

Chapter 15

Pancreatic Differentiation from Human Pluripotent Stem Cells

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Abstract The ability to produce human pancreatic cells *in vitro* would open new possibilities for developing improved therapies through cell transplantation, disease modeling, and drug screening. Of particular medical importance are the insulin-producing beta cells of the pancreas, which are lost or dysfunctional in diabetes. Furthermore, an *in vitro* model of human exocrine cells could help devise new therapies for pancreatic exocrine disease, most notably pancreatic cancer. In the past decade much progress has been made in developing protocols to generate multipotent pancreatic progenitor cells from human pluripotent stem cells (hPSCs) that are capable of differentiating into both endocrine and exocrine cells. The sole approach that has proven successful is to reproduce essential steps of *in vivo* development *in vitro* through directed step-wise differentiation of hPSCs. The directed differentiation entails sequential exposure of hPSCs to different signaling factors, thereby moving cells through several developmental intermediates towards the pancreatic fate. Upon implantation into mice, hPSC-derived pancreatic progenitor cells spontaneously differentiate into endocrine and exocrine cells. Here, we describe a detailed protocol for the generation of pancreatic progenitor cells from hPSCs. We provide methods for the directed differentiation as well as the characterization of pancreatic progenitor cells and lineage intermediates by immunofluorescence staining and flow cytometry. With recently developed protocols, these pancreatic progenitor cells can be further differentiated *in vitro* into beta-like cells that functionally resemble immature human beta cells.

Keywords Human pluripotent stem cell (hPSC) • Human embryonic stem cell (hESC) • Definitive endoderm (DE) • Gut tube (GT) • Posterior foregut (FG) • Pancreatic endoderm (PE) • SOX17 • HNF4A • PDX1 • SOX9

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15.1 Introduction

The development of protocols for deriving mature cell types from hPSCs has opened opportunities for modeling human disease *ex vivo* as well as for discovery of novel drugs (Merkle and Eggen 2013). In addition, functional cell types derived from hPSCs could provide an unlimited source to replace tissue cells lost in disease through cell transplantation. hPSC-based cell models are particularly promising in the context of diseases where a single cell type is affected. One such example is diabetes, which is characterized by loss or dysfunction of the insulin-producing beta cells in the pancreas (Nathan 2015). There are two major forms of diabetes, type 1 and type 2 diabetes. While the causes of both types of diabetes are distinct, the manifestation is similar: insufficient production of insulin results in inadequate glucose uptake by peripheral tissues, which in turn leads to elevated blood glucose levels. Type 1 diabetes is caused by autoimmune-mediated beta cell destruction and comprises 5% of diabetes cases (Cnop et al. 2005). The remaining 95% of diabetes cases are classified as type 2, and arise due to a combination of insufficient insulin production by the beta cells and insulin resistance of insulin target tissues (Johnson and Luciani 2010; Kahn et al. 2006; Stumvoll et al. 2005). Because both type 1 and type 2 diabetes eventually result in beta cell loss, transplantation of beta cells produced *in vitro* from hPSCs could help normalize blood glucose levels in patients with diabetes. In the pancreas, beta cells reside in so-called islets of Langerhans, where beta cells form functional units with other hormone-producing cells involved in blood glucose regulation, most notably the glucagon-producing alpha cells and somatostatin-producing delta cells. Since there is paracrine signaling between the endocrine cell types within the islet (Caicedo 2013), transplantation of functional islets might be clinically more beneficial than transplanting beta cells alone. There is already precedence that transplantation of cadaveric islets can be curative for diabetes, at least in the short term (Meloche 2007; Robertson et al. 2000). hPSC-derived beta cells or islets could obviate both the limited supply of cadaveric islets and the need for immunosuppression owing to the allogeneic origin of cadaveric islets. In fact, the first clinical trial to determine the safety and efficacy of a hPSC-based islet cell replacement therapy is currently ongoing (ViaCyte 2014). The current clinical trial involves encapsulation of human embryonic stem cell (hESC)-derived multipotent pancreatic progenitor cells in small devices that are implanted subcutaneously. Animal studies have provided proof-of-principle that these pancreatic progenitor cells will further differentiate into islet-like structures containing all endocrine cell types when implanted under the skin (Kelly et al. 2011; Kroon et al. 2008; Nostro et al. 2015; Rezanian et al. 2012; Schulz et al. 2012; Xie et al. 2013).

While beta cell loss is the hallmark of type 1 diabetes, type 2 diabetes, at least in its early stages, is characterized by beta cell dysfunction rather than destruction. Type 2 diabetes has a strong genetic component; yet how genetic risk factors cause beta cell dysfunction remains largely unknown. While mouse models have provided important insight into the regulation of insulin production and secretion, comparisons between mouse models of diabetes and the human disease have also revealed species differences. One condition that exemplifies such differences is a monogenetic

form of type 2 diabetes, called maturity onset diabetes of the young (MODY). In humans, inheritance of one mutant allele is sufficient to cause MODY, whereas mice carrying the same heterozygous mutation do not acquire the disease (Haumaitre et al. 2006; Lee et al. 1998; Mayer et al. 2008; Yamagata et al. 1996). *In vitro*-generated beta cells and their precursors would provide a powerful model to study how MODY-associated gene mutations cause diabetes. With recent breakthroughs in genome editing technologies, known mutations, like those seen in MODY, could be introduced into hPSCs and beta cell differentiation and function could be analyzed *in vitro*. Alternatively, induced hPSCs could be derived from MODY patients to create patient-specific disease models. Similar strategies could be implemented to determine how genetic variants, identified via genome-wide association studies, affect beta cell development and/or function and cause disease.

