Characterization of polyhormonal insulin-producing cells derived in vitro from human embryonic stem cells


Laboratory of Molecular and Cellular Medicine, Department of Cellular & Physiological Sciences, Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada

Department of Surgery, University of British Columbia, Vancouver, BC, Canada

Department of Anesthesiology, Pharmacology & Therapeutics, University of British Columbia, Vancouver, BC, Canada

Department of Chemical & Biological Engineering, Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada

BetaLogics Venture, Janssen R & D LLC, Raritan, NJ, USA

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Abstract Human embryonic stem cells (hESCs) were used as a model system of human pancreas development to study characteristics of the polyhormonal cells that arise during fetal pancreas development. HESCs were differentiated into fetal-like pancreatic cells in vitro using a 33-day, 7-stage protocol. Cultures were ~90–95% PDX1-positive by day (d) 11 and 70–75% NKX6.1-positive by d17. Polyhormonal cells were scattered at d17, but developed into islet-like clusters that expressed key transcription factors by d33. Human C-peptide and glucagon secretion were first detected at d17 and increased thereafter in parallel with INS and GCG transcript levels. HESC-derived cells were responsive to KCl and arginine, but not glucose in perifusion studies. Compared to adult human islets, hESC-derived cells expressed ~10-fold higher levels of glucose transporter 1 (GLUT1) mRNA, but similar levels of glucokinase (GCK). In situ hybridization confirmed the presence of GLUT1 transcript within endocrine cells. However, GLUT1 protein was excluded from this population and was instead observed predominantly in non-endocrine cells, whereas GCK was co-expressed in insulin-positive cells. In rubidium efflux assays, hESC-derived cells displayed mild potassium channel activity, but no responsiveness to glucose, metabolic inhibitors or glibenclamide. Western blotting experiments revealed that the higher molecular weight SUR1 band was absent in hESC-derived cells, suggesting a lack of functional KATP channels at the cell surface. In addition, KATP channel subunit transcript levels were not at a 1:1 ratio, as would be expected (SUR1 levels were ~5-fold lower than Kir6.2). Various ratios of SUR1:Kir6.2 plasmids were transfected into COSM6 cells and rubidium efflux was found to be particularly sensitive to a reduction in SUR1. These data suggest that an impaired ratio of SUR1:Kir6.2 may contribute to the observed KATP channel defects in hESC-derived islet endocrine cells, and along with lack of GLUT1, may explain the absence of glucose-stimulated insulin secretion.

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Introduction

Diabetes is a chronic disease characterized by elevated circulating glucose levels and debilitating long-term health complications. Patients with either type 1 or 2 diabetes suffer from insulin insufficiency and loss of pancreatic β-cells, albeit via different mechanisms (Mathis et al., 2001; Donath and Halban, 2004). Strategies to restore regulated insulin production in diabetic patients include regeneration of endogenous pancreatic β-cells via neogenesis from a resident progenitor cell population, proliferation of pre-existing β-cells or transdifferentiation from a closely related non-β-cell type (Tuduri et al., 2012; Bonner-Weir and Weir, 2005). Alternatively, an exogenous source of insulin-secreting β-cells, such as cadaveric human islets, porcine islets or human embryonic stem cell (hESC)-derived cells could be transplanted into diabetic patients to restore insulin secretion (Tuduri et al., 2012; Guo and Hebrok, 2009; Bruin and Kieffer, 2012). Despite exciting progress in generating glucose-responsive insulin secreting cells following transplantation of hESC-derived pancreatic progenitor cells (Kroon et al., 2008; Rezania et al., 2012; Bruin et al., 2013; Rezania et al., 2013), this process has not yet been replicated in vitro. Instead, most studies report the formation of polyhormonal insulin-expressing cells from hESCs that resemble the transient endocrine cells observed in a mid-gestation human fetal pancreas (Kroon et al., 2008; Rezania et al., 2012; Bruin et al., 2013; Nostro et al., 2011; Rezania et al., 2011; J. Jiang et al., 2007; Chen et al., 2009; Xu et al., 2011). The role and fate of polyhormonal pancreas cells during human fetal development are poorly understood, although immunohistochemical characterization indicated that these cells possess an α-cell transcription factor profile (Riedel, 2011). Moreover, several reports describe the formation of α-cells in vivo following transplantation of hESC-derived polyhormonal cells (Rezania et al., 2011; Kelly et al., 2011; Basford et al., 2012; Rezania et al., 2013). Further characterization of insulin/glucagon co-expressing cells may provide insight into the possible function and fate of these cells during human pancreas development.

Here, we characterized a new differentiation protocol to model human pancreas development in vitro. Since only roughly 1% of the human pancreas is composed of endocrine cells, our goal was not to maximize the endocrine compartment, but rather to assess how closely the mixed population of hESC-derived cells resembled the developing whole human pancreas. Secondly, we utilized this model system to study characteristics of hESC-derived polyhormonal cells. Although insight has been gained into the RNA profile of hESC-derived polyhormonal cells from previous studies (D’Amour et al., 2006; Kelly et al., 2011; Basford et al., 2012), there is still much to be learned about the protein profile and function of these cells. For instance, we still do not understand which features of mature β-cells are lacking in hESC-derived insulin/glucagon co-positive cells and preventing them from secreting insulin in response to a glucose stimulus. Previous studies characterized polyhormonal cells following cell sorting, which has the advantage of a pure population for RNA analysis (Kelly et al., 2011; Basford et al., 2012). To complement studies in isolated cells, we examined the functional and morphological characteristics of intact polyhormonal cells within their culture environment, thus maintaining the normal cell–cell interactions and preserving their cellular niche.

