Production of Functional Glucagon-Secreting Alpha Cells from Human Embryonic Stem Cells.

Alireza Rezania1*, Michael J. Riedel2*, Rhonda D. Wideman2*, Francis Karanu1, Ziliang Ao3, Garth L. Warnock3, and Timothy J. Kieffer2,3

1BetaLogics Venture, Centocor Research & Development, Skillman, NJ, USA; 2Laboratory of Molecular and Cellular Medicine, Department of Cellular & Physiological Sciences, Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada, V6T 1Z3; 3Department of Surgery, University of British Columbia, Vancouver, BC, Canada, V5Z 1M9

* A. R., M. J. R., and R. D. W. contributed equally to this work.

Address correspondence to:
Dr. Timothy Kieffer
Email: tim.kieffer@ubc.ca
or
Dr. Alireza Rezania
Email: ARezani@its.jnj.com

Submitted 22 April 2010 and accepted 20 September 2010.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

This is an uncopyedited electronic version of an article accepted for publication in Diabetes. The American Diabetes Association, publisher of Diabetes, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes in print and online at http://diabetes.diabetesjournals.org.
Objective - Differentiation of human embryonic stem (hES) cells to fully developed cell types holds great therapeutic promise. Despite significant progress, the conversion of hES cells to stable, fully differentiated endocrine cells that exhibit physiologically regulated hormone secretion has not yet been achieved. Here we describe an efficient differentiation protocol for the in vitro conversion of hES cells to functional glucagon-producing alpha cells.

Research Design and Methods - Using a combination of high-throughput small molecule screening and empirical testing, we developed a six-stage differentiation protocol for creating functional alpha cells. An extensive in vitro and in vivo characterization of the differentiated cells was performed.

Results - A high rate of synaptophysin expression (>75%) and robust expression of glucagon and the alpha cell-specific transcription factor ARX was achieved. Following a transient polyhormonal state in which cells co-express glucagon and insulin, maturation in vitro or in vivo resulted in depletion of insulin and other beta cell markers with concomitant enrichment of alpha cell markers. Following transplantation, these cells secreted fully processed, biologically active glucagon in response to physiological stimuli including prolonged fasting and amino acid challenge. Moreover, glucagon release from transplanted cells was sufficient to reduce demand for pancreatic glucagon, resulting in a significant decrease in pancreatic alpha cell mass.

Conclusions - These results indicate that fully differentiated pancreatic endocrine cells can be created via step-wise differentiation of hES cells. These cells may serve as a useful screening tool for the identification of compounds that modulate glucagon secretion as well as those that promote the transdifferentiation of alpha cells to beta cells.

Human embryonic stem (hES) cells hold great potential for the development of replacement therapies for conditions including heart disease, spinal cord injury, and diabetes. With the recent FDA approval of the first United States-based clinical trial for the use of cells derived from hES cells (1), there is renewed optimism that other stem cell-based therapies may soon be developed and tested clinically. Treatment of type 1 diabetes with cadaveric human islets has been promising suggesting that a cell-based therapy for this disease may be possible given sufficient availability of transplant material. hES cells can be efficiently differentiated to definitive endoderm (2;3) and further to endocrine-like polyhormonal cells that are capable of hormone secretion in response to some physiological and pharmacological stimuli (4-6). However, the formation of mature, single hormone-expressing endocrine cells in culture remains a major hurdle. Recent efforts have been focused on the maturation of partially differentiated cells towards beta cells in vivo following transplantation into model animals (7;8); however, the clinical use of partially differentiated cells may present an unacceptable risk of tumor formation. We therefore sought to develop a protocol for the in vitro differentiation of a functional, terminally differentiated endocrine cell type from hES cells. In pursuing our goal of ultimately developing a scalable protocol to produce beta cells, we established a method to convert hES cells to functional glucagon-expressing cells that resemble mature pancreatic alpha cells.
RESEARCH DESIGN AND METHODS

Differentiation of hES cells. The H1 hES cell line was obtained from WiCell Research Institute, Inc. (Madison, WI), and cultured according to instructions provided by the source institute. Briefly, cells were cultured on 1:30 diluted, growth factor reduced Matrigel (Invitrogen; Carlsbad, CA) coated plates in mouse embryonic fibroblast (MEF)-conditioned media as previously described (9). When ~80% confluent (approximately 5-7 days after plating), hES cells were treated with 1 mg/mL Dispase (Invitrogen) for 5 min and then gently scraped off the surface using a 5 mL pipette. Cells were spun at 900 rpm for 3 min, and the pellet was resuspended and re-plated at a 1:3 to 1:4 ratio of hES cells in MEF-conditioned media supplemented with 16 ng/mL of FGF2 (R&D Systems; Minneapolis, MN). Details of stage-specific treatments are described in the supplemental data in the online appendix available at http://diabetes.diabetesjournals.org.