A scalable differentiation system for human beta cells or islets would also provide an ideal drug-screening platform. In addition to enabling new drug discovery, such a system could help customize diabetes treatment in a patient-specific manner. It is known that not all patients with type 2 diabetes respond similarly to existing anti-diabetic drugs (Standl and Fuchtenbusch 2003). Responsiveness to different drugs could be tested by deriving induced hPSCs from patients and producing patient-specific beta cells or islets *in vitro*. In the long term, the collective data from such experiments might allow us to predict drug responsiveness based on the absence or presence of specific genetic markers, as already used to predict responsiveness to cancer therapy.

The past decade has seen remarkable progress in the development of protocols to generate pancreatic cells from hPSCs (Mfopou et al. 2010; Pagliuca and Melton 2013). Multiple differentiation protocols aimed at deriving pancreatic progenitors and beta cells have been published with mounting levels of success. The underlying principle of all successful approaches is to closely mimic embryonic development *in vitro*. Efficacious protocols have taken a step-wise approach in which cells are guided through defined developmental stages by sequential exposure to the growth factors that are also relevant during *in vivo* development. Earlier protocols yielded insulin-expressing cells *in vitro* that were not functional, expressed multiple hormones, and failed to restore blood glucose levels when implanted into diabetic mice (D'Amour et al. 2006; Kelly et al. 2011; Kroon et al. 2008; Nostro et al. 2011; Rezanian et al. 2012). In contrast, when pancreatic progenitor cells were implanted into mice, the implants rescued diabetes within ~16 weeks after implantation (Kroon et al. 2008; Nostro et al. 2015; Rezanian et al. 2012; Schulz et al. 2012). During this time, the progenitors differentiate into islet-like structures, containing mature functional beta cells as well as other endocrine cell types. A small fraction of pancreatic progenitors also differentiates into exocrine acinar and ductal cells after implantation (Kroon et al. 2008; Xie et al. 2013).

Here, we present a step-wise protocol for the generation of pancreatic progenitor cells by directed differentiation of hESCs. The protocol was adapted from several previously published protocols (Schulz et al. 2012; Rezanian et al. 2013, 2014). The protocol is optimized for the H1 hESC line from WiCell (WiCell Research Institute, WA01) and employs a planar culture system in which the cells are adhered to Matrigel-coated tissue culture plates throughout differentiation. Recently, protocols have been published

that report strategies to further differentiate hPSC-derived pancreatic progenitor cells into functional beta-like cells entirely *in vitro* (Pagliuca et al. 2014; Rezanian et al. 2014; Russ et al. 2015). After implantation into diabetic mice, these beta-like cells lead to normalization of blood glucose levels within 2 weeks.

15.2 Materials

15.2.1 Cell Culture Supplies and Equipment

- Biological safety cabinet.
- CO₂ incubator capable of maintaining 5% CO₂ and >95% humidity at 37 °C.
- 37 °C water bath.
- Sterile plastic tissue culture treated cell culture dishes:
 - 6-Well plate; 9.5 cm² cell growth area (Corning, Cat. No. 3516).
 - 12-Well plate; 3.8 cm² cell growth area (Corning, Cat. No. 3513).
 - 10 cm plate; 56.5 cm² cell growth area (Corning, Cat. No. 351029).
- Sterile ultra-low attachment 6-well plates (Corning, Cat. No. 3471), for alternative suspension culture (see *Sect. 15.3.5* and *Note 5*).
- Cell lifter/scrapper (VWR, Cat. No. 3008).
- Hemocytometer or other cell counting tool.
- Sterile 15 ml conical tubes.
- Benchtop centrifuge capable of spinning 15 ml conical tubes.
- Sterile 1.5 ml microcentrifuge tubes.
- Benchtop microcentrifuge capable of spinning 1.5 ml microcentrifuge tubes.
- Serological pipette controller and 10 ml sterile pipettes.
- 2, 20, 200, and 1000 µl pipettes and accompanying sterile pipette tips.
- Stericup-GP, 0.22 µm, polyethersulfone, 500 ml, radio-sterilized vacuum filters (Millipore, SCGPU05RE).
- Falcon 5 ml round bottom polystyrene test tube with cell strainer snap cap (Corning, Cat. No. 352235).
- Inverted light microscope (Zeiss Axio Vert or equivalent).
- Inverted fluorescence microscope (Zeiss Axio Observer or equivalent).
- Flow cytometer (BD FACSCanto™ or equivalent).

15.2.2 Reagents

- Matrigel Growth Factor Reduced Basement Membrane Matrix (Corning, Cat. No. 356231).
- RHO/ROCK Pathway Inhibitor Y-27632 (StemCell, Cat. No. 72307).
- TrypLE™ Express Enzyme (Life Technologies, Cat. No. 12604-021).