Materials and methods

In vitro differentiation of hESCs

All differentiation experiments for the protocol development (Fig. 1) were performed with H1 hESCs, obtained from WiCell Research Institute, Inc. (Madison, WI). The optimized 33-day, 7-stage differentiation protocol (Fig. 2A, Supplementary Tables 1–2) was then applied to the CA1S hESC line, derived from the parental CA1 line (Amps et al., 2011). These cells maintain pluripotency markers and the ability to generate all germ layers when transplanted into nude mice (Caron et al., 2013). CA1S cells are more conducive to single cell seeding than the parental CA1 cells and as such, are an excellent line for generating homogenous and uniform cultures (Caron et al., 2013; Schulze et al., 2010). All experiments at UBC with CA1 and CA1S cells were approved by the Canadian Stem Cell Oversight Committee and UBC Clinical Research Ethics Board.

H1 and CA1S cells were both cultured in mTesR1 media (Stem Cell Technologies, Vancouver BC, Canada) on 1:30 Matrigel coated surfaces (Stem Cell Technologies). Once cells reached ~90–95% confluence, hESCs were directed through 7 stages of pancreatic development over a period of 33 days according to the details provided in Supplementary Tables 1–2 and as summarized in Fig. 2A. Differentiation culture media was changed daily during stages 1–6 and every other day during stage 7.

Flow cytometry

HESC-derived cells were released into single-cell suspension by incubation in Accutase (Stem Cell Technologies) at 37 °C for 10 min. For surface marker staining, cells were washed twice in staining/wash buffer with 5% FBS (BD Biosciences). Cells were washed and immediately fixed in 1% paraformaldehyde (PFA) and permeabilized with 0.2% TritonX-100 for 20 min and incubated with protein blocking solution (Dako; Glostrup, Denmark) for 1 h at RT. Primary antibodies were

Immunofluorescent staining

In situ staining

hESC-derived cells were washed with PBS and immediately fixed with 4% PFA on the plate at 4 °C overnight. Cells were washed with PBS, permeabilized with 0.2% TritonX-100 for 20 min and incubated with protein blocking solution (Dako; Glostrup, Denmark) for 1 h at RT. Primary antibodies were
incubated overnight at 4 °C (Supplementary Table 3). Cells were washed 5× with PBS and incubated in secondary antibodies for 90 min at RT: goat anti-guinea pig AF488 (1:500; Life Technologies) or donkey anti-rabbit AF555 (1:500; Life Technologies). Cells were washed 5× with PBS and counterstained with Hoechst 33342 (10 μg/ml; Invitrogen) for 30 min at RT. PBS was added to the wells and images were captured using the ImageXpressMicro™ Imaging System and analyzed using MetaXpress Software (Molecular Devices Corporation, Sunnyvale, CA, USA).

Paraffin-embedded sections
HESC-derived cells were incubated in Accutase for ~2 min and cell sheets were gently lifted from the well with a cell scraper. The intact cell sheet was fixed overnight in 4% PFA at 4 °C, embedded in 1% agarose and then stored in 70% ethanol prior to paraffin-embedding and sectioning (5 μm thickness; Wax-it Histology Services, Vancouver, BC Canada). Paraffin-embedded human pancreas sections were used as a positive control for antibody conditions (Irving K. Barber Human Islet Isolation Laboratory; Vancouver, BC). Immunofluorescent staining was performed as previously described (Rezania et al., 2012).

Primary antibodies are detailed in Supplementary Table 3. Images were captured as described above.

In situ hybridization
Paraffin embedded hESC-derived cells were prepared and sectioned as above (see immunofluorescent staining). In situ hybridization was performed with the Ishyb In Situ Hybridization kit (Biochain, San Francisco, California). Briefly, slides were de-paraffinized with three consecutive xylene washes, rehydrated with graded ethanol washes. Sections were fixed with 4% PFA for 20 min, and treated with 10 μg/ml Proteinase K (Sigma Aldrich) at 37 °C for 15 min. Sections were incubated for 4 h at 50 °C in pre-hybridization solution (Biochain). Probe for human SLC2A1 mRNA (NM_006516) (GLUT1) was designed and custom synthesized with a 5′ DIG label at IDT Oligo (Vancouver, Canada). Slides were incubated with the 5′ DIG labeled GLUT-1 probe (AAGCACATGCCCAC AATGAAATTTGAGGTCC) in hybridization solution (Biochain) for 14 h at 50 °C. Control sections were incubated in hybridization solution alone. Slides were then washed in different concentration of saline-sodium citrate (SSC) buffers (2×, 1.5×,
Figure 2  Summary of in vitro differentiation protocol checkpoints in CA1S cells. A) Schematic summary of the 7-stage, 33-day differentiation protocol. B) Expression of key checkpoint proteins throughout the differentiation, illustrating the typical efficiency of pancreatic cell development at various stages. C) Images of in situ immunofluorescent staining for key checkpoint markers in differentiating 12-well cultures at day 11, 19 and 26. Hoescht (blue) marks nuclei and is shown only in the top image at day 11 and the top left images at days 19 and 26. At day 11 and 19 scale bars = 50 μm. At day 26 rare cells co-expressing both NKX6.1 and insulin (white arrow) are shown; scale bars = 100 μm.
0.2× (Biochain) and sections were incubated with 1X blocking solution (Biochain) for 1 h. Slides were incubated overnight with alkaline-phosphate conjugated anti-digoxinogen antibody (diluted 1:500 in PBS; Biochain) at 4 °C. The following day, slides were washed with PBS and alkaline phosphatase buffer (Biochain) and incubated with nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3’-indolylphosphate p-toluidine salt (BCIP) solution (6.6 μl NBT and 3.3 μl BCIP were diluted in 1 ml alkaline phosphatase buffer; Biochain) for 48 h at 4 °C. Sections were then immunostained for insulin, glucagon and DAPI as indicated above using the following antibodies: guinea pig anti-insulin (Sigma Aldrich), mouse anti-glucagon (Sigma Aldrich), Alexa Fluor® 594 goat anti-guinea pig IgG (Life Technologies, Carlsbad, California) and Alexa Fluor® 488 donkey anti-mouse IgG (Life Technologies). Fluorescent and bright field images were obtained with Openlab software (PerkinElmer).