Human Islets. Human islets were obtained from the Irving K. Barber Human Islet Isolation Laboratory (Vancouver, BC) and were maintained in Final Wash Media (Mediatech, Inc., Herndon, VA). For dithizone staining, islets were washed with PBS(-), then incubated in a filter-sterilized 78 μM dithizone (Sigma-Aldrich) solution in DMSO for 1 h. Islets were then washed with DMEM/F12 media to remove excess dithizone and examined under light microscope.

qRT-PCR. Total RNA was extracted with the RNeasy Mini Kit (Qiagen; Valencia, CA) and reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. cDNA was amplified by PCR using Taqman Universal Master Mix and Taqman Gene Expression Assays (see supplemental table 2) which were pre-loaded onto custom Taqman Arrays (Applied Biosystems). Data was analyzed using Sequence Detection Software (Applied Biosystems) and normalized to undifferentiated ES cells using the ΔΔCt method.

Insulin, glucagon and DNA content. Cells were lysed by suspension in Tris-EDTA (pH 7.4) followed by sonication until cell membranes were dispersed. DNA content was determined using the Quant-IT® PicoGreen® kit (Invitrogen), while insulin and glucagon content were determined using Insulin and Glucagon ELISA, respectively (Alpco Diagnostics; Salem, NH).

Flow Cytometry. Differentiated cells were released into single-cell suspensions by incubation in TrypLE Express (Invitrogen), fixed and stained using antibodies directed against intracellular proteins as indicated in the text. A detailed description of staining can be found in the supplemental data.

Immunocytochemistry. Isolated human islets and stage 6 differentiated hES cell clusters were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, then embedded in a 1% agarose in PBS gel prior to being paraffin embedded and sectioned for immunostaining. Graft-bearing kidneys and pancreata were fixed in 4% PFA overnight at 4°C prior to being paraffin embedded and sectioned. All sections were cut at a thickness of 5 μm. Staining procedures and antibodies used are detailed in the supplemental data.

Alpha Cell Mass Quantification. Following DAB staining, pancreas sections were digitally rendered using a ScanScope CS digital slide scanner (Aperio Technologies, Inc., Vista, CA) and analyzed using the ImageScope positive pixel count, version 9 algorithm (Aperio Technologies).

Perfusion. Equal volumes of human islets or stage 6 hES cell-derived cells were loaded into temperature-/CO₂-controlled chambers of an Endotronics Acu-syst S Perifusion apparatus. HEPES-buffered Krebs Ringers
Bicarbonate Buffer (KRBB) containing 0.5% BSA was pumped through the chambers at ~350 µl/min following a 1 h preincubation under basal conditions. Fractions were collected every 5 min and assayed for insulin and glucagon via radioimmunoassay (Millipore; Billerica, MA).

**Animal Studies and Transplantation of hES Cell-Derived Cells.** All experiments were approved by the UBC Animal Care Committee. Male B6.129S7-Rag<sup>Tm1Mom/J</sup> mice (stock 2216) were obtained from the Jackson Laboratories (Bar Harbor, ME) at 8-10 weeks of age. Mice were maintained on a 12 h light/dark cycle and had ad libitum access to a standard irradiated diet (PicoLab 20; #5058l PMI International; St. Louis, MO). Blood glucose and body weight were monitored 2-3 times weekly following a 4 h morning fast. Blood glucose was measured via the saphenous vein using a handheld glucometer (Lifescan; Burnaby, BC). Mice were anaesthetized with inhalable isoflurane and received transplants of ~1.9 million stage 6 hES cell-derived cells beneath the left kidney capsule. In some cases, transplants were performed in diabetic mice (blood glucose >18 mM) following treatment with streptozotocin (STZ; 175 mg/kg). Following transplantation all mice were treated with oral enrofloxacin (Bayer Animal Health; Shawnee Mission, KS) for one week (50 mg/500 mL in drinking water). In diabetic animals a 30 d, slow-release insulin pellet (LinBit; Linshin Canada; Toronto, ON) was implanted subcutaneously to maintain normoglycemia.

**In vivo analysis of transplanted cells.** Metabolic analyses were performed in conscious, restrained mice on the indicated days. Details of metabolic studies can be found in the supplemental data.

**RESULTS**

Development of an alpha cell differentiation protocol. Significant advances in the formation of pancreatic endocrine cells from hES cells have been recently achieved by attempting to mimic the natural step-wise development of the endocrine pancreas *in vitro* (4-6). Using a similar step-wise approach, we performed a combination of small molecule screening and empirical testing of known endocrine morphogens to develop a differentiation protocol leading to the formation of mature alpha cells (Fig. 1A). To improve upon previous attempts at *in vitro* endocrine cell formation, adherent cultures were differentiated under feeder-free conditions in the absence of fetal bovine serum. Our protocol is divided into 6 distinct stages, mimicking the step-wise development of endogenous human islets. To specify the anterior primitive streak region, referred to here as mesoendoderm, stage 1 cells were cultured in the presence of Wnt-3A, FGF2, and activin A (10-12). Robust expression of CD184 (CXCR4), FOXA2, and SOX17 suggested mesoendoderm formation (13-15), (Figs. 1B and S1A, B) while low expression levels of α-fetoprotein, Sox7, and brachyury suggested a lack of visceral endoderm (16) and mesoderm formation (Fig. S1A). Promoting FGF and retinoic acid signaling while restricting sonic hedgehog (SHH) and BMP signaling can specify the pancreatic domain from the gut tube (17-20) and may reduce formation of hepatocytes (21). Furthermore, inhibition of BMP signaling can promote the formation of endocrine cells in the developing Zebrafish (22). Thus during stages 2 and 3, cells were exposed to FGF7, cyclopamine, retinoic acid, and noggin, resulting in the upregulation of foregut endoderm and pancreatic precursor markers including HNF4α and PDX-1 (Fig. S1C). Removal of Noggin at later stages of the protocol may allow for sufficient BMP signaling to facilitate the formation and maturation of alpha cells (23).