- Accutase (eBioscience, Cat. No. 00-4555-56).
- Sterile DPBS (Dulbecco's Phosphate Buffered Saline) without Ca^{2+} and Mg^{2+} (VWR, Cat. No. 45000-434).
- DMEM/F12 50:50 mix without glutamine (VWR, Cat. No. 45000-346).
- Bovine Serum Albumin (BSA) Fraction V (7.5 % solution) (Life Technologies, Cat. No. 15260-037), for alternative suspension culture (see *Sect. 15.3.5* and *Note 6*).
- Reagent Grade BSA—pH 7.0, >98 % purity (Lampire Biological Laboratories, Cat. No. 7500804), for use in differentiation media.
- H1 hESC line (WiCell Research Institute, WA01). Other hPSC lines may be used, but may require further optimization.
- Essential 8 (E8) pluripotency maintenance medium (Life Technologies, Cat. No. A1517001). The E8 culture medium is a xeno-free pluripotency maintenance medium (Chen et al. 2011) used here for feeder-free growth and expansion of hESCs.
- MCDB 131 cell culture medium (Life Technologies, Cat. No. 10372-019). The MCDB 131 medium is used here as the base medium throughout differentiation and is supplemented with different components and factors depending on the stage of differentiation. See *Sect. 15.2.3* for proper stage-specific base-medium formulations and Table 15.1 for additional factors to be added on each day of differentiation.
- Supplements and factors used for endocrine differentiation:
 - Sodium bicarbonate (NaHCO_3) (Sigma, Cat. No. S6297).
 - Recombinant Activin A (AA) (R&D Systems, Cat. No. 338-AC/CF).
 - Recombinant Wnt-3a (R&D Systems, Cat. No. 1324-WN/CF).
 - GlutaMAX (Life Technologies, Cat. No. 35050061).
 - BSA (Fisher, Cat. No. 7500804).
 - L-Ascorbic Acid (Vitamin C, VIT-C) (Sigma, Cat. No. A4544).
 - Recombinant KGF/FGF-7 (R&D Systems, Cat. No. 251-KG/CF).
 - D-Glucose (Fisher Scientific, Cat. No. D161).
 - Retinoic Acid (RA) (Sigma, Cat. No. R2625).
 - LDN193189 (Stemgent, Cat. No. 04-0074).
 - ITS-X (Life Technologies, Cat. No. 51500056).
 - SANT-1 (Sigma, Cat. No. S4572).
 - TPB (EMD Millipore, Cat. No. 565740).
- Cell fixation solution for immunofluorescence (IF) analysis: 4 % paraformaldehyde (PFA) (96 % extra pure, Acros Organics, Cat. No. 41678) in DPBS.
- Cell permeabilization and blocking buffer for IF analysis: 0.15 % Triton X-100 (Fisher, Cat. No. BP151) and 1 % donkey serum (Gemini Bio-Products, Cat. No. 100-151) in DPBS.
- Superfrost Plus microscope slides (Fisher Scientific, Cat. No. 12-550-15).
- VECTASHIELD Antifade mounting medium (Vector Laboratories, Cat. No. H-1000).
- FACS buffer: 0.2 % (w/v) BSA (Lampire, Cat. No. 7500804) in DPBS, keep at 2–8 °C.
- BD fixation/permeabilization solution (BD Biosciences, Cat. No. 554714) for flow cytometry analysis, keep at 2–8 °C.

Table 15.1 Medium formulations used for each day of differentiation. See Sect. 15.2.3 for stage-specific base-medium formulations

Day	Base medium	Added factors
1	Stage 1 medium	100 ng/ml activin A 25 ng/ml Wnt-3a
2–3	Stage 1 medium	100 ng/ml activin A
4–5	Stage 2 medium	0.25 mM VIT-C 50 ng/ml FGF7
6–7	Stage 3 medium	0.25 mM VIT-C 50 ng/ml FGF7 0.25 μ M SANT-1 1 μ M retinoic acid 100 nM LDN193189 1:200 ITS-X 200 nM TPB
8–10	Stage 4 medium	0.25 mM VIT-C 2 ng/ μ l FGF7 0.25 μ M SANT-1 0.1 μ M retinoic acid 200 nM LDN193189 1:200 ITS-X 100 nM TPB

The same base media formulations and factors are used for either adherent cultures or suspension cultures

- BD perm/wash buffer (BD Biosciences, Cat. No. 554723) for flow cytometry analysis, keep at 2–8 °C.

15.2.3 Stage-Specific Medium Formulations

The supplements listed below are stable in the MCDB 131 medium for 1 month if kept at 2–8 °C. Because sodium bicarbonate (NaHCO_3) and BSA are supplied as a non-sterile powder, the medium must be sterile filtered (0.22 μ m vacuum filter) after NaHCO_3 and BSA are added.

Stage 1 Medium

- MCDB 131 medium.
- 1.5 g/l NaHCO_3 .
- 1 \times GlutaMAX.
- 10 mM D-Glucose (final concentration; MCDB 131 already contains 5.55 mM).
- 0.5 % BSA.

Stage 2 Medium

- MCDB 131 medium.
- 1.5 g/l NaHCO₃.
- 1× GlutaMAX.
- 10 mM D-Glucose (final concentration; MCDB 131 already contains 5.55 mM).
- 0.5 % BSA.

Stage 3/4 Medium

- MCDB 131 medium.
- 2.5 g/l NaHCO₃.
- 1× GlutaMAX.
- 10 mM D-glucose (final concentration; MCDB 131 already contains 5.55 mM).
- 2 % BSA.

15.2.4 Antibodies Used for Cell Characterization**Primary Antibodies for Flow Cytometry**

- Mouse anti-SOX17-PE (1:20, BD Biosciences, Cat. No. 561591).
- Mouse anti-PDX1-PE (1:20, BD Biosciences, Cat. No. 562161).
- Mouse anti-NKX6.1-Alexa Fluor 647 (1:20, BD Biosciences, Cat. No. 563338).
- Rabbit anti-SOX9 (1:50, Millipore, Cat. No. AB5535).
- Mouse anti-IgG1, κ-PE isotype control (1:20, BD Biosciences, Cat. No. 556650).
- Mouse anti-IgG1, κ-Alexa Fluor 647 isotype control (1:20, BD Biosciences, Cat. No. 557732).

Secondary Antibodies for Flow Cytometry

- Donkey anti-rabbit IgG-PE (1:20, eBioscience, Cat. No. 12-4739-81).

Primary Antibodies for Immunofluorescence

- Goat anti-SOX17 (1:1000, R&D Systems, Cat. No. AF1924).
- Rabbit anti-OCT4 (1:1000, Cell Signaling Technology, Cat. No. 2840).
- Rabbit anti-HNF4A (1:500, Santa Cruz, Cat. No. SC-6556).
- Rabbit anti-SOX9 (1:1000, Millipore, Cat. No. AB5535).
- Goat anti-PDX1 (1:500, Abcam, Cat. No. ab47383).
- Mouse anti-NKX6.1 (1:300, Developmental Studies Hybridoma Bank, Cat. No. F55A10).