Quantitative RT-PCR

qPCR
At each stage of differentiation hESC-derived cells were incubated in Accutase for 10 min, washed with PBS and stored in Buffer RLT (Qiagen, Hilden, Germany) at −80 °C. RNA extraction was performed using the RNeasy kit (Qiagen) with on-column DNase digestion according to manufacturer’s instructions. cDNA synthesis was performed using the iSCRIPT kit (Biorad, Mississauga, ON Canada). Quantitative PCR was performed using the SsoFast EvaGreen Supermix with Low ROX master mix (Bio-Rad) and run on a StepOne Plus Real Time PCR machine (Applied Biosystems). HPRT was used as a reference gene. Data were analyzed using the ΔΔCT method. Primer sequences are listed in Supplementary Table 4. Gene expression in hESC-derived cells was normalized to undifferentiated CA1S cells (Supplementary Fig. 1A-F), adult human islets (Supplementary Figs. 1G, 1H and Fig. 7D) or adult human pancreas (Supplementary Fig. 1H). Human islets were kindly provided by the Irving K. Barber Human Islet Isolation Laboratory (Vancouver, BC). Human pancreas total RNA was purchased from Life Technologies (Cat# AN7954).

SA biosciences array
RNA isolation, cDNA synthesis and qPCR were performed in day (d) 33 cells as described above, but using a custom designed PCR array (primers provided in Supplementary Table 5; SA Biosciences). Data analysis was performed using the online RT2 Profiler PCR Array Data Analysis Tool. Gene expression is presented as the fold change relative to adult human islets (n = 3).

Applied biosysytems array
Gene expression profiles of H1 cells were assessed by custom Taqman qPCR Arrays (Applied Biosystems, Foster City, CA), as described previously (Rezania et al., 2012). Data were analyzed using Sequence Detection Software (Applied Biosystems) and normalized to a housekeeping gene (GAPDH) using the ΔΔCT method. Primers are provided in Supplementary Table 6.

Total cell extraction and western blotting

CA1S cells from different stages of differentiation (d7-d33) and HL-1 cells were lysed in RIPA buffer (50 mM Tris pH 8, 400 mM NaCl, 0.5% Triton NP-40, 1% DOC, 0.1% SDS, 1 μg/ml pepstatin, 1 μg/ml bestatin, 2 μg/ml leupeptin, 2 mM PMSF, 10 mM β-Glycerophosphate, 1 mM NaF, 1 mM DTT), homogenized with a needle and syringe, and rotated for 20 min at 4 °C. Lysate was centrifuged for 20 min at 4 °C at 14,000 rpm. Total proteins were loaded on a 7.5% SDS gel and transferred to nitrocellulose membrane and blotted with anti-SUR1 antibody (NeuroMab, clone N289/16), goat anti-mouse secondary antibody (ABM, Vancouver, BC). Signal was detected with ECL (Femto ECL reagent, Pierce).

Hormone secretion

Basal hormone secretion
Media was collected from cells after a 24 h incubation period on the indicated days of differentiation (Figs. 5A,B), centrifuged briefly and stored at −30 °C. Samples were assayed for C-peptide and glucagon via radioimmunoassay (Millipore, Billerica, MA).

Perfusion protocol 1
D33 cells were loaded into temperature-/CO2-controlled chambers of an Endotronics Acu-syst S Perifusion apparatus. Cells from 4 wells of a 6-well plate were pooled for each individual column. HEPES-buffered Krebs Ringers Bicarbonate Buffer (KRBB) containing 0.5% BSA and 3 mM glucose was pumped through the chambers at −350 μl/min following a 35 min preincubation under basal conditions. Fractions were then collected throughout the 170 min perfusion protocol, as indicated in Figs. 5C,D. All samples were assayed for C-peptide and glucagon via radioimmunoassay (Millipore).

Perfusion protocol 2
To determine if the cells were desensitized to glucose after long-term culture in 25 mM glucose, cells were cultured throughout from d20 to 33 in stage 7 media (Supplementary Table 1) containing low (8 mM) or high (25 mM) glucose. D33 cells (low or high glucose) were transferred to stage 7 culture media containing 3 mM glucose for 2 h before transfer to perfusion columns (4 wells per column). Once contained within the perfusion apparatus, cells were incubated under basal conditions for 1 h and then fractions were collected throughout the 250 min perfusion protocol, as indicated in Fig. 6B. All samples were assayed for insulin via radioimmunoassay (Millipore).

Non-radioactive rubidium efflux assay

β-cell lines and hESC-derived cells
A non-radioactive rubidium (Rb+) efflux assay was used as a measure of KATP channel activity (Li et al., 2013). Cells were loaded for 1 h with Rb+ loading media (5.4 mM RbCl, 150 mM NaCl, 2 mM CaCl2, 0.8 mM NaH2PO4, 1 mM MgCl2, 5 mM glucose, 25 mM HEPES, pH 7.4), washed 2X with PBS, and incubated in assay buffer (118 mM NaCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 4.7 mM KCl, 25 mM NaHCO3, 1.2 mM MgSO4, 10 mM HEPES, pH 7.4) supplemented with glucose, glibenclamide, or...
metabolic inhibitors (2.5 μg/ml oligomycin, 1 mM 2-deoxy-D-glucose) as indicated. Aliquots of the assay buffer were removed at multiple time points (5, 10, 20, 40 min) and the Rb⁺ concentration in the supernatant was determined by flame atomic absorption spectroscopy using an Aurora Biomed ICR8000 instrument. Rubidium efflux was represented as a fraction of total loaded Rb⁺ (determined as the sum of extruded Rb⁺ and Rb⁺ left in lysed cells at the end of the assay).