Disruption of Notch and TGF-β signaling has been shown to promote the
pancreatic endocrine cell lineage, partially through upregulation of *NGN3* expression (24) and by redirecting pancreatic epithelial cells that would otherwise differentiate into ductal cells towards an endocrine fate (25). We performed a targeted screening of 160 cell permeable kinase inhibitors at stages 3 and 4 of differentiation and identified 2-(3-(6-Methylpyridin-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine (ALK5 inhibitor II) as a potent inducer of insulin and glucagon message (Fig. S2A, C), likely via upregulation of the transcription factors *NeuroD* and *NGN3* (Fig. S1D and S2B, D, E). Similar results were obtained using 3-(Pyridin-2-yl)-4-(4-quinonyl)-1H-pyrazole (ALK5 inhibitor I), although the upregulation of these markers was not as dramatic (data not shown). Addition of the notch inhibitor DAPT at stage 4 resulted in a small increase in expression of *NeuroD* and *NGN3* expression, but no significant effect on the expression of insulin and glucagon (data not shown). Thus in stage 4, inhibition of TGF-β signaling using ALK5 inhibitor II as well as Notch signaling using DAPT progressed cells to a pancreatic endocrine phenotype. The endocrine cell markers *PAX4*, *PAX6*, and *NKX2.2* were highly upregulated at stage 4 (Fig. S1C) as were the number of *NGN3*-positive cells within the culture (Fig. S2E). Furthermore, ALK5 inhibitor II resulted in a concentration-dependent increase in expression of the alpha cell-enriched transcription factor *ARX* (Fig. S2D) thus potentially contributing to the eventual maturation of the cells towards an alpha cell phenotype. As further evidence of the transition of the differentiating cells away from a beta cell phenotype, *NKX6.1* message was undetectable in the differentiating cell population (Fig S1C) and we were unable to detect *NKX6.1* immunofluorescence in PDX-1-positive cells within the culture at this stage (Fig. S1E). Previous reports have shown that *NKX6.1* null mice lack pancreatic beta cells (26) and as early as 10 weeks of gestation and in the adult human islet, expression of *NKX6.1* becomes limited to beta cells and is absent from glucagon-expressing alpha cells (27).

The expression of both glucagon (*GCG*) and insulin (*INS*) was upregulated at stage 5, with continued inhibition of TGF-β receptor signaling (Figs. 1C and S2C). During stage 6, we observed the spontaneously formation of clusters that resembled human islets in both size and shape (Fig. 1D). These clusters contained significantly higher levels of *INS* and *GCG* mRNA compared to stage 6 cells that remained part of the monolayer (Fig. 1C) and were highly enriched for the pan-endocrine cell marker synaptophysin (Fig. 2A). In contrast to adult human islets (Fig. 2B), significant co-expression of *INS* and *GCG* was detected by both flow cytometry and immunofluorescence (Fig. 2C). Co-localization of C-peptide and insulin immunoreactivities (Fig S3A) indicates the observed insulin immunoreactivity is unlikely to be due to uptake of insulin from the culture medium. In stage 6, cells within the clusters expressed additional islet hormones including somatostatin and ghrelin (Fig. S4B, C). Glucagon-positive cells exhibited a subcellular morphology similar to that of human alpha cells (Fig. S4D, E).

**In vitro characterization of differentiated cells.** Glucagon and insulin protein content were approximately 10-fold higher and 10-fold lower in the stage 6 clusters than in human islets, respectively (Fig. 3A). Biologically active glucagon secretion was first detected at stage 5 and increased in stage 6 (Fig. 3B). Stage 6 clusters exhibited insulin secretion in response to KCl, and arginine, albeit at quite low levels (Fig. 3D), with no significant response to glucose (Fig. 3D). In some cases, basal secretion of glucagon in low glucose was significantly greater than that observed in human islets (Fig. 3C), while in some cases basal glucagon secretion was
similar (Fig. 3E). Both KCl and arginine stimulated glucagon secretion (Fig. 3C and E) as did the acetylcholine analogue carbachol (Fig. 3E). Glucagon release was diminished in the presence of the somatostatin analogue octreotide or high glucose, suggesting that glucagon secretion can be physiologically regulated in these cells (Fig. 3E). Following extended culture of stage 6 clusters in vitro, the percentage of insulin-positive and insulin/glucagon co-positive cells decreased markedly. Conversely, the percentage of glucagon-positive cells was increased (Fig. 2D), while the proportion of cells expressing the endocrine marker synaptophysin remained high at approximately 75% (Fig. 2E).

