insulin secretion, particularly if there are concerns about insulin being taken up into the cells from the environment. Furthermore, measuring C-peptide levels can also be useful during transplantation studies, as it persists longer *in vivo* than insulin. However, there is no insulin in the ESFM formulation used in stage 6 or the KrB used during GSIS, and we prefer to measure insulin protein levels directly when possible.

Intracellular flow cytometry •TIMING 2 days (2.5 hrs sample preparation and primary antibody staining on day 1, 3 hrs sample preparation and staining secondary antibodies on day 2)

1. At the end of the desired stage, aspirate the media and add approximately 0.2 mL TrypLE/cm^2. For aggregated clusters at the end of stage 6, pipette the clusters into a microcentrifuge tube with a P1000 and add 1 mL TrypLE. Incubate for 10 minutes at 37°C.
2. Pipette up and down gently to disperse the cells, transfer the solution to a microcentrifuge tube, and centrifuge at 300g for 3 minutes at room temperature.
3. Aspirate the TrypLE and add 0.5 mL of 4% (wt/vol) PFA solution for 30 minutes at 4°C.

   ▲**CRITICAL STEP**: To avoid cells sticking together, be sure to disperse the cell pellet immediately after adding the 4% PFA by either pipetting up and down or vigorously shaking the microcentrifuge tube.

   !CAUTION: We advise using the 4% PFA in a chemical fume hood to avoid repeated exposure.
4. Centrifuge at 1000g for 3 minutes at room temperature. Remove and appropriately dispose of the PFA.
5. Resuspend in 1 mL PBS and mix well to disperse the cell pellet.

- ■**PAUSE POINT** These fixed cells can be stored at 4°C for at least a month.

6. Centrifuge at 1000g for 3 minutes at room temperature.

7. Discard the PBS, add 0.5 mL of ICC solution (see “Reagent Setup”), mix well, and incubate for 45 minutes at 4°C.

8. Prepare the primary antibodies by diluting them in ICC solution as described in Table 9. Cells can be co-stained with multiple markers depending on the capabilities of the flow cytometer and compatibility of the antibodies.

9. Centrifuge the samples at 1000g for 3 minutes at room temperature.

10. Remove the ICC solution. Resuspend cells in 0.3 mL of the primary antibody solutions and incubate overnight at 4°C.

11. Prepare the appropriate secondary antibodies by diluting them in ICC solution at a 1:300 dilution.

12. Centrifuge the samples at 1000g for 3 minutes at room temperature.

13. Remove the ICC solution containing the primary antibodies. Resuspend cells in 0.5 mL ICC solution, mix well, and centrifuge the samples at 1000g for 3 minutes at room temperature.

14. Remove the ICC solution. Resuspend cells in 0.3 mL of the prepared secondary antibodies, mix well, and incubate for 2 hours at 4°C.

15. Centrifuge the samples at 1000g for 3 minutes at room temperature. Remove the ICC solution and resuspend cells in 0.3 mL ICC solution.

16. Pipette the stained cell solution through the strainer caps on polystyrene test tubes used for flow cytometry, and run samples on a flow cytometer the same day. Record approximately 50,000 cells for each sample.

    ▲**CRITICAL STEP**: Because the markers measured during the differentiation protocol only turn on after the start of differentiation, it is useful to include stem cells as a negative biological control when running the samples on the flow cytometer (Figure 3a–c). Perform this staining procedure on fixed stem cells in parallel with the other samples. A stock of fixed stem cells can be kept at 4°C for at least one month for this purpose.

    ▲**CRITICAL STEP**: The results can be analyzed with an appropriate software package, such as FlowJo. Cell debris and doublets can be gated out (Supplementary Figure 4a), and then the negative stem cell control can be used to gate the cell populations (Figure 3a–c). A secondary antibody only control can also be run (Supplementary Figure 4b). Human islets can be used as a positive control for PDX1, NKX6–1, CHGA, GCG, SST, C-peptide, and FOXA2 (Supplementary Figure 5a–f). The HepG2 or OVCAR-3 cell line can be used as a positive control for SOX17 (Supplementary Figure 5g).

Immunocytochemistry •TIMING 2 days (2 hrs sample preparation and primary antibody staining on day 1, 3 hrs sample preparation and staining secondary antibodies on day 2)

1. Throughout the protocol, cells can be fixed and immunostained directly on the plate for markers of various differentiation stages (Table 9). At the desired timepoint, aspirate the media and wash each well with PBS.
2. Aspirate the PBS and add enough 4% (wt/vol) PFA to cover the cells. Incubate at room temperature for 30 minutes.
3. Remove and properly dispose of the PFA.
4. Wash the wells once with PBS and add ICC solution (see “Reagent Setup”) for 45 minutes at room temperature to block and permeabilize the cells.
5. Prepare the primary antibodies by diluting them in ICC solution as described in Table 9.
6. Remove the ICC solution, add the diluted primary antibodies, and incubate overnight at 4°C.
7. Prepare the appropriate secondary antibodies by diluting them in ICC solution at a 1:300 dilution.
8. Remove the primary antibodies, wash once with ICC solution, and add the secondary antibodies. Incubate for 2 hours at room temperature, protecting samples from light.
9. Remove the secondary antibodies, wash once with ICC solution, and add the DAPI nuclear stain. Incubate for 12 minutes at room temperature, protecting samples from light.
10. Remove the DAPI nuclear stain, wash once with ICC solution, and then once with PBS. Store samples in PBS at 4°C until they are imaged on a confocal or wide-field fluorescence microscope. The images can be processed with an appropriate software package, such as ImageJ. Examples of secondary antibody only controls are given in Supplementary Figure 6.

Histology for stage 6 aggregated clusters •TIMING 2 days (8 hrs sample preparation and primary antibody staining on day 1, 3 hrs sample preparation and secondary staining on day 2)

1. In order to immunostain stage 6 SC-β cells after aggregation, the clusters must be embedded in paraffin and sectioned into slides. To begin this procedure, pipette the clusters into a microcentrifuge tube with a P1000. Allow the clusters to settle and remove the media. Add 1 mL PBS and gently invert the tube to wash the clusters.
2. Allow the clusters to settle, remove the PBS, and add 0.5 mL of 4% (wt/vol) PFA solution for 30 minutes at room temperature.
3. Remove and properly dispose of the PFA.

4. Wash once with PBS, suspend the clusters in 20 μL of warmed HistoGel according to the manufacturer's instructions, and allow the HistoGel to solidfy for 30 minutes at 4°C.

5. Remove the samples from the microcentrifuge tube with a 200 μL pipette, place them in a histoscreen cassette, and store it in 70% (vol/vol) ethanol at 4°C.

6. Paraffin-embed samples and section onto glass slides using standard histological techniques.[50]

   ■**PAUSE POINT** The sectioned samples can be stored for at least 1 year in a slide box at room temperature.

7. To remove the paraffin from the slides in preparation for immunostaining, fill 9 containers with 100 mL of the following liquids and transfer the slides between them for the time indicated in Table 10.

8. Rinse the glass slides with Milli-Q water until no ethanol remains.

9. Perform antigen retrieval by placing slides into a 0.05% (vol/vol) EDTA solution and run in a pressure cooker for 2 hours.

10. Rinse glass slides carefully and slowly at least two times with Milli-Q water and once with PBS.

11. Block histology samples for 30 minutes at room temperature by pipetting 300 μL of ICC solution onto the slide and placing a coverslip on top.

12. Remove the coverslip and ICC solution. Apply primary antibodies diluted in ICC as detailed in Table 9. Note, however, that we use different GCG and SST antibodies for immunohistochemistry. This GCG antibody comes prediluted but can be further diluted 1:2.

13. Place coverslips on the slides and incubate in a humidified box overnight at 4°C by laying the slides on top of wet paper towels inside a slide box.

14. Remove the coverslips and wash the slides with PBS.

15. Remove the liquid and apply the appropriate secondary antibodies at a 1:300 dilution in ICC solution.

16. Place coverslips on the slides and incubate in the humidified box for 2 hours at room temperature.

17. Remove the coverslips and wash slides in PBS for 5 minutes.

    ▲**CRITICAL STEP**: Do not let samples dry out at this point.

18. Remove the liquid and use a P1000 pipette to place 2–3 drops of DAPI Fluoromount-G solution to each slide.

19. Place a coverslip onto each slide and allow the solution to dry, protecting the slides from light.

▲**CRITICAL STEP**: Be careful to not introduce bubbles under the coverslip, especially where samples are located on the slide.