**Transfected COSM6 cells**

COSM6 cells were maintained in culture in a 5% CO₂ incubator at 37 °C in DMEM supplemented with 10% FBS and penicillin/streptomycin. COSM6 cells in 24 well plates were transiently transfected with mouse SUR1 and hamster Kir6.2 cDNAs (expressed in pcDNA3.1-) in various ratios (400 ng total DNA per well) using jetPRIME (Polyplus), and Rb⁺ efflux assays were performed after 2 days. As the total amounts of Kir6.2 and SUR1 plasmids were reduced, additional GFP vector was added to match the total transfected DNA in each well. For efflux assays, cells were washed overnight by adding 3 mM RbCl to the cell culture medium. Just before the experiment, cells were washed twice quickly with PBS. Cells were then incubated in assay buffer supplemented with metabolic inhibitors (as described above). Aliquots of the assay buffer were removed at multiple time points (5, 10, 20, 40 min) and the Rb⁺ concentration was determined as described above. For clarity of figure presentation, data was normalized to efflux from WT Kir6.2 transfected cells (normalized Rb⁺ efflux = (EffluxMutant – EffluxUntransfected)/ (EffluxWT Kir6.2 – EffluxUntransfected). However, raw efflux data was used for statistical tests. For simplicity, only data from the 40 min time point has been presented.

**Trypsin activity assay**

At day 33, cell pellets from hESC-derived cells were harvested and stored at –80 °C. Trypsin activity was measured using a commercial colorimetric Trypsin Activity Assay Kit according to manufacturer’s instructions (Abcam Inc., Toronto, ON Canada).

**Statistical analysis**

Graphpad Software (La Jolla, CA) was used for all statistical analysis. Specific statistical tests are described in figure legends. Statistical significance was considered p < 0.05. All data are presented as mean ± s.e.m.

**Results**

**Modification of the differentiation protocol in H1 cells**

Our previous protocol (Rezania et al., 2012) was revised to incorporate a number of changes designed to improve the overall quality of cultures and enhance efficiency of definitive endoderm, pancreatic endoderm and endocrine progenitor formation in H1 cells. In our previous protocol as well as most previously published studies (Kroon et al., 2008; Nostro et al., 2011; D’Amour et al., 2006; Xu et al., 2011; W. Jiang et al., 2007; Cai et al., 2010; Mfopou et al., 2010) various media formulations were used at different stages of differentiation. To simplify the protocol, we empirically tested many commercially available media formulations (data not shown) and identified that the MCDB 131 media formulation could be used throughout the entire protocol. At stage 1, we replaced activin A (AA)/WNT3A with GDF8/GSK3β inhibitor to further enhance efficiency of definitive endoderm production, as measured by expression of CXCR4 (AA/WNT3A vs GDF8/GSK3β): 84.8 ± 2.55 vs 96.8 ± 0.95% CXCR4, p < 0.05). Consistent with previous results (Bone et al., 2011), addition of a GSK3β inhibitor instead of WNT3A improved efficiency of definitive endoderm generation. Furthermore, GDF8, a TGFβ family member, proved as effective as activin A for inducing definitive endoderm and is a more economical option. At stage 3 onwards we tested the effect of various glucose concentrations and demonstrated that high glucose (15–30 mM) enhanced gene expression of pancreatic endoderm and endocrine markers compared to low glucose conditions (Fig. 1A). For the final differentiation protocol, we opted to increase the glucose concentration from 7.5 mM during stages 1–2 to 25 mM during stages 3–7. We also replaced Noggin with a small molecule inhibitor of BMP receptor and added AA and FGF7 to enhance expression of foregut endoderm markers while suppressing endocrine precursor markers at stage 3 (Fig. 1B). The rationale for suppressing endocrine precursor markers at stage 3 was to potentially reduce number of polyhormonal cells at later stages of differentiation (Johansson et al., 2007). Previous reports (Kinkel et al., 2009) indicated that inhibition of CYP26A enzyme could expand the pancreatic domain during regionalization of endoderm fields. Indeed, addition of a small molecule inhibitor of CYP26A at stage 4 resulted in enhanced expression of pancreatic endoderm and endocrine precursor markers; 100 nM was selected as the optimal concentration of the CYP26A inhibitor for the final protocol (Fig. 1C). Vitamin A (a precursor of retinoic acid) was included during the final stage of differentiation because low doses of retinoic acid were previously shown to enhance insulin expression in e10.5 pancreas explants from mice (Ostrom et al., 2008). We consistently observed a slight reduction in the number of polyhormonal cells with the addition of retinoic acid during stage 7 (A Rezania, unpublished observations). Lastly, we replaced FBS at stages 1–2 and B27 at stages 3–7 with BSA and BSA plus ITS-X, respectively.

**Assessment of pancreas markers throughout differentiation in CA1S cells**

The revised 33-day differentiation protocol (Fig. 2A) was next characterized in CA1S cells. We first sought to determine checkpoints for key markers of developing pancreas lineage cells throughout differentiation. Representative flow cytometry plots are shown in Fig. 2B. At the end of stage 1 (d5), cultures typically expressed ~95–97% CXCR4, a marker of definitive endoderm, but cultures with ~90% CXCR4-positive cells were considered acceptable to proceed with differentiation (Fig. 2B). At the end of stage 3 (d11), cultures of pancreatic endoderm cells were typically between 90 and 95% PDX1-positive, although cultures containing >90% PDX1 were accepted. At the end of stage 5 (d17–18), the pancreatic endocrine progenitor population maintained robust PDX1 expression...
(illustrated in Fig. 2C) and 70–75% of cells expressed NKX6.1 (cultures containing >70% NKX6.1-positive cells were accepted). During the final stage of differentiation (d20–33), insulin was typically expressed in 6–10% of cells (Fig. 2B).

