CULTURING OF HUMAN EMBRYONIC STEM CELLS AT THE AIR-LIQUID INTERFACE FOR DIFFERENTIATION INTO PANCREATIC ENDOCRINE CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application 61/747,662 (filed on December 31, 2012) which is incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention is in the field of cell differentiation. More specifically, the present invention provides methods, cell cultures and media for generating pancreatic endoderm, pancreatic endocrine precursor cells, and single-hormone pancreatic endocrine cells from human pluripotent stem cells by culturing cells at the air-liquid interface.

BACKGROUND

[0003] Advances in cell-replacement therapy for Type I diabetes mellitus and a shortage of transplantable islets of Langerhans have focused interest on developing sources of insulin-producing cells, or beta (β) cells, appropriate for engraftment. One approach is the generation of functional β cells from pluripotent stem cells, such as, embryonic stem cells.

[0004] In vertebrate embryonic development, a pluripotent cell gives rise to a group of cells comprising three germ layers (ectoderm, mesoderm, and endoderm) in a process known as gastrulation. Tissues such as, thyroid, thymus, pancreas, gut, and liver, will develop from the endoderm, via an intermediate stage. The intermediate stage in this process is the formation of definitive endoderm.

[0005] By the end of gastrulation, the endoderm is partitioned into anterior-posterior domains that can be recognized by the expression of a panel of factors that uniquely mark anterior, mid, and posterior regions of the endoderm. For example, HHEX, and SOX2 identify the anterior region while CDX1, 2, and 4 identify the posterior region of the endoderm.

[0006] Migration of endoderm tissue brings the endoderm into close proximity with different mesodermal tissues that help in regionalization of the gut tube. This is accomplished by a plethora of secreted factors, such as FGFs, WNTS, TGF-βs, retinoic acid (RA), and BMP ligands

and their antagonists. For example, FGF4 and BMP promote CDX2 expression in the presumptive hindgut endoderm and repress expression of the anterior genes Hhex and SOX2 (2000 *Development*, 127:1563–1567). WNT signaling has also been shown to work in parallel to FGF signaling to promote hindgut development and inhibit foregut fate (2007 *Development*, 134:2207–2217). Lastly, secreted retinoic acid by mesenchyme regulates the foregut-hindgut boundary (2002 *Curr Biol*, 12:1215-1220).

[0007] The level of expression of specific transcription factors may be used to designate the identity of a tissue. During transformation of the definitive endoderm into a primitive gut tube, the gut tube becomes regionalized into broad domains that can be observed at the molecular level by restricted gene expression patterns. The regionalized pancreas domain in the gut tube shows a very high expression of PDX1 and very low expression of CDX2 and SOX2. PDX1, NKX6.1, PTF1A, and NKX2.2 are highly expressed in pancreatic tissue; and expression of CDX2 is high in intestinal tissue.

[0008] Formation of the pancreas arises from the differentiation of definitive endoderm into pancreatic endoderm. Dorsal and ventral pancreatic domains arise from the foregut epithelium. Foregut also gives rise to the esophagus, trachea, lungs, thyroid, stomach, liver, and bile duct system.

[0009] Cells of the pancreatic endoderm express the pancreatic-duodenal homeobox gene PDX1. In the absence of PDX1, the pancreas fails to develop beyond the formation of ventral and dorsal buds. Thus, PDX1 expression marks a critical step in pancreatic organogenesis. The mature pancreas contains, both exocrine and endocrine tissues arising from the differentiation of pancreatic endoderm.

[0010] D'Amour *et al.* describe the production of enriched cultures of human embryonic stem cell-derived definitive endoderm in the presence of a high concentration of activin and low serum (*Nature Biotechnology* 2005, 23:1534-1541; U.S. Patent No. 7,704,738). Transplanting these cells under the kidney capsule of mice reportedly resulted in differentiation into more mature cells with characteristics of endodermal tissue (U.S. Patent No. 7,704,738). Human embryonic stem cell-derived definitive endoderm cells can be further differentiated into PDX1 positive cells after addition of FGF10 and retinoic acid (U.S. Patent App. Pub. No. 2005/0266554). Subsequent transplantation of these pancreatic precursor cells in the fat pad of immune deficient mice resulted in the formation of functional pancreatic endocrine cells

following a 3-4 months maturation phase (U.S. Patent No. 7,993,920 and U.S. Patent No. 7,534,608).