**In vivo characterization of differentiated cells.** To test whether stage 6 clusters were lineage-restricted to become alpha cells, and to assess the physiological regulation of glucagon secretion from these cells, normoglycemic B6.129S7-RagTm1Mom/J mice were transplanted with approximately 1.9 x 10^6 stage 6 clusters. Animals were followed with routine blood glucose tracking and additional metabolic tests for up to 5 months post-transplantation. Cell recipients showed occasionally elevated 4 h fasted blood glucose levels compared to control animals (Fig. 3A). At 99 days post-transplant, prolonged (~16 h) fasting resulted in elevated plasma glucagon levels in cell recipients (399.2 ± 32.5 pg/mL) compared to control animals, where glucagon levels were largely undetectable (<40 pg/mL). Feeding significantly reduced plasma glucagon to 227.7 ± 46.3 pg/mL in cell transplant recipients (Fig. 3B). Importantly, postprandial blood glucose levels were not significantly different between groups (Fig. 3B).

We tested the ability of the engrafted hES cell-derived glucagon-expressing cells to respond to known alpha cell secretion stimuli. In response to an intraperitoneal arginine bolus performed at 62 days post-transplant, plasma glucagon levels in cell recipients rose significantly from 88.1 ± 15.3 pg/mL to 1060.4 ± 83.6 pg/mL. In contrast, control animals mounted a minimal glucagon response to this challenge (Fig. 3D; <40 pg/mL at basal to 46.7 ± 4.4 pg/mL at 7 min). Despite the robust glucagon secretion observed in cell recipients, blood glucose levels increased only minimally by 13.8 ± 6.9% within the first 7 min following arginine administration. Control animals exhibited a 47.6 ± 15.9% increase in blood glucose levels in this same timeframe (Fig. 3C). In addition, cell recipients exhibited a more marked reduction in blood glucose levels at later time points than control animals, reaching a minimum of 58.7 ± 7.7% of basal versus 71.4 ± 9.0% of basal for controls at 30 min post-injection. Pancreatic insulin secretion was not significantly different between groups (Fig. 3C, inset).

Interestingly, plasma glucagon-like peptide-1 (GLP-1) levels were significantly elevated in cell recipients following arginine administration (54.2 ± 8.2 pg/mL versus 7.5 ± 0.89 pg/mL in control animals at 7 min; Fig. S5A). GLP-1 secretion was stimulated by feeding in both groups. However, while basal plasma GLP-1 levels were higher in cell recipients, stimulated levels remained in the physiological range and were not significantly different from control animals (Fig. S5B).

In response to an oral glucose challenge performed at 77 days post-transplant, no differences were observed in peak blood glucose, glucose clearance, or insulin secretion between groups (Fig. 3E). To test whether chronic hyperglucagonemia in cell transplant recipients induced compensatory glucagon resistance, we performed an intraperitoneal glucagon tolerance test at 92 days post-transplant. Control mice exhibited a significant increase (45.5 ± 8.9%) in blood glucose at 7 min while cell recipients did not (Fig. 3F). However, both groups of mice mounted an equal pancreatic insulin response (Fig. 3F,
Furthermore, while blood glucose had returned to basal in control mice by 30 min (101.3 ± 19.5 % of basal), cell recipients exhibited an exaggerated decline in blood glucose levels at 30 min (65.0 ± 8.4 % of basal; Fig. 3F). At 114 days post-transplant, control and cell recipient mice displayed similar insulin sensitivity (Fig. 3G), and both groups exhibited a robust hypoglycemia-induced increase in plasma glucagon levels (368.9 ± 128.2 pg/mL for controls, n=5; 483.2 ± 106.8 pg/mL for cell recipients; n=7; Fig. 3H, left) in response to a similar level of hyperinsulinemia (Fig. 3H, right). Thus, the primary metabolic phenotype observed in cell recipients was chronic hyperglucagonemia and mild glucagon resistance.

Characterization of engrafted hES-derived cells. At approximately 4 months post-transplant, engrafted cells were harvested and characterized in comparison to pre-transplant stage 6 clusters. Gross histological analysis of the graft reveals that the vast majority of cells express glucagon. H&E staining reveals characteristic endocrine cell morphology within the graft, while Masson’s trichrome staining indicates that areas not occupied by glucagon-positive cells are largely occupied by connective tissue. As in stage 6 clusters that underwent extended in vitro culture, transplanted cells lost insulin expression while maintaining robust glucagon expression (Fig. 4A and S6A-C). This loss of polyhormonal islet endocrine cells is also observed in the developing human pancreas (28). Grafts were primarily composed of glucagon-expressing cells, while the majority of glucagon-negative area within the grafts was occupied by connective tissue (Fig. S6D and E).