In situ immunofluorescent staining was also performed at our key checkpoints (Fig. 2C). Cultures displayed highly uniform nuclear PDX1 expression at the end of stage 3, which was maintained throughout the differentiation. During stage 6 PDX1-positive cells co-expressed variable levels of NKX6.1, creating a mosaic pattern of pancreatic endocrine progenitors. NKX6.1 expression was maintained throughout stage 7, but only rare NKX6.1/PDX1-positive cells co-expressed insulin. Insulin-positive/NKX6.1-negative cells were dispersed throughout the cultures at d26.

We next assessed gene expression of key pancreas markers in whole cultures throughout differentiation. Pancreatic-specific transcription factors were initially detected at the end of stage 3 and maintained until the end of differentiation; PDX1 was detected first, beginning at d11, followed next by NKX6.1 and ARX beginning at d14 (Supplementary Fig. 1A). Gene expression of INS and GCG was detected at d17 and increased throughout stages 5–7 (Supplementary Fig. 1A). Amylase (AMY2A) was consistently expressed at relatively low levels throughout the differentiation (Supplementary Fig. 1A). To put these transcript levels in perspective, gene expression in hESC-derived d33 cells was compared to adult human islets. Notably, a marked upregulation (~60-fold) of the pancreatic endocrine progenitor marker NGN3 was observed in the hESC-derived cultures relative to adult islets (Supplementary Fig. 1B). PDX1, SOX9, MAFB and ARX were also substantially upregulated. Most other pancreas transcription factors were expressed at similar levels to adult islets, with the exception of MAFA and PTF1A, which were markedly downregulated (Supplementary Fig. 1B). Pancreatic hormones, INS, IAPP, and PP were much lower than in islets, whereas GCG and SST were similar, and GHRGL levels were ~10-fold higher (Supplementary Fig. 1B). Interestingly, β-cell markers INS, NKX6.1 and PDX1 were expressed at levels more similar to whole human pancreas than isolated human islets (Supplementary Fig. 1C) and INS transcript levels were equivalent to that of a 10 week gestational age human fetal pancreas (J Bruin and S Erener, unpublished observations). In contrast, α-cell markers GCG and ARX were more similar to isolated human islet levels than whole pancreas (Supplementary Fig. 1C).

Immunofluorescent staining of paraffin-embedded mono-layer cultures confirmed the appearance of pancreatic endocrine cells in differentiating cells beginning at d17 and increasing in frequency thereafter (Fig. 3A). Distinct islet-like endocrine clusters were observed at d33 and were generally polyhormonal, with most cells expressing either two or three of insulin, glucagon and/or somatostatin (Fig. 3B). Ghrelin-positive cells were present within the bud structures, but surprisingly did not co-localize with chromogranin A (Supplementary Fig. 2A). Rare cells expressing pancreatic polypeptide were also detected in the cultures (Supplementary Fig. 2B). NKX6.1 and NKX2.2 were generally localized within the endocrine buds, but in distinct compartments; NKX6.1 was expressed in non-endocrine cells (Fig. 3C), whereas NKX2.2 was expressed in insulin/glucagon co-positive cells (Fig. 3D).

A pro-insulin antibody specific for the intact B/C junction of pro-insulin revealed pro-insulin immunoreactivity in the majority of insulin expressing cells at d33 (Fig. 3E), suggesting that insulin produced by hESCs was incompletely processed. To further investigate, we next examined expression of pro-hormone processing enzymes, which are required for production of functional pancreatic hormones. Gene array results revealed significantly lower levels of both prohormone convertase (PC) 1/3 and 2 compared to human islets (Supplementary Fig. 3). PC2 protein was detected in nearly all insulin-positive cells at d33 (Fig. 3F), whereas PC1/3 expression was heterogeneous (Fig. 3G). Similarly, the majority of insulin/glucagon double-positive cells expressed PC2 but not PC1/3. A substantial number of insulin-positive/PC1/3-negative cells and insulin-negative/glucagon-negative/PC1/3-positive cells were observed within the endocrine clusters (Fig. 3G), suggesting that lack of PC1/3 could be the underlying cause of incomplete insulin processing in hESC-derived cells.

Lastly, non-endocrine pancreas markers were examined in the d33 cultures (Fig. 4). Immunoactivity for trypsin, an enzyme found in developing and mature pancreatic acinar cells, was expressed throughout the cultures, including within the endocrine cells (Fig. 4A). Pancreatic secretory trypsin inhibitor (PSTI) was also co-expressed in the endocrine clusters (Fig. 4B). Yet, the surrounding trypsin-positive cells did not express other markers of mature exocrine tissue, such as carboxypeptidase A1 (CPA1; Figs. 4C-D) suggesting that these acinar cells were immature. Indeed a trypsin activity assay revealed that media secreted from d33 cultures had no detectable trypsin activity (data not shown). Interestingly, rare CPA1-positive cells were found to be co-localized with synaptophysin (Fig. 4C, white arrows). CK19, a ductal marker, was widely expressed throughout the cultures and also within the bud structures, but in a separate compartment from the endocrine cells (Figs. 4D–F). This pattern of CK19 expression (adjacent to endocrine cells within the bud structures) resembles that of NKX6.1 (Fig. 3C), indicating that NKX6.1 was co-expressed within this subset of ductal cells. Indeed, immunostaining for transcription factors PDX1 and NKX6.1 revealed that CK19 was co-localized with the majority of NKX6.1 expressing cells (Fig. 4E) and also with PDX1 expressing cells (Fig. 4F) albeit more heterogeneously.

**Regulation of hormone secretion by hESC-derived endocrine cells**

To assess the function of hESC-derived pancreatic endocrine cells, we first measured basal hormone secretion at all stages of differentiation. Corresponding to the timeline of detectable RNA and protein expression (Supplementary Fig. 1A and Fig. 3A, respectively), human C-peptide and glucagon were first detected in the culture media at d17 (Figs. 5A and B, respectively). Hormone secretion increased steadily throughout stages 6 and 7, and peak hormone levels were detected at d26 (Figs. 5A–B). Perfusion experiments at d33 revealed that C-peptide and glucagon secretion were both regulated by 30 mM KCl and 15 mM arginine, but not 15 mM glucose (Figs. 5C and D).