Secondary Antibodies for Immunofluorescence

- Donkey anti-goat Cy3 (1:1000, Jackson Immuno, Cat. No. 705-165-147).
- Donkey anti-rabbit Cy3 (1:1000, Jackson Immuno, Cat. No. 711-165-152).
- Donkey anti-goat Alexa Fluor 488 (1:1000, Jackson Immuno, Cat. No. 705-545-147).

15.3 Methods

Cell culture and passaging of hESCs

Proper handling and preparation of hESCs is important to ensure successful and efficient differentiation. The protocol is optimized for H1 cells, which can be obtained from WiCell Research Institute (WA01). Below are the recommended methods for proper culture and expansion of H1 cells prior to differentiation.

15.3.1 Preparation of Matrigel-Coated Tissue Culture Dishes

Expansion is performed in 6-well plates and differentiation in 12-well plates. If different sizes of culture dishes are desired, adjust volumes as necessary.

1. Thaw Matrigel on ice and keep cold at all times;
2. Following the manufacturer's instructions dilute Matrigel in the appropriate volume of cold DMEM/F12 medium and keep on ice. The dilution used in this protocol is 1:100, but may vary from lot to lot. Unused diluted Matrigel can be stored at 2–8 °C, but should be used within 2 weeks from the time of dilution;
3. Load diluted Matrigel into each well of the tissue culture plate to be used. Ensure there is sufficient volume to completely cover the entire well surface (~2 ml per well of a 6-well plate and ~1 ml per well of a 12-well plate);
4. Incubate the plate at 37 °C for 30 min before use.

15.3.2 Culturing hESCs

1. Aspirate excess Matrigel from pre-coated plates before seeding cells;
2. Thaw frozen cells in 37 °C water bath until just a small amount of ice is left in the vial;
3. Wash cells by resuspending in ~5 ml of E8 medium and spin at 200 × g for 4 min at room temperature;
4. Aspirate supernatant carefully to not disrupt the cell pellet;
5. Resuspend cells in E8 medium (~5 × 10⁵ cells/ml) containing freshly added ROCK inhibitor (10 μM);
6. Add 2 ml cell suspension per well of the Matrigel-coated 6-well plate and place in a 37 °C incubator at 5 % CO₂;
7. Replace medium with fresh E8 medium (w/o ROCK inhibitor) every 24 h;
8. Cells are passaged at ~80 % confluency.

15.3.3 Passaging hESCs with TrypLE

1. Aspirate medium from wells and wash with DPBS without Ca²⁺ and Mg²⁺;
2. Aspirate DPBS and load 0.5–1 ml of room temperature TrypLE in each well and incubate at 37 °C for 1 min;

3. After 1 min examine cells under a microscope to ensure sufficient detachment from plate. Cells should appear balled up at the edges of colonies, but not free-floating. If more time is required for cell detachment, place cells back in 37 °C and examine under microscope every 1 min until cells are sufficiently detached (see *Note 1*);
4. Stop enzymatic reaction by adding 4 volumes of E8 medium;
5. Use a cell scraper to lift cell clusters off plate;
6. Use a serological pipette to transfer the cell suspension into sterile 15 ml conical tubes;
7. Centrifuge for 4 min at 200× *g* to pellet cells;
8. Aspirate media and resuspend cells in appropriate volume of E8 medium containing 10 μM ROCK inhibitor (Y-27632) to achieve desired dilution (see *Note 2*);
9. Add 2 ml of cell suspension per well of a Matrigel-coated 6-well plate;
10. Replace medium with fresh E8 medium (w/o ROCK inhibitor) every 24 h.

15.3.4 Cell Differentiation

We describe a step-wise protocol for the directed differentiation of H1 hESCs into pancreatic progenitor cells, which we refer to as pancreatic endoderm (Fig. 15.1). During *in vitro* differentiation, the cells progress through different lineage intermediates, resembling cell populations found in the developing embryo. The first step is the differentiation to definitive endoderm, from which endodermal organs, such as liver, lungs, thymus, intestine, and pancreas are derived (Wells and Melton 1999). In subsequent steps, definitive endoderm cells are directed to acquire primitive gut tube, posterior foregut, and finally pancreatic endoderm identity. Expression of early pancreatic transcription factors, such as PDX1 and SOX9, is observed as early as the end of the

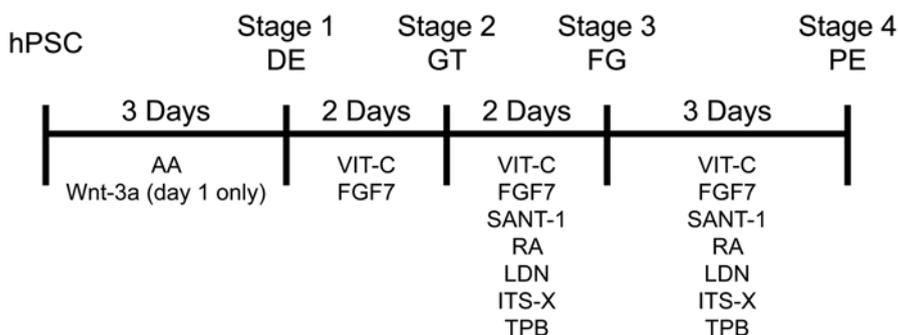


Fig. 15.1 Schematic outlining the protocol for differentiation of human pluripotent stem cells into pancreatic progenitor cells (pancreatic endoderm). The duration of each differentiation step and the required factors are depicted. Exact concentrations of factors and medium composition can be found in *Sects. 15.2.2* and *15.2.3*. *hPSC* human pluripotent stem cell, *DE* definitive endoderm, *GT* primitive gut tube, *FG* posterior foregut, *PE* pancreatic endoderm, *AA* activin A, *VIT-C* vitamin C, *RA* retinoic acid

posterior foregut stage. When implanted subcutaneously or under the kidney capsule into mice, pancreatic endoderm differentiates into fully functional beta cells, other endocrine cell types, as well as a small percentage of pancreatic ductal cells (Kelly et al. 2011; Kroon et al. 2008; Rezania et al. 2013; Schulz et al. 2012; Xie et al. 2013). The *in vivo* differentiation and maturation process of implanted pancreatic endoderm takes ~16 weeks, regardless of the protocol and cell line used to generate pancreatic endoderm. Although the application of the protocol we describe is not limited to H1 hESCs, when other hPSC lines are used, the protocol needs to be adapted to achieve similar differentiation efficiencies as observed with H1 hESCs. Key variables include the concentration of the factors and the duration of each differentiation step.