20. Use a sealing solution, such as clear nail polish, to secure the coverslip. Store the samples in a slide box at 4°C until they are imaged on a confocal or wide-field fluorescence microscope.

qRT-PCR •TIMING 7 hours (1.5 hrs for RNA extraction, 2.5 hrs for reverse transcription, 3 hrs for plate setup and PCR reaction)

1. To process planar cells for qRT-PCR directly on the plate during differentiation, aspirate the media, add RLT lysis buffer into each well, incubate at room temperature for 5 minutes, pipette vigorously to disperse the cells, and transfer to a microcentrifuge tube. Alternatively, collect aggregated stage 6 clusters in a microcentrifuge tube, aspirate the media, add 350 μL RLT lysis buffer, incubate at room temperature for 5 minutes, and pipette vigorously to disperse cells.

   ■**PAUSE POINT** Lysed cells can be stored in RLT buffer at −20°C for several months.

2. Continue with the mRNA extraction, reverse transcription reaction, and PCR reaction according to the manufacturer's instructions.

3. There are large changes in gene expression as cells progress through the various stages that can be helpful to track the success of a differentiation. Table 2 details markers for each stage with validated primers.

Insulin content and proinsulin/insulin ratio •TIMING 3 days (1 hr on day 1, 10 min on day 2, 2 hrs on day 3 for each ELISA)

1. Around day 14 of stage 6, aspirate the media from cells still attached to the plate and wash with PBS. For aggregated clusters, pipette the clusters into a microcentrifuge tube with a P1000 and wash with PBS.

2. For normalization of total insulin content, disperse either an equivalent but separate well of planar cells or half of the aggregated clusters with TrypLE. Perform cell counts on these samples.

   ▲**CRITICAL STEP**: Cells should not be single-cell dispersed with TrypLE before adding the acid-ethanol solution, as this can interfere with the assay. Thus, to obtain cell counts for insulin content when cells are still attached to the plate, use a separate well from the same differentiation. This approximation works well since cell numbers are consistent across similar wells of the same differentiation. For aggregated clusters within a microcentrifuge tube, gently pipette up and down to evenly suspend the clusters, transfer half of the volume to a new microcentrifuge tube, and disperse these cells for counting.

3. Remove the PBS from the washed cells and add 0.5 mL acid-ethanol solution (see "Reagent Setup") per well of a 24-well plate or to clusters in a single microcentrifuge tube. For cells still attached to the plate, pipette up and down

vigorously to disperse the cells and move to a microcentrifuge tube. Vortex the tubes vigorously and incubate overnight at −20°C.

4. After 24 hours, vortex the microcentrifuge tubes vigorously and place them back at −20°C.

5. After an additional 24 hours, vortex the microcentrifuge tubes vigorously and centrifuge them at 2,100g for 15 minutes at room temperature.

6. Transfer the 0.5 mL supernatant to a new tube and neutralize it by adding 0.5 mL of 1M TRIS.

7. To determine insulin content, run this solution on a human insulin ELISA according to the manufacturer's instructions. Normalize to cell counts to get the total insulin content per cell.

   ▲**CRITICAL STEP**: Due to the high levels of insulin within SC-β cells generated with this protocol, we often have to dilute our samples in KrB by 500x or more in order to avoid saturating the insulin ELISA.

8. To determine the proinsulin/insulin ratio, run the same samples on a human proinsulin ELISA according to the manufacturer's instructions. To get the proinsulin/insulin ratio, divide the value obtained from the proinsulin ELISA by the value calculated from the insulin ELISA from the same sample.

   ▲**CRITICAL STEP**: Due to the high levels of insulin and proinsulin within SC-β cells generated with this protocol, we often have to dilute our samples in KrB by 100x or more in order to avoid saturating the proinsulin ELISA. Be sure to adjust both the calculated insulin and proinsulin values by their respective dilution factors before calculating the proinsulin/insulin ratio.

Dynamic GSIS •TIMING 8 hours (5 hrs for setup and sample collection, 2 hrs to quantify insulin with ELISA, 1 hr to quantify DNA content)

1. Prepare the perfusion setup by first connecting the 0.015 inch inlet and outlet tubing to the 275-μL cell chambers with 0.04 inch connection tubing (Figure 4a). Connect the other end of the outlet tubing to a dispensing nozzle. Connect the inlet tubing to a high precision 8-channel dispenser pump.

   ▲**CRITICAL STEP**: Here, we describe a custom setup for performing a dynamic GSIS assay in order to provide an accessible and cost-efficient system. Alternatively, this dynamic GSIS procedure can also be adapted to work on the commercially available Biorep Perfusion System (BioRep Diabetes, PERI5-FA-115), following the manufacturer's instructions for operation.

2. Scoop approximately 5 mL of Bio-Gel beads into a 50 mL tube. To hydrate the beads, add 40 mL of Milli-Q water and incubate at 75°C with gentle agitation for 90 minutes.

!CAUTION: Opening and handling of the dry beads should be done in a fume hood or biosafety cabinet to avoid inhalation of the beads.

3. While the beads are hydrating, prepare 500 mL of KrB as described in Table 6. With this KrB, prepare 150 mL of 2 mM glucose and 50 mL of 20 mM glucose. Store these solutions in the 37°C water bath with their caps loose.

4. Place the inlet tubing into a 50 mL tube containing Milli-Q water. With the empty cell chambers connected, turn on the perfusion pump at a flow rate of 200 μL/min for 15 minutes to purge air from the tubes and rinse the tubing.

5. After flushing the system, replace the Milli-Q water with KrB and continue to pump for 15 minutes to replace the Milli-Q water with KrB in the lines and chamber.

6. Use a hole punch to cut out one piece of filter paper per chamber. Stop the pump and unscrew the outlet cap from the chamber body. Using forceps, remove the orange gasket in the outlet cap, place the filter paper into the outlet cap, replace the orange gasket, and reattach the outlet cap to the chamber body (Figure 4a).

7. After the Bio-Gel beads have incubated for at least 90 minutes, wash them twice with Milli-Q water and then twice with KrB, resuspending the beads finally in KrB.

8. Unscrew the chamber body from the inlet cap and pipette approximately 200 μL of settled beads into each chamber (Figure 4b). Allow beads to settle for 5 minutes.

9. Collect approximately 15–30 cell clusters per chamber and wash them twice with KrB. Pipette these clusters into the chambers on top of the settled beads (Figure 4b). After cells have settled, add an additional 200 μL of beads into the chamber (Figure 4c).

10. Turn the pump on at a flow rate of 100 μL/min and reattach the chamber to inlet cap. Ensure that KrB is coming out at the metal outlet nozzles and that there are no leaks in the tubing. Once confirmed that there are no leaks, place the chambers in the water bath (Figure 4d).

    ▲**CRITICAL STEP**: Immediately check to ensure that beads and cells are not leaking into the outlet tubing. If this occurs, stop the pump immediately and replace the filter paper.

11. Move the inlet tubing from the KrB into the 2 mM glucose KrB solution in the water bath (Figure 4d). Run for 80 minutes, refilling the glucose solution periodically to ensure that it does not run out. The flow-through may be discarded.

12. Arrange each metal outlet nozzle into a holder or rack so that the nozzles will drip easily into a 96-well plate (Figure 4d). Label two 96-well plates long-ways, corresponding to the 8 cell chambers.

13. After the 80 minute pre-incubation, move the metal outlet nozzles over the first 96-well plate and start collecting the effluent. After 2 minutes, manually move the 96-well plate to the next row and collect the effluent. Each well should collect 200 μL of sample. Continue this procedure until the end of the experiment. We typically collect 4 time points (every 2 minutes over an 8 minute interval) at 2 mM glucose, 12 time points (over 24 minutes) at 20 mM glucose, and 8 time points (over 16 minutes) at 2 mM glucose. This is a total of 24 time points (48 minutes) that will fit on two 96-well plates long-ways.

    ▲**CRITICAL STEP**: Set a timer and be very exact when moving the plate every 2 minutes.

    ▲**CRITICAL STEP**: In order to switch from one glucose concentration to another, stop the pump, move the inlet tubing into the appropriate 50 mL tube, then start the pump. It is important to do this as fast as possible and to minimize the introduction of bubbles.

14. At the end of the experiment, cover the 96-well collection plates with an adhesive cover and place in the −80°C freezer.

15. To collect the cells for DNA content quantification, unscrew the inlet and outlet caps and place the chamber body in a microcentrifuge tube. Add 1 mL of the DNA content lysis buffer (see “Reagent Setup”) to the chamber body to flush the beads containing the cells into the microcentrifuge tube. Vortex for 30 seconds every 10 minutes for a total of 30 minutes, keeping the tubes on ice. Store the samples at −80°C until ready to quantify them with the Quant-iT Picogreen dsDNA assay kit according to the manufacturer’s instructions.