Since cells had been cultured in media containing 25 mM glucose throughout stages 3–7, we speculated that the hESC-derived endocrine cells might be desensitized to high glucose and consequently, the switch from 3 to 15 mM...
glucose during perifusion may simply have been inadequate to stimulate insulin secretion. Therefore, stage 7 cells were cultured in either high (25 mM) or low (8 mM) glucose media. At d26, two independent experiments demonstrated that basal human C-peptide secretion was significantly higher in cells exposed to 8 mM glucose compared to 25 mM glucose (Fig. 5E). Perifusion at d33 showed a trend towards elevated insulin secretion in response to KCl and arginine by cells cultured in low glucose stage 7 media, but no change in response to glucose (Fig. 5F), suggesting that desensitization alone does not explain the lack of glucose-stimulated insulin secretion.

Characteristics of hESC-derived insulin-secreting cells

Glucose transport and sensing components

To further characterize the hESC-derived insulin/glucagon co-positive cells, we next assessed various components of the
Figure 4  Characterization of exocrine and ductal markers in hESC-derived cells at day 33. A) Trypsin was weakly expressed throughout cultures and also co-expressed within endocrine cells (marked by synaptophysin). B) Pancreatic secretory trypsin inhibitor (PSTI) was also expressed in hESC-derived insulin-positive cells. C–D) Rare carboxypeptidase A1 (CPA1)-positive cells were detected at day 33, but these were generally co-localized in endocrine cells (panel C insets; white arrows). C–F) Ductal marker, CK19, was widely expressed throughout the culture and also located within the bud structures. E–F) Costaining of CK19 with transcription factors NKX6.1 (panel E) and PDX1 (panel F). All scale bars = 100 μm.
glucose sensing machinery required for β-cell function. Glucose is transported into β-cells by the glucose transporter (predominantly GLUT1 in humans (De Vos et al., 1995; Coppieters et al., 2011); Fig. 6B) and then phosphorylated by glucokinase (GCK) prior to entering the glycolysis pathway (MacDonald et al., 2005). We observed that GLUT1 transcript levels were ~10-fold higher than in adult human islets, whereas GCK levels were similar (Fig. 6A). However, immunofluorescent staining revealed that GCK was co-expressed in insulin-positive cell clusters (Fig. 6D), whereas GLUT1 protein was not (Fig. 6C). Notably, the same GLUT1 antibody displayed strong immunoreactivity in β-cells from adult human pancreas tissue (Fig. 6B). Given the striking absence of GLUT1 protein in the endocrine population, we next sought to determine if the transcript was present in these cells. In situ hybridization experiments revealed that GLUT1 mRNA was present in both the polyhormonal endocrine cells and the surrounding non-endocrine population (Fig. 6E). Therefore, it appears that the glucose transporter in hESC-derived insulin/glucagon co-positive cells may be transcribed, but not translated.
ATP-sensitive potassium channels
Glucose metabolism elevates ATP levels in mature β-cells, triggering closure of ATP-sensitive K+ (K_{ATP}) channels, membrane depolarization, opening of voltage-sensitive Ca^{2+} channels and Ca^{2+}-induced insulin secretion (MacDonald et al., 2005). To assess K_{ATP} channel function, we performed a non-radioactive rubidium (Rb+) efflux assay (Li et al., 2013). At d26, hESC-derived cells showed mild Rb+ efflux, but to a

Figure 6 Characterization of glucose transport and sensing components in stage 7 cells. A) Gene expression of glucose transporter 1 and glucokinase (GLUT1 and GCK) at day 33 relative to levels in isolated adult human islets (indicated by dashed red line). B) GLUT1 is expressed in insulin-positive cells within adult human pancreas. C) GLUT1 was highly expressed in hESC-derived cells, but completely absent within the insulin/glucagon co-positive endocrine cells (scale bars = 50 μm). D) In contrast, glucokinase was co-expressed in polyhormonal insulin/glucagon-positive cells within the bud structures (scale bars = 50 μm). E) In situ hybridization revealed that GLUT1 mRNA is expressed in pancreatic endocrine cells within the bud structures (scale bars = 75 μm).
lesser degree than \( \beta \)-TC3 or MIN6 cells (Fig. 7A). Moreover, unlike \( \beta \)-cell lines, \( \text{Rb}^+ \) efflux in hESC-derived cells was not inhibited by exposure to glucose or the sulfonylurea receptor blocker, glibenclamide (Fig. 7A). A metabolic inhibitor cocktail, which reduces cellular ATP levels and thus causes opening of \( K_{\text{ATP}} \) channels, increased \( \text{Rb}^+ \) efflux in cultured \( \beta \)-cells but had no effect on \( \text{Rb}^+ \) efflux in hESC-derived cells. These data suggest that functional \( K_{\text{ATP}} \) channels were either not expressed in hESC-derived cells or only present in a small subset of cells.

\( K_{\text{ATP}} \) channels are composed of two subunits: the regulatory sulfonylurea receptors (SUR1) and pore-forming Kir6.2 subunits. We examined the expression of Kir6.2 in hESC-derived insulin-positive cells and detected little to no immunoreactivity for Kir6.2 (Fig. 7B). We also examined the formation of \( K_{\text{ATP}} \) channels using Western blot detection of SUR1. Different stages of maturation and trafficking of SUR1 generate multiple molecular weight bands on SDS-PAGE gels. In HL-1 cells, a cardiomyocyte derived cell line with significant endogenous \( K_{\text{ATP}} \) activity, both the higher and lower molecular weight SUR1 bands were detected, consistent with formation of functional, mature, \( K_{\text{ATP}} \) channels (Fig. 7C). However, in the hESC-derived cells (d26–d33), only the lower molecular weight band was observed. This finding is consistent with a lack of functional \( K_{\text{ATP}} \) channels at the cell surface, in line with our observation of little or no \( K_{\text{ATP}} \)-mediated \( \text{Rb}^+ \) efflux.