[0011] Fisk *et al.* report a system for producing pancreatic islet cells from human embryonic stem cells (U.S. Patent No. 7,033,831). In this case, the differentiation pathway was divided into three stages. Human embryonic stem cells were first differentiated to endoderm using a combination of sodium butyrate and activin A (U.S. Patent No. 7,326,572). The cells were then cultured with BMP antagonists, such as Noggin, in combination with EGF or betacellulin to generate PDX1 positive cells. The terminal differentiation was induced by nicotinamide.

[0012] Small molecule inhibitors have also been used for induction of pancreatic endocrine precursor cells. For example, small molecule inhibitors of TGF-β receptor and BMP receptors (*Development* 2011, 138:861-871; *Diabetes* 2011, 60:239-247) have been used to significantly enhance the number of pancreatic endocrine cells. In addition, small molecule activators have also been used to generate definitive endoderm cells or pancreatic precursor cells (*Curr Opin Cell Biol* 2009, 21:727-732; *Nature Chem Biol* 2009, 5:258-265).

[0013] HB9 (also known as HIXB9 and MNX1) is a BHLH transcriptional activator protein expressed early in pancreas development starting at approximately embryonic day 8. HB9 is also expressed in notochord and spinal cord. Expression of HB9 is transient and peaks at about day 10.5 in pancreatic epithelium being expressed in PDX1 and NKX6.1 expressing cells. At about day 12.5, HB9 expression declines and at later stages it becomes restricted only to β cells. In mice homozygous for a null mutation of HB9, the dorsal lobe of the pancreas fails to develop (*Nat Genet* 23:67-70, 1999; *Nat Genet* 23:71-75, 1999). HB9-/- β-cells express low levels of the glucose transporter, GLUT2, and NKX6.1. Furthermore, HB9 -/- pancreas shows a significant reduction in the number of insulin positive cells while not significantly affecting expression of other pancreatic hormones. Thus, temporal control of HB9 is essential to normal β cell development and function. While not much is known about factors regulating HB9 expression in β cells, a recent study in zebrafish suggests that retinoic acid can positively regulate expression of HB9 (*Development*, 138, 4597-4608, 2011).

[0014] The thyroid hormones, thyroxine ("T4") and triiodothyronine ("T3"), are tyrosine-based hormones produced by the thyroid gland and are primarily responsible for regulation of metabolism. The major form of thyroid hormone in the blood is T4, which has a longer half-life

than T3. The ratio of T4 to T3 released into the blood is roughly 20 to 1. T4 is converted to the more active T3 (three to four times more potent than T4) within cells by deiodinase.

[0015] T3 binds to thyroid hormone receptors, TRα1 and TRβ1 (TR). TR is a nuclear hormone receptor, which heterodimerizes with retinoid X receptor. The dimers bind to the thyroid response elements (TREs) in the absence of ligand and act as transcriptional repressors. Binding of T3 to TR reduces the repression of TRE dependent genes and induces the expression of various target genes. While numerous studies have suggested a role for T3 in increasing β cell proliferation, reducing apoptosis, and improving insulin secretion, its role in cell differentiation is undefined.

[0016] Transforming growth factor β ("TGF-β") is a member of a large family of pleiotropic cytokines that are involved in many biological processes, including growth control, differentiation, migration, cell survival, fibrosis and specification of developmental fate. TGF-β superfamily members signal through a receptor complex comprising a type II and type I receptor. TGF-B ligands (such as activins, and growth differentiation factors ("GDF"s)) bring together a type II receptor with a type I receptor. The type II receptor phosphorylates and activates the type I receptor in the complex. There are five mammalian type II receptors: TβR-II, ActR-II, ActR-IIB, BMPR-II, and AMHR-II and seven type I receptors (ALKs 1–7). Activin and related ligands signal via combinations of ActR-II or ActR-IIB and ALK4 or ALK5, and BMPs signal through combinations of ALK2, ALK3, and ALK6 with ActR-II, ActR-IIB, or BMPR-II. AMH signals through a complex of AMHR-II with ALK6, and nodal has been shown to signal through a complex of ActR-IIB and ALK7 (*Cell.* 2003,113(6):685-700). Following binding of the TGF-B ligand to the appropriate receptor, the ensuing signals are transduced to the nucleus primarily through activation of complexes of Smads. Upon activation, the type I receptors phosphorylate members of the receptor-regulated subfamily of Smads. This activates them and enables them to form complexes with a common mediator Smad, Smad4. Smads 1, 5, and 8 are substrates for ALKs 1, 2, 3, and 6, whereas Smads 2 and 3 are substrates for ALKs 4, 5, and 7 (*FASEB J* 13:2105–2124). The activated Smad complexes accumulate in the nucleus, where they are directly involved in the transcription of target genes, usually in association with other specific DNA-binding transcription factors. Compounds that selectively inhibit the receptors for TGF-β, have been developed for therapeutic applications and for modulating cell fate in the context of reprogramming and differentiation from various stem cell populations. In particular, ALK5