15.3.4.1 Preparation of Coverslips for Culturing hESCs

Optional: If analysis will be performed later, it is necessary to load coverslips into the culture dish wells prior to coating with Matrigel. To prepare coverslips for culturing hESCs follow the procedure below, otherwise continue with the next Section.

1. Put coverslips into a sterile 10 cm petri dish with lid;
2. Pour enough 70% ethanol into the dish to completely submerge all coverslips and place the lid on the petri dish;
3. Allow coverslips to soak in 70% ethanol overnight;
4. The following day, aspirate 70% ethanol and replace it with 100% ethanol, ensuring all coverslips are submerged;
5. Allow coverslips to soak for 5 min;
6. Aspirate 100% ethanol and replace it with fresh 100% ethanol, again submerging all coverslips;
7. Allow coverslips to soak for 5 min;
8. Place petri dish lid upside down inside the biological safety cabinet;
9. Using sterilized tweezers, remove coverslips from 100% ethanol and place inside the inverted lid;
10. Lean each coverslip against the inside wall of the lid at an angle so that both sides of the coverslip are exposed to air;
11. Allow the coverslips to dry completely;
12. Once coverslips have dried, using sterilized tweezers, place one coverslip on the bottom of each well of the 12-well culture dish;
13. Proceed to coat the now sterilized coverslips with Matrigel as previously explained.

15.3.4.2 Seeding hESCs for Differentiation

H1 cells must be plated at an appropriate density before beginning *in vitro* differentiation. Proper starting density will ensure efficient and reproducible differentiations. After expanding cells to reach the desired cell numbers, the cells are passaged

and plated on Matrigel-coated 12-well plates. To allow for accurate counting, the cells must be dissociated into a single cell suspension before seeding wells to prepare for the *in vitro* differentiation.

1. Aspirate medium from wells and rinse with DPBS without Ca^{2+} and Mg^{2+} (~2 ml);
2. Place 0.5–1 ml of room temperature TrypLE in each well and incubate at 37 °C for 3–5 min;
3. After the first 2 min, examine cells under a microscope to ensure sufficient detachment from plate (see **Note 3**);
4. Continue to incubate cells at 37 °C and examine under microscope every 1 min until cells appear balled and many are free-floating;
5. Stop enzymatic reaction by adding 4 volumes of E8 medium;
6. Use a 1 ml pipette to triturate the detached cells several times to break up remaining clumps, leaving a single-cell suspension (see **Note 4**);
7. Use a serological pipette to transfer the cell suspension into sterile 15 ml conical tubes;
8. Reserve a small aliquot of cell suspension for cell counting;
9. Centrifuge for 4 min at 200×g to pellet cells and count cells during centrifugation;
10. Aspirate media and resuspend cells in E8 medium (~3 × 10⁵ cells/ml) containing freshly added ROCK inhibitor (10 μM);
11. Add 1.5 ml of cell suspension per well of a Matrigel-coated 12-well plate;
12. After 24–48 h cells should be roughly 90% confluent and ready to begin *in vitro* differentiation.

15.3.5 Differentiation to Pancreatic Endoderm (Adherent Culture)

Figure 15.1 outlines the step-wise differentiation process and key factors contained in the differentiation medium at each stage of differentiation. Once cells have reached 90% confluency (Fig. 15.2a), the differentiation process can be started. Figure 15.2b shows an example of cells that need to be expanded further before differentiation can be initiated. Stage-specific base-media are stable at 2–8 °C for up to 1 month and can therefore be mixed ahead of time. The formulations for stage-specific base-media are listed in Sect. 15.2.3. Table 15.1 shows the final concentrations of the added factors at each day of differentiation. *These factors are not stable at 2–8 °C for long periods of time and must be added to stage-specific base-medium immediately prior to warming the medium on each day of differentiation.*

Described here is a method in which the cells are adhered to 12-well plates in planar culture. However, this protocol can also be performed in suspension culture (Fig. 15.2c), as previously described in detail (Schulz et al. 2012). Suspension culture requires scaling up medium volumes and cell numbers, as well as orbital rotation (see **Notes 5** and **6**).

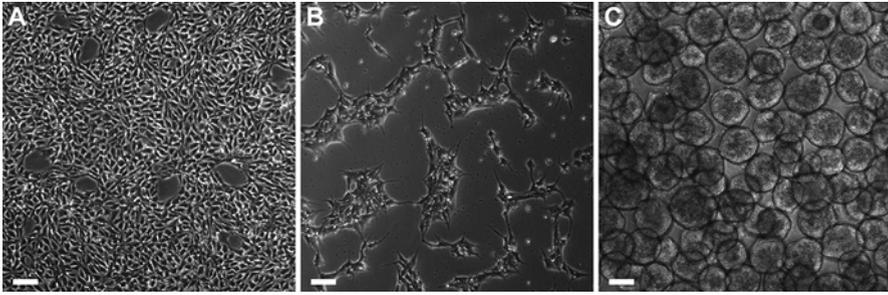


Fig. 15.2 Brightfield images of H1 human embryonic stem cells. H1 cells cultured under adherent conditions (**A**, **B**) or as suspension aggregates (**C**). (**A**) Image showing adherent H1 cells at the correct density to initiate *in vitro* differentiation. (**B**) Image showing cells that are of insufficient density to initiate differentiation. (**C**) Image showing aggregated H1 cells prior to induction of directed differentiation. Scale bars = 50 μM in **A**, **B** and 100 μM in **C**