Our gene array indicated that mRNA levels of Kir6.2 were similar to that of adult human islets, but that ABCCB (SUR1) levels were significantly downregulated (Fig. 7D). Given that these subunits should be expected at a 1:1 ratio within mature \( \beta \)-cells, we speculated that the observed discrepancy in the subunit proportions (~5:1 Kir6.2:SUR1 transcript levels; Fig. 7D) may also contribute to the defect in \( K_{\text{ATP}} \) channel activity (Fig. 7A). Therefore, COSM6 cells were transfected with varying ratios of Kir6.2:SUR1 plasmid (ensuring that the total DNA transfected was the same in each condition) and \( \text{Rb}^+ \) efflux was assessed. Our findings demonstrate that \( K_{\text{ATP}} \) channel activity was detrimentally affected by a reduction in the SUR1 subunit (Fig. 7E). In contrast, reducing the Kir6.2 plasmid did not substantially impact \( \text{Rb}^+ \) efflux, even when a 10:1 ratio of SUR1:Kir6.2 plasmid was transfected (Fig. 7E). These findings highlight that an appropriate stoichiometric balance of Kir6.2 and SUR1 is required to generate adequate \( K_{\text{ATP}} \) channel activity (Flagg et al., 2005), and that stoichiometric mismatch may contribute to the absence of functional \( K_{\text{ATP}} \) channels in hESC-derived cells.

**Discussion**

In the present study, we characterize a differentiation protocol that converts pluripotent hESCs into a mixed cell population of immature pancreas cells, including budding structures of NKX6.1-positive ductal cells adjacent to islet-like endocrine clusters. These hESC-derived cells resembled a mid-gestation human fetal pancreas and thus may be a useful model for studying human pancreas development. Our studies provide novel insights into several key elements of mature \( \beta \)-cells that appear to be deficient in hESC-derived polynuclear cells, such as GLUT1 protein expression, \( K_{\text{ATP}} \) channel activity and prohormone processing enzymes. Until hESC-derived insulin-secreting cells gain these features, glucose-stimulated insulin secretion will not be achieved in vitro.

There are several lines of evidence to suggest that our in vitro differentiation protocol provides a meaningful model of human pancreas development. Firstly, our modified protocol for inducing definitive endoderm formation, using GDF8 and a GSK3\( \beta \) inhibitor, consistently proved to be more efficient than the typical AA/WNT3A combination. Unlike our other studies (Rezania et al., 2012; Bruin et al., 2013; Rezania et al., 2013) this definitive endoderm protocol is also serum-free, which is preferable for reproducibility. Similar to previously published differentiation protocols designed to produce insulin-secreting cells in vitro (Nostro et al., 2011; D’Amour et al., 2006), we observed robust induction of PDX1 in more than 90% of cells at d11 and then NKX6.1 in over 70% of cells at d17. As with human fetal pancreas (Riedel, 2011), the developing endocrine cells were initially scattered and sparse, but increased in number over time, as demonstrated by qPCR, immunofluorescent staining of in situ cultures and paraffin sections, and hormone secretion into the media. Moreover, the developing endocrine cells transitioned into distinct islet-like clusters that resembled the endocrine clusters in a mid gestation human pancreas (Riedel, 2011). For instance, the hESC-derived insulin-positive cells co-expressed glucagon and NKX2.2, but were negative for NKX6.1, which was instead expressed in neighbouring CK19-positive/hormone-negative cells within the budding clusters. Yet, unlike a mid-gestation human fetal pancreas, the rare hESC-derived insulin-only cells lacked expression of NKX6.1 and PDX1. Approximately 6–10% of cells in our cultures expressed insulin at d33 and INS gene levels were only 2-fold less than whole adult pancreas, but comparable to levels in a mid gestation human fetal pancreas (J Bruin and S Erener, unpublished observations). These data suggest that our differentiation protocol more accurately modeled whole pancreas development rather than simply islet development.

We next examined whether our highly homogenous PDX1-positive pancreatic progenitor cells differentiated into both endocrine and exocrine lineages in vitro. Trypsin, an early marker of developing acinar cells, was weakly expressed throughout the cultures, including within the developing pancreatic endocrine cells. This was unexpected since trypsin and synaptophysin are not typically co-expressed during human fetal pancreas development from 12w4d to 21w (A Asadi and J Bruin, unpublished observations). Interestingly, PSTI was also highly expressed in the insulin-positive cells, similar to human fetal islets (Fukayama et al., 1986), suggesting that trypsin activity was likely inhibited in the developing endocrine cells. Given that trypsin-positive cells did not co-express other exocrine markers, such as amylase or CPA1, and the cultures showed no detectable trypsin activity (data not shown), we concluded that these cells were unlikely to be comparable to developing exocrine cells.