inhibitors have been previously used to direct differentiation of embryonic stem cells to an endocrine fate (*Diabetes*, 2011, 60(1):239-47).

[0017] In general, the process of differentiating progenitor cells to functional β cells goes through various stages; and great strides have been made in improving protocols to generate pancreatic cells from progenitor cells such as human pluripotent stem cells. Despite these advances in research, each step in the process of differentiating progenitor cells presents a unique challenge. As such, there is still a need for a protocol resulting in functional endocrine cells and, in particular, functional β cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIGS. 1A to 1H show phase contrast images of cells cultured at the air-liquid interface using the methods described in Example 1 at the following time points: Day 1 (FIG. 1A); Day 5 (FIG. 1B); Day 6 (FIG. 1C); Day 7(FIG. 1D); Day 9 (FIG. 1E); Day 13 (FIG. 1F); Day 16 (FIG. 1G); and Day 21 (FIG. 1H).

[0019] FIGS. 2A to 2K show images of cells differentiated for one week at the air-liquid interface using the methods described in Example 1 and immunostained for the following: DAPI (FIG. 2A); insulin (FIG. 2B); HB9 (FIG. 2C); DAPI (FIG. 2D); glucagon (FIG. 2E); insulin (FIG. 2F); DAPI (FIG. 2G); insulin (FIG. 2H); somatostatin (FIG. 2I); NKX6.1 (FIG. 2J); and insulin (FIG. 2K). Panels A-C, D-F, G-I and J-K were taken from the same field.

[0020] FIGS. 3A to 3H show images of cells differentiated for two weeks at the air-liquid interface using the methods described in Example 1 and immunostained for the following: insulin (FIG. 3A); glucagon (FIG. 3B); insulin (FIG. 3C); somatostatin (FIG. 3D); insulin (FIG. 3E); NKX6.1 (FIG. 3F); HB9(FIG. 3G); and NKX6.1 (FIG. 2H). Panels A-B, C-D, E-F, and G-H were taken from the same field.

[0021] FIGS. 4A to 4D show images of cells differentiated for three weeks at the air-liquid interface using the methods described in Example 1 and immune stained for insulin (FIG. 4A), glucagon (FIG. 4B), insulin (FIG. 4C), and somatostatin (FIG. 4D). Panels A-B and C-D were taken from the same field.

[0022] FIGS. 5A to 5R depict data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 1: PDX1 (FIG. 5A); NKX6.1 (FIG. 5B); PAX4 (FIG. 5C); PAX6 (FIG. 5D); NGN3

(FIG. 5E); NKX2.2 (FIG. 5F); ABCC8 (FIG. 5G); chromogranin-A (FIG. 5H); PCSK1 (FIG. 5I); IAPP (FIG. 5J); insulin (FIG. 5K); glucagon (FIG. 5L); somatostatin (FIG. 5M); ghrelin (FIG. 5N); PTF1A (FIG. 5O); ZIC1 (FIG. 5P); CDX2 (FIG. 5Q); and SOX9 (FIG. 5R). Cells were cultured at the air-liquid interface after Stage 5.