- **Day 1:** Aspirate E8 medium and wash cells with DPBS without Ca^{2+} and Mg^{2+} .
- To each well add 1.5 ml of prewarmed Stage 1 Medium with freshly added Activin A (100 ng/ml) and Wnt-3a (25 ng/ml);
- **Day 2:** Aspirate medium and replace with 1.5 ml of prewarmed Stage 1 Medium with freshly added Activin A (100 ng/ml);
- **Day 3:** Repeat the previous step. At this point (or the following day) it is recommended to analyze the cells for differentiation efficiency to the definitive endoderm (DE) stage. The success of downstream differentiation is largely dependent upon highly efficient DE induction. Figure 15.3a provides an example of efficient DE induction (data generated using protocols in Sect. 15.3.6.1);
- **Day 4:** Aspirate Stage 1 Medium and wash cells with MCDB 131 base medium without added supplements or factors. To each well add 1.5 ml of prewarmed Stage 2 Medium with freshly added L-Ascorbic Acid (VIT-C) (0.25 mM) and FGF7 (50 ng/ml);
- **Day 5:** Repeat the previous step;
- **Day 6:** Aspirate medium and replace with 1.5 ml of fresh, prewarmed Stage 3 Medium with freshly added L-Ascorbic Acid (VIT-C) (0.25 mM), FGF7 (50 ng/ml), SANT-1 (0.25 μM), Retinoic Acid (1 μM), LDN193189 (100 nM), ITS-X (1:200) and TPB (200 nM);
- **Day 7:** Repeat the previous step;
- **Day 8:** Aspirate medium and replace with 1.5 ml of fresh, prewarmed Stage 4 Medium with freshly added L-Ascorbic Acid (VIT-C) (0.25 mM), FGF7 (2 ng/ml), SANT-1 (0.25 μM), Retinoic Acid (0.1 μM), LDN193189 (200 nM), ITS-X (1:200) and TPB (100 nM);
- **Day 9:** Repeat the previous step;
- **Day 10:** Repeat the previous step (same as Day 8).

At the end of the differentiation protocol the cells have adopted characteristics of pancreatic progenitor cells. A recent report by Rezania et al. describes a method to further differentiate these H1-derived pancreatic endoderm cells into beta-like cells *in vitro* (Rezania et al. 2014). The method involves culturing the cells in a liquid-air interface after the pancreatic endoderm stage. An alternative method to the liquid-air interface culture is to perform the differentiation into beta-like cells in suspension culture. However, the differentiation efficiency is reported to be lower in suspension culture than in liquid-air interface culture (Rezania et al. 2014). Two other recent reports utilized suspension culture methods to derive beta-like cells, similar to the ones reported by Rezania et al. from hPSC-derived pancreatic endoderm (Pagliuca et al. 2014; Russ et al. 2015).

15.3.6 Cell Characterization

15.3.6.1 Characterization of Cells by Flow Cytometry and Immunofluorescence Analysis

To ensure efficient differentiation, it is important to characterize the cells at various stages throughout the differentiation. Flow cytometry analysis and IF staining for stage-specific proteins are the hallmark methods to assess differentiation efficiencies. Figure 15.3 shows example images of IF analysis as well as flow cytometry plots of stage-specific markers from a successful differentiation. For a complete list of antibodies used for flow cytometry and IF analysis see *Sect. 15.2.4*. Below are recommended protocols for both flow cytometry and IF analysis.

Flow cytometry analysis protocol

1. Thaw Accutase at room temperature or 2–8 °C prior to use;
2. Aspirate medium from each well to be analyzed;
3. Wash cells with DPBS (~1.5 ml per well of 12-well plate) and aspirate;
4. Add enough Accutase® to cover the surface of each well (0.3–0.5 ml per well of 12-well plate);
5. Incubate at 37 °C for 5 min, then check briefly under microscope to ensure cells appear balled up and many are detached. If needed, place back at 37 °C and check every 1 min for good detachment (see *Note 7*);
6. Add ~1 ml cold FACS buffer (0.2% BSA in DPBS) to each well (final volume ~1.5 ml) to stop Accutase activity;
7. Using a 1000 µl pipette triturate cells to break up any clumps and obtain a single cell suspension;
8. Label a 5 ml polystyrene tube with cell strainer cap for each sample and add ~250 µl FACS buffer to each cap to pre-wet the cell strainer. Ensure the buffer flows through the cap into the tube;
9. Load each sample into the cap of the appropriately labeled tube and allow the entire suspension to flow through the cell strainer. This will ensure good cell