We next utilized our culture model of pancreas development to study the phenotype of hESC-derived polynuclear cells, as the role of these cells in fetal pancreas remains controversial. For instance, it is currently unclear why insulin/glucagon co-expressing cells seem to be more “\( \alpha \)-like” than “\( \beta \)-like”, both in terms of their transcription factor profile (Riedel, 2011) and their apparent preference to differentiate into \( \alpha \)-cells following transplantation (Rezania et al., 2011; Kelly et al., 2011; Basford et al., 2012; Rezania et al., 2013). We first
Figure 7  Characterization of ATP-sensitive potassium channels in stage 7 cells. A) \( K_{\text{ATP}} \) channel activity was determined by measuring rubidium (Rb+) efflux over time. hESC-derived cells were not responsive to either \( K_{\text{ATP}} \) channel inhibitors (glibenclamide and glucose) or activators (metabolic inhibitors: oligomycin and 2-deoxy-D-glucose). In contrast, \( K_{\text{ATP}} \) channel activity in MIN6 \( \beta \)-cells was appropriately stimulated by metabolic inhibitors and inhibited by the addition of glibenclamide; both glibenclamide and glucose inhibited channel activity in another \( \beta \)-cell line, beta-TC3 cells. B) Immunofluorescent staining of \( K_{\text{ATP}} \) channel subunit Kir6.2 in stage 7 cells (scale bars = 100 \( \mu \)m). C) Total cell extracts from different stages of hESC cultures (d7-d33) and HL-1 cells were prepared and proteins were analyzed by Western blot with an antibody against SUR1. D) Gene expression of \( K_{\text{ATP}} \) channel subunits, \( \text{SUR1} \) and Kir6.2, in hESC-derived cells at day 33, relative to levels in isolated adult human islets. E) \( \text{SUR1} \) and Kir6.2 DNA was transfected into COS cells to determine if the subunit ratio affected channel activity. Rb+ efflux in each condition was normalized to efflux in cells transfected with a 1:1 ratio after 40 min.
examined the hormone secretion kinetics of our hESC-derived endocrine cells and established that although both insulin and glucagon secretion increased throughout differentiation, hormone secretion was not regulated by glucose, similar to previous reports (D’Amour et al., 2006; Basford et al., 2012). We also confirmed that our cells released both C-peptide and glucagon in response to direct depolarization by both KCl and arginine. We speculated that the absence of glucose-induced insulin secretion could be due to the high glucose concentrations in our stages 3–7 media and thus, desensitization of cells to glucose. Indeed, when cells were cultured during stage 7 under lower glucose conditions, we observed increased insulin secretion but no improvement in the sensitivity of endocrine cells to glucose. Therefore, several markers of mature β-cells were examined to identify possible defect(s) in the glucose-stimulated insulin secretion pathway, including: a) glucose transport and sensing mechanisms, b) KATP channels, and c) proinsulin processing.

Our hESC-derived insulin-positive cells appear to be similar to those produced by Nostro and colleagues (Nostro et al., 2011). In a recent report, their cells were thoroughly characterized by FACS-purifying the GFP/insulin-positive cell population, which provided the advantage of a pure population for analysis (Basford et al., 2012). In contrast, our experiments were performed in whole, intact cultures, thus preserving the endocrine cell microenvironment and contact with neighbouring cells. Since transcript levels were measured in mixed cultures, we also carefully assessed the cellular localization of proteins and found key discrepancies between gene and protein expression patterns. For instance, in the mixed cultures at d33, GLUT1 mRNA levels were ~10-fold higher than in human islets, but immunofluorescent staining revealed that GLUT1 protein was virtually absent from the insulin-positive cell population. Interestingly, Basford et al. did not detect a difference in GLUT1 gene expression between sorted GFP/insulin-positive and GFP/insulin-negative cells (Basford et al., 2012), raising the possibility that GLUT1 expression may be discrepant at the protein level. Indeed, in situ hybridization experiments revealed that GLUT1 transcript was present in our hESC-derived endocrine cells, suggesting that the glucose transporter was transcribed but not translated. These data indicate that our hESC-derived insulin-secreting cells may be lacking the mechanism to transport glucose into the cell, the crucial first step for glucose-stimulated insulin secretion.

Our studies also demonstrate that hESC-derived cells only displayed mild K+ channel activity, which did not appear to be mediated by functional KATP channels. HESC-derived cells contained little immunoreactivity for Kir6.2. Although SUR1 could be detected in the hESC-derived cells (d26–d33), it was not assembled with Kir6.2. Basford et al. demonstrated that KATP channels were only active 45% of the time in isolated hESC-derived insulin-positive cells, despite enriched gene expression of the KATP channel subunits (Basford et al., 2012). We also detected gene expression of KATP channel subunits in our hESC-derived cell population, similar to that in human islets, but noted that Kir6.2 transcript levels were ~5-times higher than SUR1. Since these subunits should be expressed in equal proportion within functional KATP channels, we hypothesized that an inappropriate subunit ratio could affect channel activity. Indeed, transfection experiments revealed that Rb+ efflux was particularly sensitive to a reduction in SUR1 plasmid, whereas decreasing Kir6.2 had little effect on channel activity. It remains to be determined whether the ratio of SUR1:Kir6.2 is inappropriate at the single cell level. Regardless, our findings illustrate that appropriate ratios of KATP channel subunits are critical for its activity and this may be a factor contributing to the lack of glucose-responsiveness in hESC-derived insulin-producing cells.

Finally, we also demonstrate that there may be defects in proinsulin processing in the hESC-derived polychromal cell population. The formation of mature insulin hormone involves translation of a single-chain proinsulin peptide, which is then processed by prohormone convertases (PC1/3 and PC2) and carboxypeptidase (CPE) into equimolar amounts of the mature disulphide-linked insulin along with the cleaved connecting peptide (C-peptide). While most insulin-positive cells expressed PC2, there was heterogeneous expression of PC1/3 in this population, resulting in many insulin-positive/PC1/3-negative cells that would presumably produce incompletely processed proinsulin. Defective PC1/3 expression could be particularly problematic, as lack of PC1/3 has been associated with more severe defects in proinsulin processing than loss of PC2 in transgenic mouse models (Zhu et al., 2002; Furuta et al., 1998).

In summary these studies characterize a new differentiation protocol that models human fetal pancreas development in vitro. We demonstrated the utility of our hESC-derived cells for understanding important characteristics of human fetal pancreas cells that are difficult to study due to inaccessibility of human fetal tissues. Our studies revealed several key features of polychromal insulin-positive cells that differ from mature pancreatic β-cells, including defects in glucose transporter expression, KATP channel function and prohormone processing enzymes. These deficiencies will require addressing with further protocol modifications in order to achieve glucose-stimulated secretion of mature insulin by hESC-derived pancreatic endocrine cells in vitro.

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Appendix A. Supplementary data

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References