[0023] FIGS. 6A to 6L depict data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 2: PDX1 (FIG.6A); NKX6.1 (FIG. 6B); PAX4 (FIG. 6C); PAX6 (FIG. 6D); NGN3 (FIG. 6E); NKX2.2 (FIG. 6F); ABCC8 (FIG. 6G); chromogranin-A (FIG. 6H); PCSK1 (FIG. 6I); IAPP (FIG. 6J); insulin (FIG. 6K); and glucagon (FIG. 6L).

[0024] FIGS. 7A to 7L depict data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 3: PDX1 (FIG.7A); NKX6.1 (FIG. 7B); PAX4 (FIG. 7C); PAX6 (FIG. 7D); NGN3 (FIG. 7E); NKX2.2 (FIG. 7F); ABCC8 (FIG. 7G); chromogranin-A (FIG. 7H); PCSK1 (FIG. 7I); IAPP (FIG. 7J); insulin (FIG. 7K); and glucagon (FIG. 7L).

[0025] FIGS. 8A to 8H depict data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 4: PDX1 (FIG.8A); NKX6.1 (FIG. 8B); NGN3 (FIG. 8C); ABCC8 (FIG. 8D); PCSK1(FIG. 8E); Ghrelin (FIG. 8F); glucagon (FIG. 8G); and insulin (FIG. 8H).

[0026] FIGS. 9A to 9F depict data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 4: PDX1 (FIG.9A); NKX6.1 (FIG. 9B); NGN3 (FIG. 9C); ABCC8 (FIG. 9D); glucagon (FIG. 9E); and insulin (FIG. 9F).

[0027] FIGS. 10A to 10B depict the results of immunostaining Stage 6 cells cultured at the air-liquid interface according to Example 4 and treated either with 1 micro molar SD208 inhibitor (FIG. 10A) or 1 micro molar ALK5 inhibitor II (FIG. 10B) and stained for chromogranin-A (pan-endocrine marker) and NKX6.1 (Pancreatic precursor marker and β cell specific marker).

[0028] FIGS. 11A to 11H show data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as described in Example 6: ABCC8 (FIG. 11A); glucagon (FIG. 11B); amylin (FIG. 11C); insulin (FIG. 11D); NGN3 (FIG. 11E); NKX2.2 (Fig. 11F); NKX6.1 (FIG. 11G); and PDX1 (FIG. 11H). The data is shown as fold increase versus undifferentiated H1 line.

[0029] FIGS. 12A to 12H depict data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 7 and cultured at the ALI: ABCC8 (FIG.12A); glucagon (FIG. 12B); amylin (FIG. 12C); insulin (FIG. 12D); NGN3 (FIG. 12E); NKX2.2 (Fig. 12F); NKX6.1 (FIG. 12G); and PDX1 (FIG. 12H).

[0030] FIGS. 13A to 13H depicts data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 8 and cultured at the ALI: ABCC8 (FIG. 13A); glucagon (FIG. 13B); amylin (FIG. 13C); insulin (FIG. 13D); NGN3 (FIG. 13E); NKX2.2 (Fig. 13F); NKX6.1 (FIG. 13G); and PDX1 (FIG. 13H).

[0031] FIGS. 14A to 14H depict data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 9 and cultured at the ALI: ABCC8 (FIG. 14A); glucagon (FIG. 14B); amylin (FIG. 14C); insulin (FIG. 14D); ISL-1 (FIG. 14E); MNX1 (FIG. 14F); NKX6.1 (FIG. 14G); and SLC30A8 (FIG. 14H).

[0032] FIGS. 15A to 15J show FACS profile of Stage 5 day 3 cells, differentiated according to Example 10, and stained for: Isotype control (FIG. 15A); NKX6.1 (FIG. 15B); NKX2.2 (FIG. 15C); NKX6.1 (Y-axis) co-stained with insulin (X-axis) (FIG. 15D); PDX1 (X-axis) co-stained with KI-67 (Y-axis) (FIG. 15E); PAX6 (FIG. 15F); ISL-1 (FIG. 15G); FOXA2 (FIG. 15H); NeuroD (FIG. 15I); and glucagon (Y-axis) co-stained with insulin (X-axis) (FIG. 15J).