16. To clean the components, rinse the chamber body, outlet cap, and gaskets in a beaker of Milli-Q water. Start pumping 50 mL Milli-Q water through the system and reattach the chambers. Continue to run the pump for approximately 30 minutes, which will drain the water and then pump air through the system to dry the tubing.

    ▲**CRITICAL STEP**: It is important to thoroughly clean the components in order to avoid residual salts from drying and clogging the tubing before the next use.

17. Run each well of the collected samples on a human insulin ELISA according to the manufacturer’s instructions, yielding the amount of insulin secreted into 200 μL every 2 minutes. Normalize these values to the DNA content for each sample.

TIMING

The differentiation of stem cells to SC-β cells takes approximately 5 weeks to complete, plus 1–2 weeks for stem cell expansion before beginning differentiation and assessment of the SC-β cells at the end of the protocol. The amount of time spent each day on cell culture will depend on the number of separate differentiations and plate configurations.

Production of SC-β cells

Steps 1–9, Stem cell culture and seeding differentiations (Stage 0): ~8 days (1–2 hrs/d hands-on)

Steps 10–12, Definitive endoderm differentiation (Stage 1): 4 days (30–60 min/d hands-on)

Steps 13–15, Primitive gut tube generation (Stage 2): 2 days (30–60 min/d hands-on)

Steps 16–18, Generating pancreatic progenitor 1 cells (Stage 3): 2 days (30–60 min/d hands-on)

Steps 19–20, Generating pancreatic progenitor 2 cells (Stage 4): 4 days (30–60 min/d hands-on)

Steps 21–24, Endocrine induction (Stage 5): 7 days (30–60 min/d hands-on)

Steps 25–27, SC-β cell maturation (Stage 6): 14–30+ days (30–60 min/d hands-on)

In vitro assays to assess SC-β cell quality and function

Steps 28–29, Static GSIS: 6 hours (4 hrs for setup and sample collection, 2 hrs to quantify insulin with ELISA)

Steps 30–45, Intracellular flow cytometry: 2 days (2.5 hrs sample preparation and primary antibody staining on day 1, 3 hrs sample preparation and staining secondary antibodies on day 2)

Steps 46–55, Immunostaining: 2 days (2.5 hrs sample preparation and primary antibody staining on day 1, 3 hrs sample preparation and staining secondary antibodies on day 2)

Steps 56–75. Histology: 2 days (8 hrs sample preparation and primary antibody staining on day 1, 3 hrs sample preparation and secondary staining on day 2)

Steps 76–78, qRT-PCR: 7 hours (1.5 hrs for RNA extraction, 2.5 hrs for reverse transcription, 3 hrs for plate setup and PCR reaction)

Steps 79–86, Insulin content and proinsulin/insulin ratio, 3 days (1 hr on day 1, 10 min on day 2, 2 hrs on day 3 for each ELISA)

Steps 87–103, Dynamic GSIS: 8 hours (5 hrs for setup and sample collection, 2 hrs to quantify insulin with ELISA, 1 hr to quantify DNA content)

TROUBLESHOOTING

The main assays that can be used to check differentiation efficiency during the protocol are flow cytometry (quantitative) or immunocytochemistry imaging (qualitative) to ensure >90% of the cells co-express SOX17 and FOXA2 at the end of stage 1, >80% of the cells express PDX1 at the end of stage 3, and >40% of the cells co-express PDX1 and NKX6–1 at the end of stage 4 (Figures 1,3). Comparing gene expression between stages with qRT-PCR can also be useful for determining if a particular gene has turned on, as expression levels of these genes will change drastically between stages. Each stage depends upon efficient

induction of the previous cell type, and thus later stages will not produce the desired cells if the previous stage does not work efficiently. Therefore, monitoring these genes throughout the protocol allows the user to pinpoint where any issues are occurring. Cell morphology can also be an initial indicator of success, as various cell lines look similar at each stage (Figure 2, Supplementary Figure 1), but users should ultimately rely on marker expression to assess differentiation status. If any of these stages does not meet the specified threshold for these markers, the differentiation should be discarded, since the progenitors will not efficiently produce SC-β cells. The first troubleshooting steps that should be attempted if any of these thresholds are not met are to check the pluripotency of the starting stem cell population and adjust initial cell seeding density at the beginning of the protocol, as these parameters can have strongest effect on the success of the differentiation. A full troubleshooting workflow is outlined in Supplementary Figure 7. The culture parameters summarized in Table 11, however, should allow a user to differentiate these specific cell lines, including the commonly used HUES8 and H1 cell lines, as stated without any further optimization.

Unique cell lines will naturally have different competencies to differentiate into various cell types, and thus it is not expected that cell lines from a wide variety of genetic backgrounds will necessarily have the same differentiation efficiency. While we have had very good success with differentiating new iPSC lines with this protocol, we recommend performing some initial troubleshooting when adapting the protocol to a new cell line. First, we strongly recommend optimizing the initial seeding density of stem cells prior to executing the entire differentiation process. We suggest starting with 0.53×10^6 cells/cm^2 (i.e., 5×10^6 cells in a well of 6 well plate). Ensure that this seeding density creates a confluent monolayer of cells 24 hours after seeding. If the culture is not confluent, increase seeding density by increments of 0.1×10^6 cells/cm^2. If the stem cells are over-confluent, reduce cell seeding density in a similar manner, though over-confluency is less of an issue. Typically, if cells are not confluent enough, they begin to fall off the plate, further exacerbating the issue. Even if the cells remain on the plate, the effects of improper seeding cell density on cell fate will become apparent by stage 4 as determined by a low percentage of PDX1+ cells. We recommend testing for differentiation performance by measuring PDX1+ yields at the end of stage 3 using flow cytometry. We expect to see at least 80% PDX1 positivity for the differentiation to be successful. If low PDX1+ cell yields persist after optimizing initial cell seeding density, additional steps that have worked on certain cell lines include adding 10 ng/mL of bFGF throughout stage 1 of the differentiation or using the Stemcell Technologies Definitive Endoderm kit (catalog #05110) in place of stage 1 media and factors.

After optimizing pancreatic progenitor generation, further troubleshooting is typically not needed. However, there may be some cases where an extensive amount of cell death occurs following latrunculin A treatment during stage 5. If so, we recommend lowering the latrunculin A concentration to 0.75 or 0.5 μM. Up to 30% cell death can still occur during this stage, however, and is dependent upon the efficiency of the differentiation. On the other hand, if there are large areas of off-target cells at the end of stage 5 that look different than the expected morphology (Figure 2m,r), latrunculin A concentration may need to be increased to 1.5 or 2 μM. If problems continue during stage 5, feeding the cells every day for the entirety of stage 5 can help. Table 11 outlines the conditions used for the cell

lines presented here. Table 12 details these and other troubleshooting steps that help ensure successful differentiations.

ANTICIPATED RESULTS

SC-β cells generated with this protocol should exhibit several key characteristics, including glucose responsiveness in both static and dynamic GSIS assays as well as high expression of endocrine gene and protein markers, all of which can be assessed by the *in vitro* assays detailed here. While the extent of this functionality will depend on the particular cell line and its disease state, this protocol is very robust in generating SC-β cells from a variety of cell lines. These stem cell lines can be derived from a wide range of genetic backgrounds, including iPS lines of patients with T1D, T2D, and monogeneic forms of diabetes such as MODY and Wolfram Syndrome. Studying β cells derived from these disease states can be potentially very useful in investigating diabetic pathogenesis and testing new treatment options.

Here, we provide representative data from *in vitro* assessment assays of SC-β cells generated from 10 different human stem cell lines after aggregation into clusters. All data presented here have been freshly generated and not published previously. On average, this protocol generates approximately the same number of endocrine cells as the number of starting stem cells, though this yield will vary depending on the differentiation efficiency of a particular cell line. In other words, one well of a 6-well plate that is seeded with 5 million stem cells in Stage 0 will typically yield 5 million endocrine cells after aggregation into clusters. Around 20–60% of these will be SC-β cells (Figure 5a). This protocol was originally developed and optimized with the HUES8 cell line, and therefore it can provide a good benchmark for comparing SC-β cells generated from new lines.