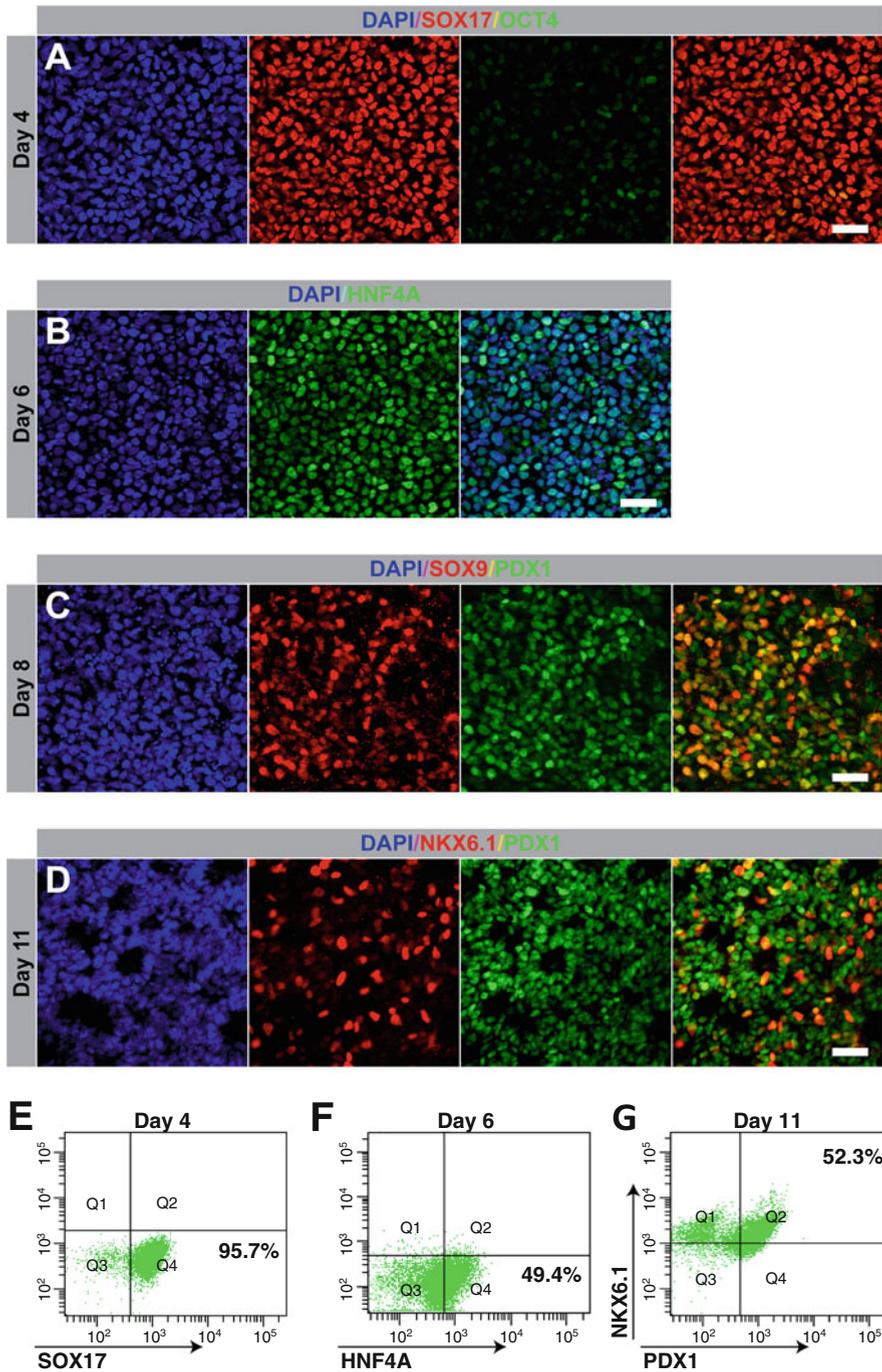


Fig. 15.3 Immunofluorescence staining and flow cytometry analysis of H1 cells throughout pancreatic differentiation. Immunofluorescent staining (A–D) or flow cytometry analysis (E–G) for stage-restricted transcription factors in H1 cells at different days of differentiation toward pancreatic endoderm. Day 4 represents definitive endoderm; day 6 primitive gut tube; day 8 posterior foregut; and day 11 pancreatic endoderm. Scale bars = 50 μ m

separation, which will provide better flow cytometry results. If the suspension does not easily flow through the cell strainer it may be necessary to pull the sample through. To do so, place the tip of a 1000 μ l pipette on the underside of the cell strainer cap containing the sample. Slowly release the pipette plunger to gently pull the sample through the filter top. Carefully eject the sample from the 1000 μ l pipette into the bottom of the tube;

10. Transfer the strained cells into new 1.5 ml microcentrifuge tubes and centrifuge at room temperature and $200\times g$ for 5 min;
11. Resuspend each cell pellet with cold BD fixation/permeabilization solution (300 μ l per tube);
12. Incubate cells for 20 min at 2–8 °C (i.e. refrigerate—*do not place on ice*);
13. Wash cells by adding ~1.1 ml cold 1 \times BD Perm/Wash™ Buffer to each tube following fixation/permeabilization (1.5 ml per well of 12-well plate) and centrifuge at 10 °C and $200\times g$ for 5 min;
14. Aspirate supernatant and repeat *step 11*;
15. Aspirate supernatant and resuspend cells in 50 μ l cold 1 \times BD Perm/Wash Buffer for each staining and isotype control to be used (e.g. add 150 μ l total for two different staining samples and one isotype control sample);
16. Aliquot 50 μ l of cell suspension into separate microcentrifuge tubes for each individual reaction. Co-staining with PE and AlexaFluor 647 conjugated antibodies can be performed together in one 50 μ l aliquot;
17. For analysis of SOX17 expression (DE marker) load 2.5 μ l SOX17-PE antibody into one 50 μ l cell suspension aliquot. Load 2.5 μ l PE isotype into a separate 50 μ l cell suspension aliquot to serve as a control;
18. For analysis of PDX1 expression (pancreatic endoderm marker) load 2.5 μ l PDX1-PE antibody into one 50 μ l cell suspension aliquot. Load 2.5 μ l PE isotype into a separate 50 μ l cell suspension aliquot to serve as a control;
19. For analysis of NKX6.1 expression (pancreatic endoderm marker) load 2.5 μ l NKX6.1-AlexaFluor 647 antibody into one 50 μ l cell suspension aliquot. Load 2.5 μ l AlexaFluor 647 isotype into a separate 50 μ l cell suspension aliquot to serve as a control;
20. After the antibodies and matching isotypes have been loaded into cell suspensions, incubate cells in the dark for 1 h at 2–8 °C (i.e. refrigerate—*do not place on ice*);
21. Wash cells by adding 1.25 ml cold 1 \times BD Wash Buffer to each tube and centrifuge at room temperature and $200\times g$ for 5 min;
22. Aspirate supernatant and resuspend each sample in 300 μ l cold FACS buffer;
23. The samples are now stained and ready for analysis on a flow cytometer, such as the FACSCanto used to generate the plots in Fig. 15.3.