The transcription factor ARX is critical for the development and maintenance of the alpha cell phenotype (29;30). ARX immunoreactivity within the stage 6 clusters and the transplanted cells (Fig. 4B). Despite evidence of insulin immunoreactivity within the stage 6 clusters, nuclear-localized PDX-1 immunoreactivity was not observed in stage 6 clusters and was completely absent within the graft (Fig. 4C). Furthermore, glucagon expression in the grafts was colocalized with the prohormone processing enzyme PC2 (Fig. 4D), which mediates glucagon release from the proglucagon precursor in endogenous alpha cells, but not with the alternate processing enzyme PC1/3. The lineage fate of stage 6 clusters was not dependent on the glycemic state of the animal, as clusters transplanted into streptozotocin-induced diabetic animals also developed an alpha cell phenotype. As such, these cells were unable to reverse hyperglycemia (Fig. S7A) but were capable of arginine-stimulated glucagon secretion (Fig. S7B, C). Moreover, these engrafted cells likewise lost insulin while maintaining glucagon expression (Fig. S7D). To further characterize the hES cell derived-cells within the graft, mRNA expression levels of 84 genes were assessed in grafts collected approximately 5 months after transplant and compared to adult human islets (Table S1). In agreement with immunostaining observations, GCG and ARX mRNA levels in the engrafted cells were up-regulated 37.14 ± 3.51 and 82.53 ± 10.09 fold, respectively, while INS message was nearly undetectable. Interestingly, MAFA mRNA was down-regulated 40.53 ± 2.32 fold and NKX6.1 message was undetectable, providing further evidence of alpha cell formation.

Tumorigenicity remains a major concern facing hES cell-based therapies and previous studies show that transplantation of endocrine precursor cells can result in teratoma formation in a significant percentage of examined grafts (8). To test the proliferative potential of our transplanted hES cells, we examined PCNA immunoreactivity within the stage 6 clusters and the engrafted kidney. Although no features of teratomas or other tumors were observed in any of the 12
examined engrafted kidneys (Fig. S6D-G), proliferating cells were observed within the clusters both prior to and after transplantation (Fig. 4E).

To assess whether bioactive glucagon release from the transplanted cells had reduced the demand for pancreatic glucagon, pancreatic alpha cell mass was quantified. We noted a significant decrease in alpha cell mass in cell recipients (241 ± 35 µg/pancreas; n=6) versus control animals (393 ± 57 µg/pancreas; n=5; Fig. S8).

**DISCUSSION**

The developmental program driving the formation of mature endocrine islets, especially in humans, is not fully understood. A number of critical signaling pathways and transcription factors have been identified and their roles elucidated through genetic knockout and pharmacological activation/inhibition experiments; however, the temporal and spatial cues that result in proper development are complex. For example, while inhibition of BMP signaling in the early mouse embryo increases *Pdx-1* and *Hnf6* expression at the expense of liver-specific genes, inhibition of BMP signaling only hours later can reduce both *Pdx-1* and *Hnf6* expression (21). Despite this temporal complexity, significant progress has recently been made in the differentiation of hES cells to definitive endoderm (2;3). This has facilitated the development of protocols that promote further differentiation to endocrine precursor cell types (4;8). Here we report the formation of mature glucagon-secreting alpha cells from hES cells. Characterizing expression levels of several endocrine cell markers at each stage of differentiation aided in the discovery of culture conditions that resulted in the efficient formation of pancreatic endocrine cells. Indeed, this protocol resulted in 75% of differentiated cells expressing the endocrine marker synaptophysin. Lineage specification towards alpha cells was determined by positive immunoreactivity using an antibody specific for fully processed glucagon and by using a cell-based bioassay that recognizes activation of the human glucagon receptor. The observation that stage 6 clusters matured to alpha cells *in vitro* and *in vivo*, regardless of the glycemic state of the cell recipient suggests that lineage specification likely occurs at or before this stage of the differentiation protocol. Therefore, the creation of beta cells from a similar differentiation protocol may require culture manipulations preceding this last differentiation stage.

Pancreatic alpha and beta cells likely arise from the same *NGN3*-expressing progenitor cell population (31) and the co-expression of insulin and glucagon within the developing human and rodent pancreas has been reported by several groups (28;32). Although one study has suggested that these dual hormone expressing cells are unlikely to contribute to the adult alpha or beta cell pool, this conclusion was based on potentially incomplete labeling during lineage tracing of developing alpha and beta cells (33). The existence of a sub-population of dual expressing cells contributing to the mature alpha cell population in mice cannot be ruled out based on this study alone, nor does any evidence exist for the inability of these dual expressing cells to contribute to the development of mature endocrine cells in the human islet. While the fate of these dual-expressing cells remains controversial, it is clear that these cells arise from the same progenitor pool as the adult uni-hormonal cells as *NGN3* expression is likely absolutely required for the formation of pancreatic endocrine cells (34;35). Temporal regulation of *NGN3* expression can greatly influence the alpha to beta cell ratio, with early expression preferentially forming alpha cells (36). Given the short duration of our differentiation protocol (26 days through the end of stage 6),
subtle changes in the temporal activation of transcription factors could dramatically alter the ratio of alpha to beta cells created. Furthermore, inhibition of TGF-beta signaling, which resulted in an upregulation of NGN3 and ARX expression in our model, has been implicated in shifting the balanced formation of alpha and beta cells towards a predominately glucagon-positive population (37). Our data suggest that there exists a narrow competence window at stage 4 for the inhibition of TGF-beta signaling to induce the expression of NGN3 as well as the endocrine hormones insulin and glucagon, with Alk5 inhibitor II increasing expression of these genes when added at stage 4 (Fig. S2A, C) but not at stage 3 (data not shown) or stage 5 alone.