All lines presented here secrete insulin in a static GSIS assay, though the degree of functionality is dependent on the specific cell line and its disease state (Figure 5b). For example, an iPSC line with a pathogenic variant in the WFS1 gene ($WS4^{unedit}$) generates SC-β cells that are not glucose-responsive, but CRISPR-correcting this variant ($WS4^{corr}$) allows these stem cells to produce highly functional SC-β cells (Figure 5b).[42] The best functioning lines can even approach the insulin secretion levels observed in human islets (Figure 5b), and SC-β cells from all lines are able to be aggregated into islet-like clusters (Figure 5c). These clusters contain more than 80% CHGA+ endocrine cells, with a majority being β cells in addition to small numbers of glucagon (GCG) and somatostatin (SST) positive cells (Figure 6a,b). The ratio of these endocrine cell types is similar across cell lines, but the number of alpha and delta cells present in these clusters are lower than in a human islet (Figure 6b). These islet-like clusters also exhibit the first and second phase dynamic insulin secretion kinetics that is observed in primary human islets, though the magnitude of dynamic response is often lower (Figure 6c). Furthermore, these SC-β cells have high levels of insulin content (Figure 6d) and a low ratio (<0.1) of proinsulin/insulin content (Figure 6e), indicating proper insulin processing. Gene expression for many endocrine genes is close to that of human islets across cell lines (Figure 6f). In contrast to stem cells, they do not express high levels of genes that are known to be repressed in β cells, such as LDHA. One notable difference from primary human islets that we consistently

observe is that both CHGA and NEUROG3 are much higher in freshly generated SC-β cells. Furthermore, while MAFB is expressed similarly in SC-β cells, MAFA is much lower than in human islets. However, we have recently demonstrated that the transcriptional profile of SC-β cells becomes much more similar to native human islets after long-term transplantation into mice, demonstrating that *in vitro* generated SC-β cells have the potential for further maturation.[51]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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DATA AVAILABILITY

The original data for Figures 5 and 6 as well as sample calculations for these data are available in the Source Data section. Any other data are available from the corresponding author upon reasonable request.

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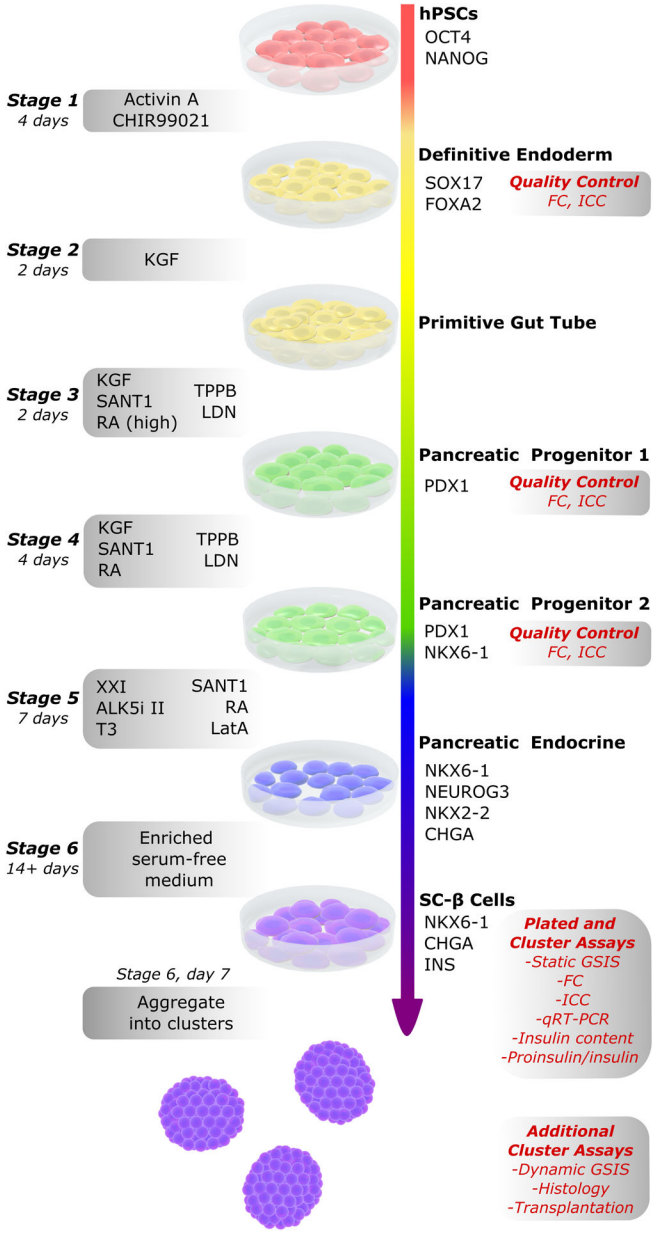

Figure 1). Overview of the 6-stage SC-β cell differentiation protocol.

HPSCs are first seeded onto Matrigel-coated plates. Over the next 5 weeks, these stem cells are driven through intermediate cell types toward a β cell fate by progressive stages of specific growth factor and small molecule combinations in serum-free media. This stepwise approach attempts to recreate stages of embryonic development in order to achieve high differentiation efficiency and SC-β cell maturity. At the end of stages 1, 3, and 4, the current quality of the differentiation can be assessed by flow cytometry (FC) and immunocytochemistry (ICC) of the indicated markers. After two weeks into stage 6, the presence of SC-β cells can be determined by measuring marker expression with FC, ICC, and qRT-PCR. Furthermore, the functionality of these cells can be assessed by a static glucose-stimulated insulin secretion (GSIS) assay as well as measuring both insulin content

and the ratio of proinsulin/insulin content. These assays can be completed on SC-β cells still attached to the plate or after they have been aggregated into islet-like clusters during stage 6. Additional assays can be performed on these clusters, including a dynamic GSIS assay to further profile their functional characteristics and transplantation into diabetic mice to assess the cell clusters' *in vivo* performance.

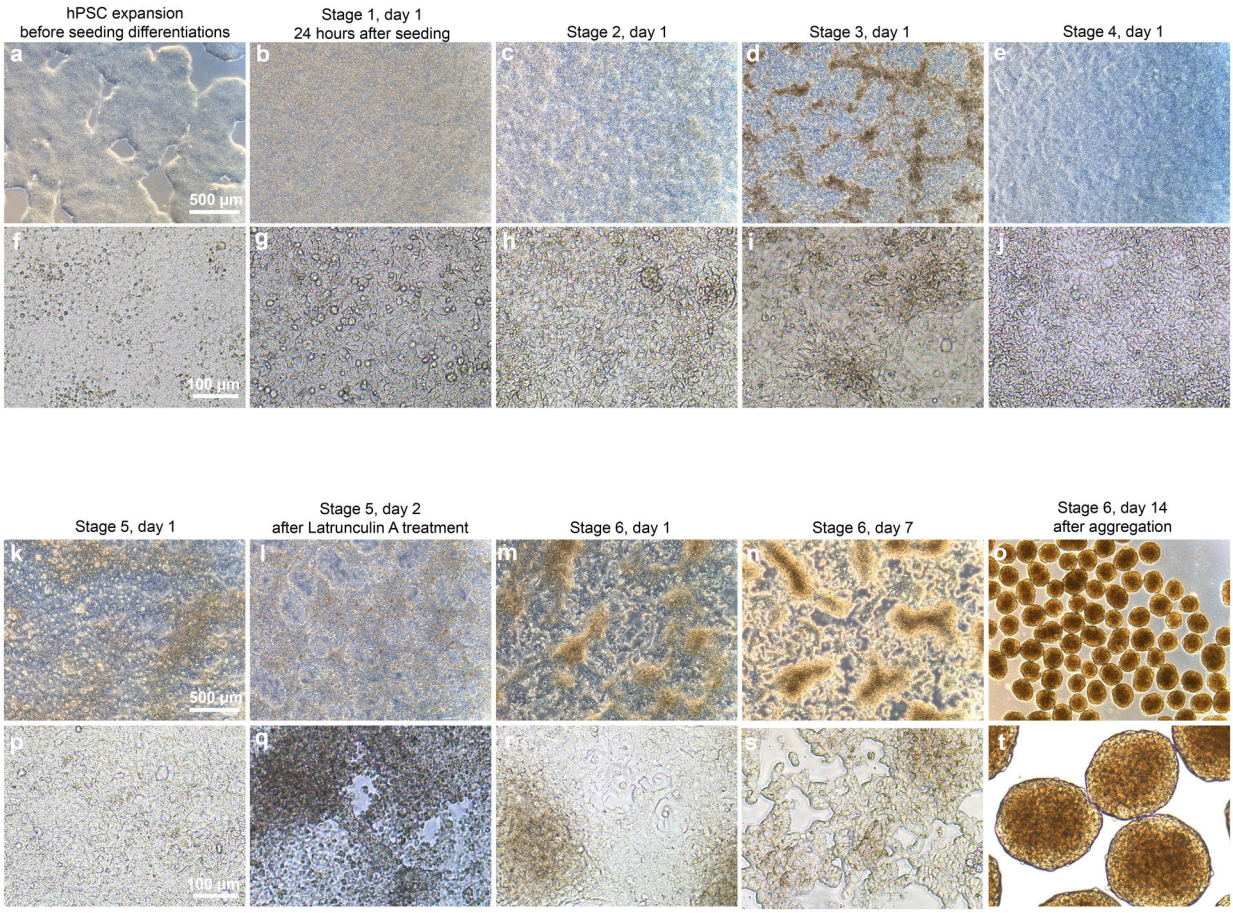

Figure 2). Morphology of differentiating cells.