Immunofluorescence analysis protocol

1. Aspirate medium from each well to be analyzed;
2. Wash cells with DPBS (~1.5 ml per well of 12-well plate) and aspirate;
3. Repeat *step 2*;
4. Add 1 ml of 4% PFA to each well and incubate at 2–8 °C overnight;
5. The following day, aspirate PFA and wash with DPBS (~1.5 ml per well of 12-well plate);

6. Aspirate DPBS and add 1 ml of blocking buffer (0.15 % Triton X-100, 1 % normal donkey serum in DPBS) and incubate at room temperature for 1 h;
7. Prepare primary antibody solutions by mixing appropriate volumes of each antibody in blocking buffer (1 ml per well of a 12 well plate) and vortex gently. See *Sect. 15.2.4* for appropriate working dilutions. For example, to analyze SOX17 expression, add 1 μ l of goat anti-SOX17 antibody to 1 ml of blocking buffer;
8. Aspirate buffer from each well and load 1 ml of pre-mixed primary antibody solution to the appropriate wells and incubate at 2–8 °C overnight;
9. The following day, aspirate antibody solution and wash with DPBS (~1.5 ml per well of 12-well plate);
10. Aspirate DPBS and repeat *step 9* three times;
11. Prepare secondary antibody solutions by mixing appropriate volumes of each antibody in blocking buffer (1 ml per well of 12 well plate) and vortex gently. See *Sect. 15.2.4* for appropriate working dilutions;
12. Aspirate DPBS from each well and add 1 ml of pre-mixed secondary antibody solution to the appropriate wells and incubate at room temperature for 1 h in the dark;
13. Aspirate antibody solution and wash with DPBS (~1.5 ml per well of 12-well plate);
14. Prepare nuclear staining solution by mixing Hoescht 33342 in DPBS (1:3000, 1 ml per well of 12-well plate);
15. Aspirate DPBS from each well and add 1 ml of nuclear staining solution and incubate at room temperature for 5 min in the dark;
16. Aspirate nuclear staining solution and wash with DPBS (~1.5 ml per well of 12-well plate);
17. Aspirate DPBS and wash once more with DPBS (~1.5 ml per well of 12-well plate);
18. The coverslips containing stained cells are now ready to be mounted on slides. Gently remove coverslips with tweezers and rinse by briefly dipping each coverslip into distilled water;
19. Allow fluid to run off the coverslip and remove excess fluid by blotting the edge of the coverslip with a paper towel. Capillary action should help remove the excess;
20. Place one drop of VECTASHIELD Antifade Mounting Medium (or desired mounting solution) on the coverslip on the side containing the cells;
21. Gently invert coverslip while placing it cell side down on a slide. Be careful to avoid any air bubbles between the slide and coverslip;
22. Seal the edges of the coverslip to the slide with clear nail polish and allow it to fully dry in the dark before analyzing the slides. The nail polish prevents the slides from drying out if stored for extended periods of time;
23. The slide containing the coverslip with the stained cells is now ready to be analyzed on an inverted fluorescent microscope, such as the Zeiss Axio Observer used to generate the images in *Fig. 15.3*;
24. Slides can be stored at 4 °C for short periods of time and at –20 °C for extended periods of time.

15.4 Notes

1. During expansion of H1 cells it is better to passage cells as clusters rather than as a single cell suspension. This will enhance health and survival of the cells.
2. Typical passaging dilutions for expanding H1 cells is 1:6 (i.e. 1 well split into 6 wells).
3. Avoid incubating with TrypLE for longer than necessary as this can reduce cell viability.
4. Avoid excessive pipetting as this can reduce cell viability.
5. *Performing differentiation in suspension culture*—To culture cells in suspension, use ultra-low attachment 6-well plates and rotate on an orbital shaker at 100 rpm. This ensures proper cell aggregation, which is necessary for cell survival and differentiation (Fig. 15.2c). The culture volume is 5.5 ml/well and base media formulations and concentrations of factors are the same as used for differentiation in adherent cultures (Table 15.1). Following expansion and dissociation of hESCs with TrypLE, seed wells with 1×10^6 cells/ml, which is a total of 5.5×10^6 cells/well. Add 10 μ M ROCK inhibitor to medium when seeding cells to promote cell survival and aggregation. 48 h following cell seeding the now aggregated cells (Fig. 15.2c) are ready to initiate the *in vitro* differentiation. The differentiation protocol is the same as described for adherent culture, but the volume of culture medium has to be adjusted.
6. *Handling cell aggregates in suspension culture*—A solution of 0.2% BSA in DPBS should be used to coat pipettes in order to prevent aggregates from adhering to plastic surfaces during medium exchanges. To properly exchange culture medium, draw PBS/BSA solution into the pipette and eject the solution. Swirl the 6-well plate in a circular motion to bring all aggregates to the center. Tilt the plate and draw medium off the side of each well with the pre-coated pipette, removing most of the medium. Leave a very small amount of medium to ensure no aggregates are removed. Load each well with 5.5 ml of the appropriate culture medium for that day. Place the plate back on the orbital shaker at 100 rpm inside a 37 °C incubator.
7. Avoid prolonged exposure to Accutase. Detachment of monolayer H1 cells should occur after 5 min, however if the Accutase has started to become inactive, more time may be necessary. To ensure swift cell detachment use freshly thawed Accutase.

Acknowledgements The authors would like to thank Andrea Carrano and Allen Wang for comments. This work was supported by National Institutes of Health grants (U01-DK089567 and UC4-DK104202), California Institute for Regenerative Medicine grants (RB5-07236 and RB4-06144), and Helmsley Charitable Trust grant 2012PG-T1D074 to M.S. N.V. was supported by the National Cancer Institute Cancer Cell Biology training grant 5T32 CA067754.

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