Downstream of NGN3, the transcription factors PAX4 and ARX are likewise known to play critical roles in the proper development of the islet. PAX4 deficiency results in an absence of beta cells, but a striking increase in the number of alpha cells (38). Conversely, ARX deficient mice show a dramatic increase in beta and delta cell formation at the expense of alpha cells (29), suggesting that PAX4 and ARX perform opposing roles in the maturation of endocrine precursors. Indeed, these two transcription factors have been shown to inhibit the expression of each other by directly binding to the promoter region of the opposing transcription factor (30). Although we observed levels of PAX4 gene expression during hES cell differentiation that exceed that found in adult human islets (~1.5 fold; Table S1), expression of ARX was much more highly upregulated (~82 fold; Table S1), suggesting that the balance of these two transcription factors is overwhelmingly tipped towards an alpha cell phenotype. In this regard, constitutive over-expression of PAX4 in differentiating hES cells can improve the efficiency of beta cell formation (39), and conditional expression of PAX4 in endogenous alpha cells that contain normal ARX expression levels results in their transdifferentiation to beta cells (40). Thus, the high level of ARX expression coupled with low expression of INS, PDX-1, and the beta cell-specific transcription factors MafA, PAX4 and NKX6.1 ((26;41); Figs. 1C, S1B), further highlight the formation of alpha cells with this protocol.

In the pancreatic alpha cell, co-expression of proglucagon with PC2 results primarily in the formation of mature glucagon. Conversely, in PC1/3-expressing cells, such as the gastrointestinal L cells, proglucagon is processed to yield GLP-1, GLP-2, glicentin, and oxyntomodulin. In some cells in the stage 6 clusters, glucagon immunoreactivity colocalized with either PC1/3 or PC2, suggesting that any or all proglucagon-derived peptides may be produced. Indeed, cell recipients had significantly elevated plasma GLP-1 levels, suggesting that GLP-1 may be secreted from the engrafted cells. Previous studies have shown that some early mouse embryonic glucagon-expressing cells also express PC1/3 and thus likely produce GLP-1 and have suggested that production within the developing islet may be important for mature islet formation (42). GLP-1 production from the graft may be from a population of alpha cells that have yet to mature or from a separate differentiated cell lineage. The separate development of glucagon and GLP-1-expressing cells within the graft is supported by the appropriately opposite regulation of glucagon and GLP-1 secretion in cell transplant recipients following feeding. Furthermore, peak plasma GLP-1 levels following arginine administration were similar at d 9 and d 62, while peak plasma glucagon levels in response to arginine administration increased 4-fold over this timeframe, suggesting that the GLP-1-producing cells may have developed by stage
6, while glucagon-producing cells required more time to mature.

In our study, we show that glucagon secretion from hES-derived cells was increased by fasting and hypoglycemia and diminished by elevated glucose concentrations. Moreover, as with native alpha cells, arginine was a potent stimulant of glucagon secretion both in vitro and in vivo. Both fasting and fed plasma glucagon levels were significantly higher in cell recipients compared to control animals. While hypoglycemia induces glucagon secretion in vivo, glucose itself can stimulate glucagon secretion in isolated alpha cells (43). In both humans and rodents, insulin plays a key role in regulating glucagon secretion in vitro and in vivo (44;45), possibly via creation of a critical microenvironment in which insulin secreted from the beta cells acts on adjacent alpha cells to inhibit glucagon secretion. The increased basal glucagon secretion observed in our study may result from reduced or absent negative feedback due to a lack of local insulin production. This excessive basal glucagon secretion likely contributed to the observed development of mild glucagon resistance in cell recipients. Despite glucagon resistance, cell recipients maintained normal glucose regulatory responses to fasting, feeding, and nutrient administration. Taken together with our bioassay data confirming that conditioned media from stage 6 clusters activated the glucagon receptor, and our observations of decreased pancreatic alpha cell mass in cell recipients, these data confirm that the glucagon secreted from hES-derived cells was mature, bioactive, and secreted in response to physiological stimuli.

We have previously shown that transplantation of alpha cell-like αTC-1 cells into normoglycemic mice results in chronic elevation of fasting blood glucose and impaired glucose tolerance (46). In contrast, recipients of the differentiated hES cells displayed only occasionally elevated fasting blood glucose and had normal glucose tolerance, suggesting that hES-derived cells possess more physiologically-regulated secretion of glucagon than do αTC-1 cells. Given that a lack of suppression of glucagon secretion contributes to hyperglycemia in individuals with type 2 diabetes (47), the development of a physiologically regulated human alpha cell line may have significant utility as a screening tool for the identification of novel therapeutics that suppress glucagon secretion.