Representative brightfield images at each stage of the protocol for the HUES8 cell line at both low (**a-e**, **k-o**, scale bar = 500 μm) and high (**f-j**, **p-t**, scale bar = 100 μm) magnification. The specific morphology at each stage can be indicative of the quality of the differentiation.

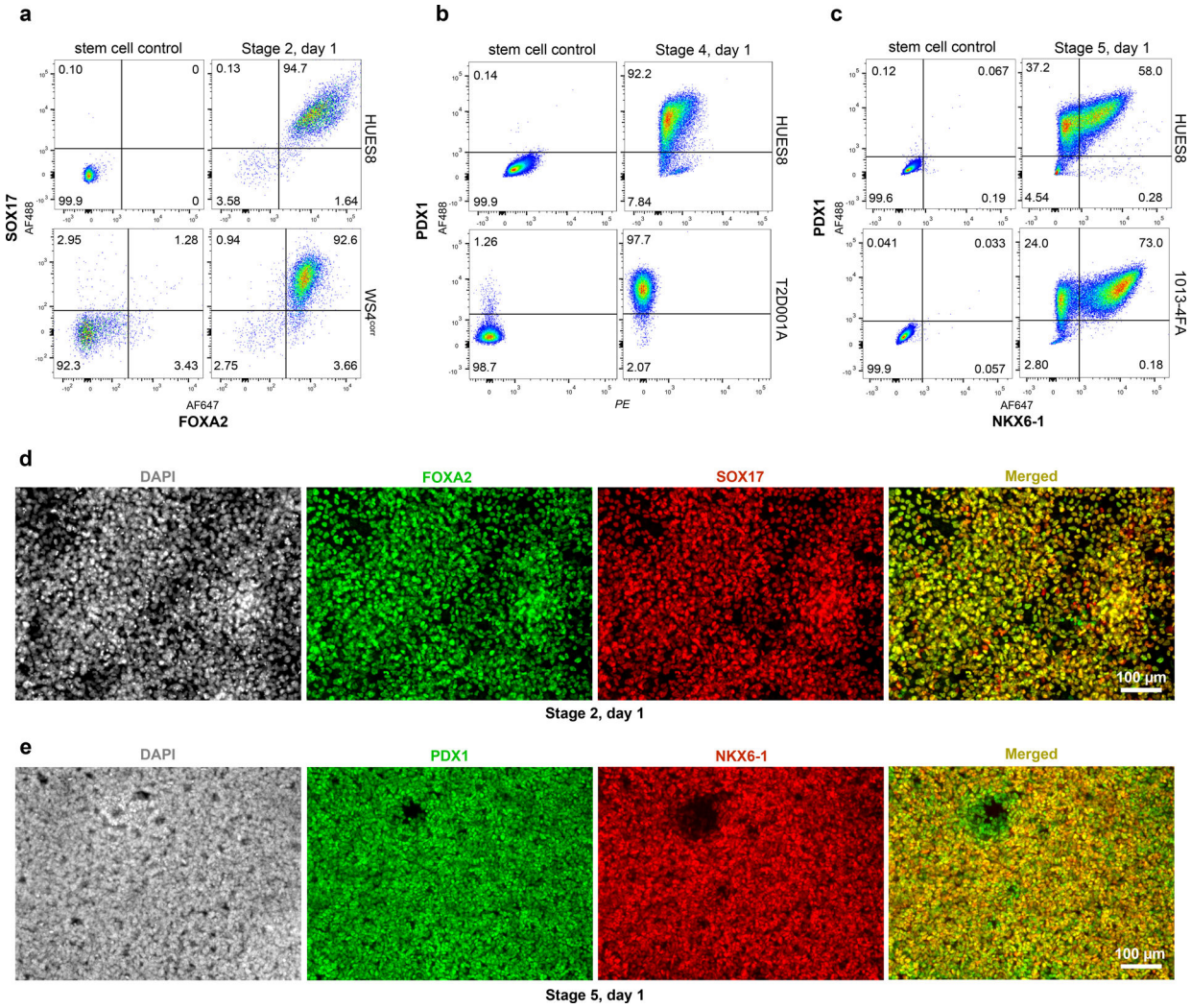

Figure 3). Quality control during differentiation.

To quantitatively assess differentiation status, flow cytometry can be used to look for key markers at (**a**) the end of stage 1 (FOXA2+/ SOX17+, >90%), (**b**) the end of stage 3 (PDX1+, >80%), and (**c**) the end of stage 4 (PDX1+/NKX6–1+, >40%). (**d-e**) These markers can also be quickly assessed qualitatively with ICC. The HUES8 line was stained (**d**) at the end of stage 1 for FOXA2/SOX17 and (**e**) at the end of stage 4 for PDX1/NKX6–1. Scale bars = 100 µm.

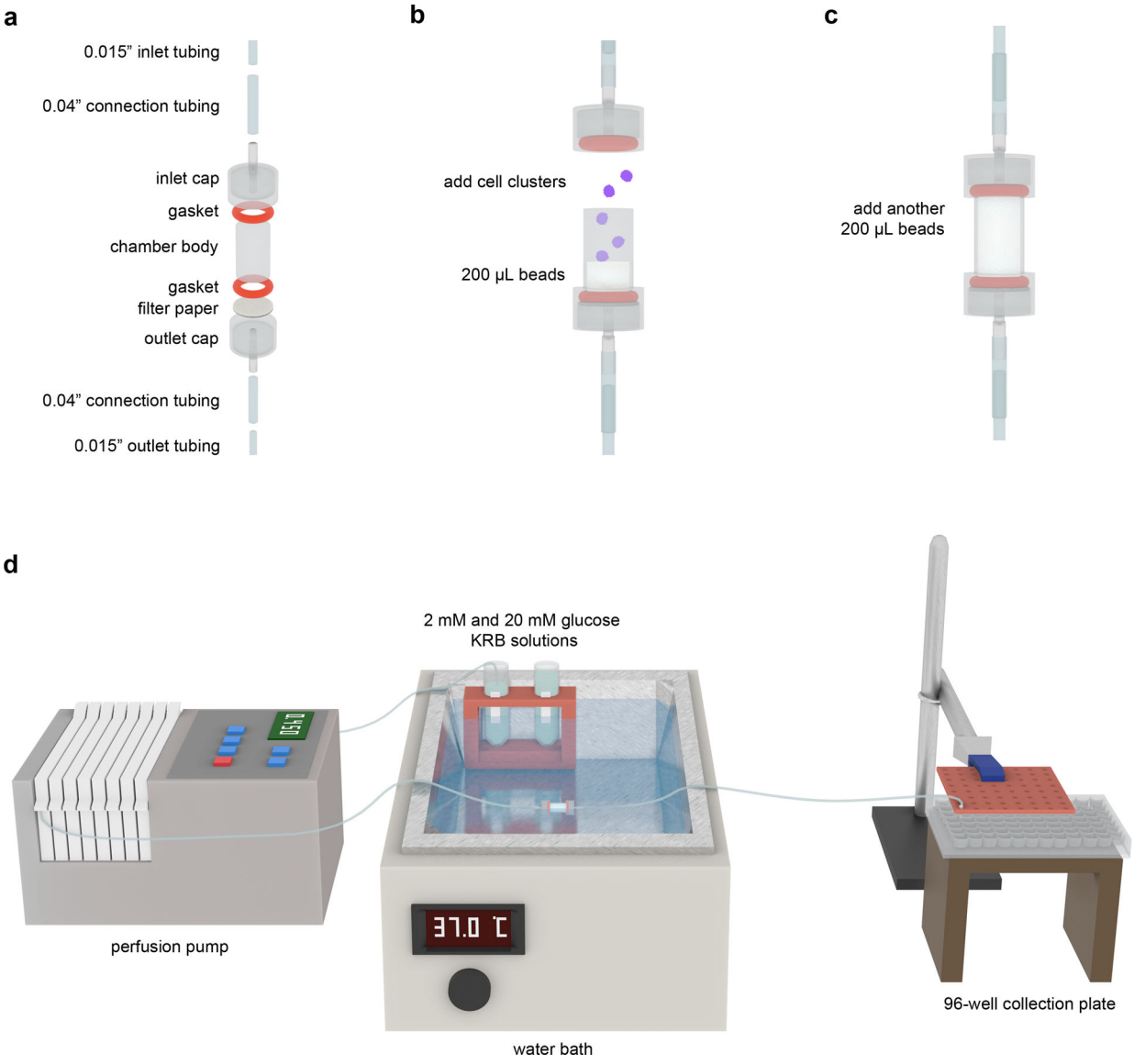

Figure 4). Setup for the dynamic GSIS assay.