As the burgeoning field of regenerative medicine continues to mature, a method for the formation of terminally differentiated, appropriately regulated, non-tumorigenic pancreatic endocrine cells is highly desirable. We demonstrate here that with appropriate and defined manipulation of culture conditions, hES cells can be differentiated into functional alpha cells. Through a rigorous in vitro and in vivo characterization, we show that these hES-derived endocrine cells secrete biologically active glucagon in response to known glucagon secretagogues and importantly are not tumorigenic. There is increasing evidence to suggest that alpha cells exhibit a unique plasticity that allows for their conversion to insulin-producing beta cells under certain conditions. The ectopic expression of pax4 (40) or ablation of men1 expression (48) in alpha cells facilitates their transdifferentiation to beta cells. Furthermore, Herrera and colleagues have recently demonstrated that near total beta cell ablation results in the conversion of pre-existing alpha cells to beta cells (49). In light of these recent findings, our hES cell-derived alpha cells may serve as a novel starting material for the identification of small molecules that promote the transdifferentiation of exocrine cells to beta cells has been recently demonstrated in vitro (50), we speculate that it may be possible
to identify compounds that convert alpha cells to beta cells as a novel strategy for the treatment of diabetes.

**Author Contributions.** A.R. researched data and reviewed/edited manuscript. M.J.R. researched data and wrote manuscript. R.D.W. researched data and reviewed/edited manuscript. F.K. researched data. Z.A. contributed human islets. G.L.W. contributed human islets and reviewed/edited manuscript. T.J.K. reviewed/edited manuscript.

**ACKNOWLEDGEMENTS**

The authors would like to acknowledge the expert technical assistance contributed by Ali Asadi, Travis Webber, Anastasia Vlasova, Christine Donald and Madeleine Speck, all from the University of British Columbia, along with Ramie Fung from Centocor Research & Development. We thank Dr. Jim Johnson and Betty Hu from the University of British Columbia for assistance with perfusion experiments. We also thank Christopher Cahill at The Joslin Diabetes Center for assistance in preparing TEM images. Financial support for this project was provided by the Stem Cell Network. M.J.R. is supported by the Michael Smith Foundation for Health Research (MSFHR), the Canadian Diabetes Association, the Stem Cell Network, and the Juvenile Diabetes Research Foundation (JDRF). T.J.K. is a MSFHR senior scholar.

**REFERENCES**


46. Wideman, RD, Covey, SD, Webb, GC, Drucker, DJ, Kieffer, TJ: A switch from prohormone convertase (PC)-2 to PC1/3 expression in transplanted alpha-cells is accompanied by differential processing of proglucagon and improved glucose homeostasis in mice. Diabetes 56:2744-2752, 2007

FIGURE LEGENDS

Figure 1: Differentiation of hES cells to maturing endocrine cells.
(A) Schematic representation of 6-stage differentiation protocol, with media and supplements shown below. (B) Representative FACS analysis of CD184 expression (green line) in hES cells at stage 1 of differentiation protocol; isotype control in red. Percentage values indicate number of cells expressing CD184 in each group. (C) GCG and INS expression as measured by qRT-PCR in hES cells at Stages 4-6, and in stage 6 clusters before (S6C) and after (S6C EC) an extended 4-week culture period. Data are expressed as fold induction versus human islet control. n=3 for each stage of differentiation. Error bars indicate SE. (D) Representative brightfield images of stage 6 hES cells, Stage 6 clusters, and dithizone-stained human islets. Scale bar, 150 µm.
Figure 2: Morphological characterization of hES cell-derived cells.
(A) Representative flow analysis of synaptophysin expression in isolated human islets (left) and stage 6 clusters (right). Isotype shown in red. (B) Representative glucagon (GCG) and insulin (INS) immunofluorescence images in paraffin-sectioned adult human islets (left) and flow data from dispersed adult human islets (right). GCG immunoreactivity is shown in green and INS immunoreactivity is shown in red. Scale bar, 50 µm. Quadrant gates set using isotype controls (not shown). (C) Representative GCG and INS immunofluorescence images in paraffin-sectioned stage 6 clusters (left) and flow data from dispersed stage 6 clusters (right). GCG immunoreactivity is shown in green and INS immunoreactivity is shown in red. Cells expressing both GCG and INS are shown in yellow. Scale bar, 50 µm. Quadrant gates set using isotype controls (not shown). (D) Representative GCG and INS flow data from dispersed stage 6 clusters after an extended 4 week culture period. Quadrant gates set using isotype controls (not shown). (E) Representative synaptophysin expression in stage 6 clusters dispersed after an extended 4 week culture period. Isotype shown in red.

Figure 3. Hormone content and secretion kinetics of hES cell-derived cells.
(A) Glucagon and insulin content of stage 6 clusters, normalized for total DNA content and expressed as fold difference over human islets (n=3 samples for ES derived cells and n=8 for human islets). Error bars indicate SE. (B) Twenty-four hour bioactive glucagon release from hES cells at indicated differentiation stages (n=2). N.D., not detected. (C and D) Glucagon (C) and insulin (D) secretion from perfused human islets and stage 6 clusters (n=4 chambers for each) in response to 15 mmol/l glucose (GLU), 30 mmol/l KCl (KCl) and 15 mmol/l arginine (ARG). (E) Glucagon secretion from perfused stage 6 clusters (n=4 chambers) in response to 3 mmol/l glucose (3GLU) or 16.7 mmol/l (16.7GLU) with or without 1 µmol/l of the somatostatin analogue octreotide (Sst), 100 µmol/l carbachol (Cch), or 15 mmol/l arginine (Arg) as indicated.

Figure 4: Transplantation of hES cell-derived cells induces hyperglucagonemia and glucagon intolerance.
(A) Blood glucose levels following a 4 h morning fast. (B) Blood glucose and plasma glucagon levels following an overnight fast and after 45 min refeeding period at 99 d post-transplant (Tx). Glucagon was below the level of detection for control and human islet Tx groups (<40 pg/mL). (C) Blood glucose and plasma mouse insulin levels (mINS; inset) at d 62 post-transplant in response to I.P. arginine injection (2 g/kg). (D) Plasma glucagon levels for I.P. arginine test shown in panel C. (E) Blood glucose and plasma mouse insulin levels (mINS; inset) after a 4 h morning fast at d 77 post-transplant in response to oral glucose challenge (2 g/kg). (F) Blood glucose and plasma mouse insulin levels (mINS; inset) at d 92 post-transplant in response to I.P. glucagon injection (1 µg/kg). (G) Whole body insulin sensitivity was assessed by injecting recombinant human insulin (0.4 U/kg) at d 114 post-transplant. (H) Plasma immunoreactive glucagon (left) and insulin (right) levels in response to insulin injection shown in panel G. n=5-7 animals/group; *p<0.05, **p<0.01, ***p<0.001 versus control; #p<0.05 versus respective 0 min time point (Student’s t-test). N.D., not detected.

Figure 5: Gross histology of transplanted hES cell-derived cells.
Grafts were harvested from hES cell recipients at d125 post-transplant. (A) Representative image of engrafted kidney section following DAB staining for glucagon. Scale bar = 1 mm. (B) Representative images of H&E staining at (left) low and (right) high magnification. Scale bars =
1 mm for low magnification image and 50 µm for high magnification image. (C) Representative images of Masson’s trichrome staining at (left) low and (right) high magnification. Collagen fibers are stained in blue. Scale bars = 100 µm. In all panels, white dotted line delineates kidney-graft border.

**Figure 6: The alpha cell phenotype is enriched following *in vivo* transplantation.** Double immunofluorescence was performed on pre-transplant stage 6 clusters and grafts collected 125 days following transplantation. Representative images are shown. (A) Insulin (INS; red) expression is largely lost following transplantation while glucagon (GCG; green) expression predominates. (B) GCG (green) and ARX (red) coexpression is maintained in stage 6 hES cell-derived cells before and following transplantation. (C) PDX-1 (red) expression is distinct from glucagon (green) expression in stage 6 clusters, and is absent in grafts. (D) Coexpression of glucagon (green) and PC2 (red) is increased in retrieved grafts compared to stage 6 clusters. (E) Robust PCNA immunoreactivity (red) is observed in many glucagon-positive cells (green) in stage 6 clusters and following transplantation (see arrows). Scale bars, 100 µm. Merged images include a DAPI nuclear stain (blue). Kidney and graft tissues are denoted by a K and G, respectively in panels B and C.
Figure 1

A

<table>
<thead>
<tr>
<th>ES Cell</th>
<th>Meso-endoderm</th>
<th>Endoderm Progenitor</th>
<th>Foregut Progenitor</th>
<th>Endocrine Precursor</th>
<th>Immature Endocrine</th>
<th>Maturing Endocrine</th>
<th>α-Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage 1</td>
<td>Stage 2</td>
<td>Stage 3</td>
<td>Stage 4</td>
<td>Stage 5</td>
<td>Stage 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 Days</td>
<td>2 Days</td>
<td>4 Days</td>
<td>3 Days</td>
<td>7 Days</td>
<td>7 Days</td>
<td></td>
</tr>
</tbody>
</table>

- RPMI 1640
- 2% BSA
- DMEM/F12
- Wnt3A
- Activin A
- FGF7
- Cyclopamine
- Noggin
- ALK5 Inhibitor
- DAPT
- RA
- AR

B

C

D

Percent Maximum Count

Fold Induction Over Human Islets

0.4%

99.6%

<table>
<thead>
<tr>
<th>Glucagon</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4</td>
<td>S5</td>
</tr>
<tr>
<td>0.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Human Islets

Stage 6 Cells

Stage 6 Clusters

Stage 6 Clusters
Figure 2

A. Human Islets vs Stage 6 Clusters

B. Human Islets vs Glucagon and Insulin

C. Stage 6 Clusters vs Glucagon and Insulin

D. Stage 6 Extended vs Glucagon and Insulin

E. Stage 6 Extended vs Synaptophysin
Figure 3

A

B

C

D

E

Content

[GCG] (nM)

Time (min)

GCG INS

S4 S5 S6

[S6 Clusters Human lslets]

[GCG] (pg/mL)

[INS] (μU/mL)

Time (min)

Glu KCl Arg

Glu KCl Arg

Sst Cch

3Glu 16.7Glu

Time (min)
Figure 4
Figure 5

A

B

C
Figure 6

A  Stage 6 Clusters  Grafts

GCG/INS

GCG/ARX

GCG/PDX-1

GCG/PC2

GCG/PCNA

Kidney

Graft