(**a**) Schematic of the chambers used to immobilize the SC-β cell clusters as fluid flows across the cells. (**b-c**) The SC-β cell clusters are sandwiched between two layers of hydrated polyacrylamide microbeads. (**d**) This chamber containing the SC-β cell clusters is attached to a perfusion pump and placed in a water bath at 37°C. 2 mM and 20 mM glucose solutions are pumped through the chambers at a flow rate of 100 µL/min, and the effluent is collected in a 96-well plate that is manually moved every 2 minutes. Up to 8 chambers can be run at once with the specified pump.

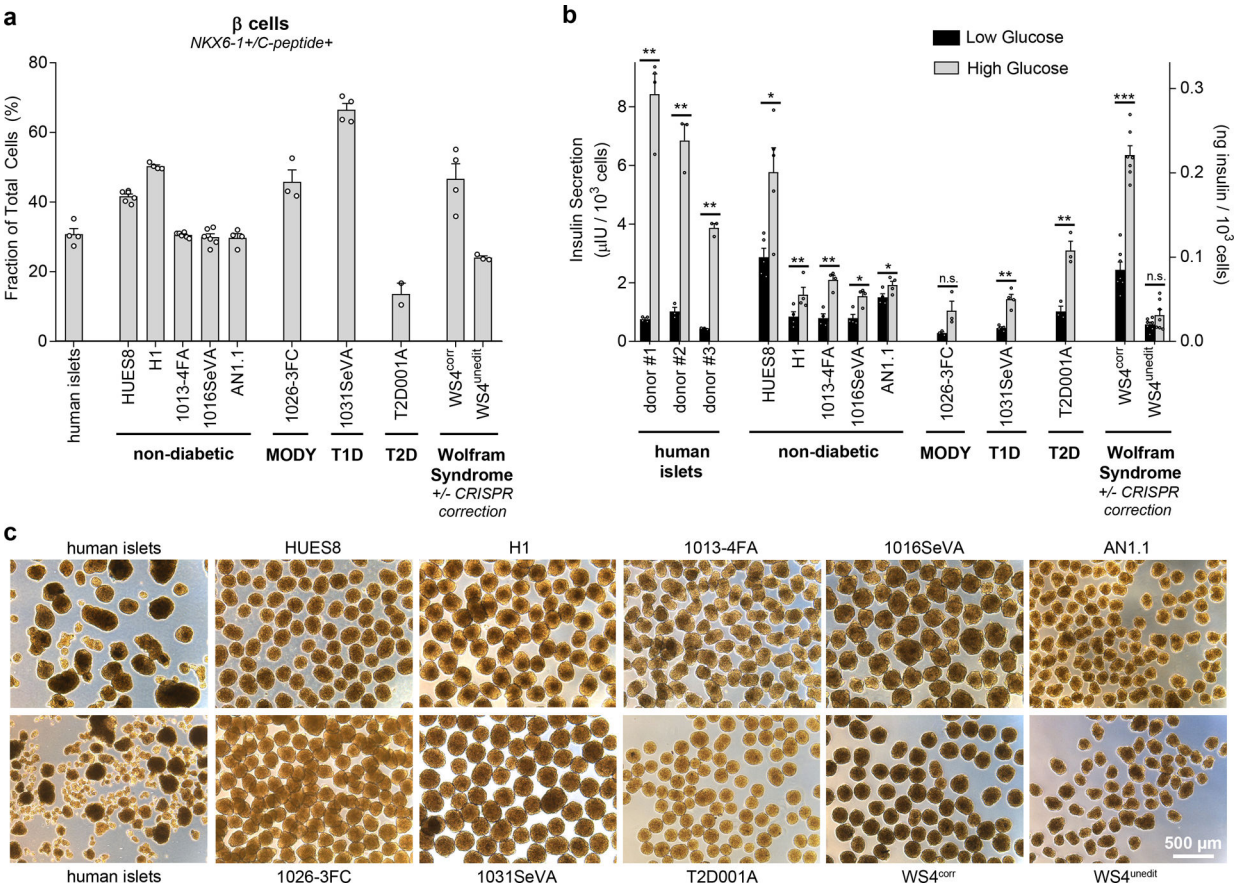

Figure 5). SC-β cells differentiated from a wide range of cell lines.

(**a**) SC-β cells, indicated by the co-expression of NKX6–1 and C-peptide as assessed by flow cytometry, can be robustly differentiated from a variety of cell lines with this protocol (n = 6 for HUES8, 1013–4FA, and 1016SeVA; n = 4 for human islets, H1, AN1.1, 1031SeVA, and WS4^{corr}; n = 3 for 1026–3FC and WS4^{unedit}; n = 2 for T2D001A). (**b**) While all these SC-β cells secrete insulin, their glucose responsiveness in a static GSIS assay is dependent upon their genetic background and disease state (n = 7 for WS4^{corr} and WS4^{unedit}; n = 5 for HUES8; n = 4 for donor #1, H1, 1013–4FA, 1016SeVA, AN1.1, and 1031SeVA; n = 3 for donor #2, donor #3, 1026–3FC, and T2D001A). A paired, two-way t-test was performed between low and high glucose for each cell line. (**c**) Representative brightfield images of human islets and the SC-β cells derived from each cell line after stage 6 aggregation into islet-like clusters. All data presented in this figure are from stage 6 cells after aggregation into clusters. All data are represented as the mean, and all error bars represent SEM. Individual data points are shown for all bar graphs, where n = number of separate wells from one or more independent differentiations. n.s. = not significant; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

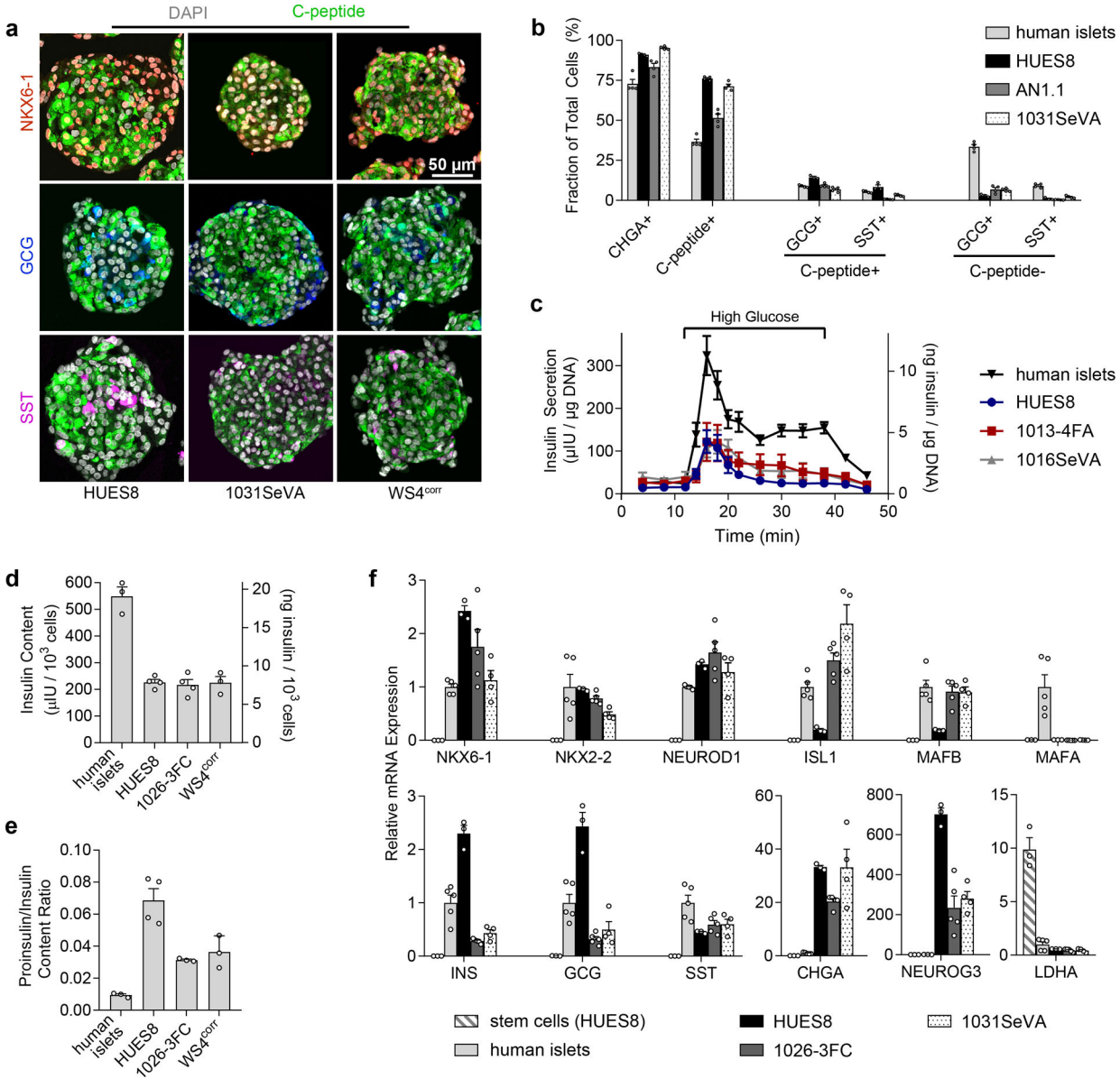

Figure 6). In-depth characterization of SC-β cells.

(**a**) Immunostaining of histological sections of SC-β cell clusters demonstrates that a majority of cells are co-positive for C-peptide and NKX6–1, while there are also a few glucagon (GCG) and somatostatin (SST) positive cells. (**b**) This ratio of C-peptide, GCG, and SST is confirmed quantitatively by flow cytometry (n = 4). (**c**) SC-β cell clusters demonstrate both first and second phase insulin secretion in a dynamic GSIS assay (n = 4). (**d-e**) SC-β cells generated with this protocol have high insulin content and a favorable proinsulin/insulin content ratio (< 0.1) (n = 4 for HUES8 and 1026–3FC; n = 3 for WS4^{corr} and human islets). (**f**) They also express similar levels of a number of islet genes compared with primary human islets, though some gene expression differences persist (n = 5 for human islets and 1026–3FC; n = 4 for 1031SeVA; n = 3 for stem cells and HUES8). The ΔΔCt method was used to calculate relative mRNA expression. For the assays shown here,

data is given for HUES8 and 2 iPSC lines compared with primary human islets. The original protocol was developed with the HUES8 cell line, and thus it can serve as a good baseline to compare SC-β cells generated from new cell lines. All data presented in this figure are from stage 6 cells after aggregation into clusters. All data are represented as the mean, and all error bars represent SEM. Individual data points are shown for all bar graphs, where n = number of separate wells from one or more independent differentiations.

Table 1:

hPSC Lines

Cell Line	RRID
HUES8	https://scicrunch.org/resolver/CVCL_B207
H1	https://scicrunch.org/resolver/CVCL_9771
1013–4FA	https://scicrunch.org/resolver/CVCL_A9M5
1016SeVA	https://scicrunch.org/resolver/CVCL_UK18
AN1.1	https://scicrunch.org/resolver/CVCL_A9K3
1026–3FC	https://scicrunch.org/resolver/CVCL_A9L4
1031SeVA	https://scicrunch.org/resolver/CVCL_A9M2
T2D001A	https://scicrunch.org/resolver/CVCL_A9K4
WS4^{corr}	https://scicrunch.org/resolver/CVCL_A9K6
WS4^{unedit}	https://scicrunch.org/resolver/CVCL_A9K5

Table 2:

qRT-PCR primers for each stage

End of Stage #	Marker	Forward Primer (5’−3’)	Reverse Primer (5’−3’)
Housekeeping genes	TBP	GCCATAAGGCATCATTGGAC	AACAACAGCCTGCCACCTTA
	GUSB	TTGCTCACAAAGGTCACAGG	CGTCCCACCTAGAATCTGCT
0	OCT4 (POU5F1)	GGTTCTCGATACTGGTTCGC	GTGGAGGAAGCTGACAACAA
	NANOG	ATGGAGGAGGGAAGAGGAGA	GATTTGTGGGCCTGAAGAAA
1	FOXA2	TACGTGTTCATGCCGTTCAT	CGACTGGAGCAGCTACTATGC
	SOX17	TCTGCCTCCTCCACGAA	CAGAATCCAGACCTGCACAA
3	PDX1	CGTCCGCTTGTTCTCCTC	CCTTTCCCATGGATGAAGTC
4	PDX1	CGTCCGCTTGTTCTCCTC	CCTTTCCCATGGATGAAGTC
	NKX6–1	CCGAGTCCTGCTTCTTCTTG	ATTCGTTGGGGATGACAGAG
	SOX9	TTAAACCCTCTTCAGAGCAAGC	GATTTAGCACACTGATCACACGA
5	NEUROG3	CTTCGTCTTCCGAGGCTCT	CTATTCTTTTGCGCCGGTAG
	NKX2–2	GGAGCTTGAGTCCTGAGGG	TCTACGACAGCAGCGACAAC
	NEUROD1	ATCAGCCCACTCTCGCTGTA	GCCCCAGGGTTATGAGACTAT
	CHGA	TGACCTCAACGATGCATTTC	CTGTCCTGGCTCTTCTGCTC
	1	CCGAGTCCTGCTTCTTCTTG	ATTCGTTGGGGATGACAGAG
	INS	CAATGCCACGCTTCTGC	TTCTACACACCCAAGACCCG
6	INS	CAATGCCACGCTTCTGC	TTCTACACACCCAAGACCCG
	GCG	AGCTGCCTTGTACCAGCATT	TGCTCTCTCTTCACCTGCTCT
	SST	TGGGTTCAGACAGCAGCTC	CCCAGACTCCGTCAGTTTCT
	CHGA	TGACCTCAACGATGCATTTC	CTGTCCTGGCTCTTCTGCTC
	IAPP	ACATGTGGCAGTGTTGCATT	TCATTGTGCTCTCTGTTGCAT
	ISL1	TCACGAAGTCGTTCTTGCTG	CATGCTTTGTTAGGGATGGG
	GCK	ATGCTGGACGACAGAGCC	CCTTCTTCAGGTCCTCCTCC
	MAFB	CATAGAGAACGTGGCAGCAA	ATGCCCGGAACTTTTTCTTT

Table 3:

Base media formulations

Media	Reagent	Amount
BE1 *stage 1*	MCDB131	500 mL
	Glucose	0.8 g
	$NaHCO_3$	0.587 g
	BSA	0.5 g
	GlutaMAX	5 mL
BE2 *stage 2*	MCDB131	500 mL
	Glucose	0.4 g
	$NaHCO_3$	0.587 g
	BSA	0.5 g
	GlutaMAX	5 mL
	Vitamin C	22 mg
BE3 *stages 3 and 4*	MCDB131	500 mL
	Glucose	0.22 g
	$NaHCO_3$	0.877 g
	BSA	10 g
	GlutaMAX	5 mL
	Vitamin C	22 mg
	ITS-X	2.5 mL
S5 *stage 5*	MCDB131	500 mL
	Glucose	1.8 g
	$NaHCO_3$	0.877 g
	BSA	10 g
	GlutaMAX	5 mL
	Vitamin C	22 mg
	ITS-X	2.5 mL
	P/S	5 mL
	Heparin (10 mg/mL stock)	500 μL
Enhanced serum-free media (ESFM) *stage 6*	MCDB131	500 mL
	Glucose	0.23 g
	BSA	10.5 g
	GlutaMAX	5.2 mL
	P/S	5.2 mL
	MEM nonessential amino acids	5.2 mL
	Trace Elements A	523 μL
	Trace Elements B	523 μL

Media	Reagent	Amount
	$ZnSO_4 \cdot 7H_2O$ (1 mM stock)	523 μL
	Heparin (10 mg/mL stock)	523 μL

Table 4:

Reconstituting differentiation factors

Factor	Molar Mass (g/mol)	Factor Amount (mg)	Solvent	Solvent Amount	Resulting Stock Concentration	Aliquot size for one 30 mL feed (µL)
Y-27632	320.26	10	molecular biology grade water	3.12 mL	10 mM	33
CHIR99021	465.34	2	DMSO	143.26 µL	30 mM	3.5
Activin A	-	0.05	0.1% BSA in PBS	0.5 mL	100 µg/mL	33
KGF	-	0.1	0.1% BSA in PBS	2 mL	50 µg/mL	33
RA	300.44	50	DMSO	16.64 mL	10 mM	6.6
LDN193189	442.94	2	DMSO	4.52 mL	1 mM	6.6
TPPB	501.54	1	DMSO	996.93 µL	2 mM	3.5
SANT1	373.49	5	DMSO	13.39 mL	1 mM	8.3
ALK5i II	287.3	5	DMSO	174.03 µL	100 mM	3.5
T3	672.96	250	DMSO	37.15 mL	10 mM	3.5
XXI	490.5	1	DMSO	203.87 µL	10 mM	3.5
Latrunculin A	421.6	-	-	-	Comes pre-mixed at 237.2 µM	130
bFGF	-	0.05	molecular biology grade water	0.5 mL	100 µg/mL	3.5

Table 5:

Stock salt solutions

Chemical	Concentration (M)	Amount (g)	Volume of water (mL)
NaCl	2.5	87.66	600
KCl	2	7.46	50
$CaCl_2 \cdot 2H_2O$	1	7.35	50
$MgSO_4$	1	6.02	50
Na_2HPO_4	0.1	0.71	50
KH_2PO_4	1	6.80	50
$NaHCO_3$	1	4.20	50

Table 6:

Krebs Buffer (KrB)

Component	Amount	Final Concentration
Milli-Q H_2O	458.1 mL	-
NaCl [2.5M]	25.6 mL	128 mM
KCL [2M]	1.25 mL	5 mM
$CaCl_2 \cdot 2H_2O$ [1M]	1.35 mL	2.7 mM
$MgSO_4$ [1M]	0.6 mL	1.2 mM
Na_2HPO_4 [0.1M]	5 mL	1 mM
KH_2PO_4 [1M]	0.6 mL	1.2 mM
$NaHCO_3$ [1M]	2.5 mL	5 mM
HEPES [1M]	5 mL	10 mM
BSA (Proliant)	0.5 g	0.1 % (wt/vol)

Table 7:

Differentiation media needed per feed

Plasticware	Feeding volume (mL)
24-well	0.6
6-well	2
T25	5
T75	15
T150	30

Table 8:

SC-β Cell Differentiation Schedule

	Day of Differentiation	Base Media	Factor	Final Concentration	Dilution from Aliquots
Stage 0	1	mTeSR1	Y-27632	10 μM	1:1,000
Stage 1	2	BE1	Activin A CHIR99021	100 ng/mL 3 μM	1:1,000 1:10,000
	3–5	BE1	Activin A	100 ng/mL	1:1,000
Stage 2	6–7	BE2	KGF	50 ng/mL	1:1,000
Stage 3	8–9	BE3	KGF TPPB SANT1 RA LDN	50 ng/mL 0.2 μM 0.25 μM 2 μM 0.2 μM	1:1,000 1:10,000 1:4,000 1:5,000 1:5,000
Stage 4	10–13	BE3	KGF TPPB SANT1 RA LDN	50 ng/mL 0.2 μM 0.25 μM 0.1 μM 0.2 μM	1:1,000 1:10,000 1:4,000 1:100,000 1:5,000
Stage 5	14	S5	XXI Alk5i II T3 SANT1 RA Latrunculin A	1 μM 10 μM 1 μM 0.25 μM 0.1 μM 1 μM	1:10,000 1:10,000 1:10,000 1:4,000 1:100,000 1:237.2
	15–20	S5	XXI Alk5i II T3 SANT1 RA	1 μM 10 μM 1 μM 0.25 μM 0.1 μM	1:10,000 1:10,000 1:10,000 1:4,000 1:100,000
Stage 6	21–27	ESFM	-	-	-
Stage 6 *After aggregating into clusters*	28–35+	ESFM	-	-	-

Table 9:

Immunostaining antibodies

End of Stage #	Primary Antibodies (dilution, compatible secondary antibody)
0	OCT4 (1:300, anti-mouse alexa fluor 488) NANOG (1:300, anti-goat alexa fluor 647)
1	SOX17 (1:300, anti-mouse alexa fluor 488) FOXA2 (1:300, anti-rabbit alexa fluor 647)
2	-
3	PDX1 (1:300, anti-goat alexa fluor 488)
4	PDX1 (1:300, anti-goat alexa fluor 488) NKX6–1 (1:100, anti-mouse alexa fluor 647)
5	C-peptide (1:300, anti-rat PE) CHGA (1:1000, anti-rabbit alexa fluor 647) NKX6–1(1:100, anti-mouse alexa fluor 488)
6	CHGA (1:1000, anti-rabbit alexa fluor 647) C-peptide (1:300, anti-rat PE) NKX6–1(1:100, anti-mouse alexa fluor 488) GCG (1:300, anti-mouse alexa fluor 488) SST (1:300, anti-rabbit alexa fluor 647)

Table 10:

Table Removing paraffin from histological sections

Solution	Submersion time (min)
Histo-clear II	5
Histo-clear II	5
100% ethanol	2
100% ethanol	2
95% ethanol	2
95% ethanol	2
70% ethanol	2
70% ethanol	2

Table 11:

Cell line optimizations

Stem cell line	Initial stem cell seeding density (10^6 cells / cm^2)	Add 10 ng/mL bFGF in stage 1	SCT Definitive Endoderm kit in stage 1	Latrunculin A concentration (μM)
HUES8	0.63	-	-	1
H1	0.53	yes	-	1
1013–4FA	0.53	-	-	1
1016SeVA	0.32	-	-	1
AN1.1	0.74	-	-	1
1026–3FC	0.53	-	yes	1.5
1031SeVA	0.53	-	-	1
T2D001A	0.53	yes	yes	0.5
$WS4^{corr}$	0.53	-	-	1
$WS4^{unedit}$	0.42	-	-	1

Table 12:

Troubleshooting poor quality differentiations

Step	Problem	Possible reason	Solution
12	Cells are falling off the plate during stage 1	Cell density is too low	Increase initial stem cell seeding density
12	Low percentage of SOX17+/FOXA2+ cells (<90%) at the end of stage 1	Cell density is not optimal	Optimize initial stem cell seeding density, typically between 0.32–0.74 × 10^6 cells/cm^2
		Variation of a particular cell line	Before beginning stage 1, ensure expression of stem cell pluripotency markers, such as OCT4 and NANOG, with flow cytometry or immuncytochemistry
			Use Accutase instead of TrypLE for passaging stem cells
			Add 10 ng/mL bFGF in stage 1
			Replace stage 1 media and factors with the SCT DE kit
18	Low percentage of PDX1+ cells (<80%) at the end of stage 3	Cell density is not optimal	Optimize initial stem cell seeding density, typically between 0.32–0.74 × 10^6 cells/cm^2
		Variation of a particular cell line	Add 10 ng/mL bFGF in stage 1
			Replace stage 1 media and factors with the SCT DE kit
20	Low percentage of PDX1+/NKX6–1+ cells (<40%) at the end of stage 4	Poor quality of pancreatic progenitor 1 cells	Optimize SOX17/FOXA2 induction in stage 1
			Optimize PDX1 induction in stage 3
21	High levels of cell death during stage 5 endocrine induction	Latrunculin A concentration is too high for this particular cell line	Decrease latrunculin A concentration to 0.75 or 0.5 μM
21	High levels of off-target cell types after stage 5 endocrine induction	Latrunculin A concentration is too low for this particular cell line	Increase latrunculin A concentration to 1.5 or 2 μM
27	After aggregation in stage 6, clusters are either very large or do not form at all	User too harsh on cells when dispersing, causing cell death	Avoid strong mechanical agitation by gently pipetting up and down to disperse cells after incubating in TrypLE
			Increase incubation time or amount of TrypLE so the cells detach from the plate more easily
			Use Accutase instead of TrypLE
27	Cells are not glucose responsive while remaining on the plate	Presence of too many off-target cell types	Perform aggregation procedure
1–27	Many stages of the differentiation are not working	Improper handling of the differentiation factors	Do not freeze/thaw the factors more than twice
		Substituted factors are not working as well	Use the exact products from the specified vendors listed in this protocol
		Edge effects of small well sizes (24-well plates and smaller) are negatively influencing the differentiation	Use a 6-well plate or larger