[0033] FIGS. 16A to 16I show FACS profile of Stage 6 day 5 cells, differentiated according to Example 10, and stained for: Isotype control (FIG. 16A); NKX6.1 (Y-axis) co-stained with chromogranin-A (X-axis) (FIG. 16B); NKX2.2 (Y-axis) co-stained with chromogranin-A (X-axis) (FIG. 16C); NKX6.1 (Y-axis) co-stained with insulin (X-axis) (FIG. 16D); PDX1 (X-axis) co-stained with KI-67 (Y-axis) (FIG. 16E); PAX6 (FIG. 16F);ISL-1 (FIG. 16G); FOXA2 (FIG. 16H); and NeuroD (FIG. 16I).

[0034] FIGS. 17A to 17I show the FACS (Fluorescence-activated cell sorting) profile of Stage 6 day 15 cells, differentiated according to Example 10, and stained for: Isotype control (FIG. 17A); NKX6.1 (Y-axis) co-stained with chromogranin-A (X-axis) (FIG. 17B); NKX2.2 (Y-axis) co-stained with chromogranin-A (X-axis) (FIG. 17C); glucagon (Y-axis) co-stained with insulin (X-axis) (FIG. 17D); NKX6.1 (Y-axis) co-stained with insulin (X-axis) (FIG. 17E); PDX1 (X-

axis) co-stained with KI-67 (Y-axis) (FIG. 17F); ISL-1(FIG. 17G); FOXA2 (FIG. 17H); and NeuroD (FIG. 17I).

[0035] FIG 18A to 18C show the FACS (Fluorescence-activated cell sorting) profile of Stage 4 day 4 cells, differentiated according to Example 1, and stained for: NKX6.1 (Y-axis) co-stained with chromogranin-A (X-axis) (FIG. 18A); PDX1 (X-axis) co-stained with KI-67 (Y-axis) (FIG. 18B); and NKX6.1 (Y-axis) co-stained with insulin (X-axis) (FIG. 18C).

[0036] FIG 19A to 19C show the FACS (Fluorescence-activated cell sorting) profile of Stage 6 day 6 cells, differentiated according to Example 11, and stained for: NKX6.1 (Y-axis) co-stained with chromogranin-A (X-axis) (FIG. 19A); PDX1 (X-axis) co-stained with KI-67 (Y-axis) (FIG. 19B); and NKX6.1 (Y-axis) co-stained with insulin (X-axis) (FIG. 19C).

[0037] FIG. 20 shows the *in vivo* kinetics of human C-peptide production in NOD-SCID mice transplanted with various populations of cells as described in Example 11.

[0038] FIGS. 21A to 21F depict data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 12: Amylin (FIG. 21A); insulin (FIG. 21B); MAFA (FIG. 21C); NKX6.1 (FIG. 21D); PTF1a (FIG. 21E); and SOX9 (FIG. 21F).

[0039] FIGS. 22A to 22D show real-time PCR data of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 13: MAFA (FIG.22A); insulin (FIG. 22B); Amylin (FIG. 22C); and NKX6.1 (FIG 22D).

[0040] FIGS. 23A to 23F depict data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 5: PDX1 (FIG.23A); NKX6.1 (FIG. 23B); NGN3 (FIG. 23C); ABCC8 (FIG. 23D); glucagon (FIG. 23E); and insulin (FIG. 23F).

DETAILED DESCRIPTION

[0041] The following detailed description of the invention will be better understood when read in conjunction with the appended figures. Figures are provided for the purpose of illustrating certain embodiments of the present invention. However, the invention is not limited to the precise arrangements, examples, and instrumentalities shown. For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into subsections that describe or illustrate certain features, embodiments, or applications of the present invention.

[0042] The present invention is directed to differentiating endoderm progenitor cells, such as pluripotent stem cells, into cells exhibiting characteristics of pancreatic endocrine cells by culturing said progenitor cells, at least in part, at the air-liquid interface that exists in an open culture vessel or a culture vessel partially filled with medium. Although referred to herein as "air" for convenience, the invention is not limited to the mixture of gasses and compositions found in the ambient environment. The invention specifically contemplates and includes gaseous mixtures having compositions different from the ambient environment including, for example, mixtures enriched for a particular component or in which a particular component has been depleted or eliminated.

[0043] Additionally, the present invention provides cell cultures for differentiating pluripotent stem cells into cells exhibiting characteristics of pancreatic endocrine cells, as well as differentiation media that initiates and facilitates such differentiation. Advantageously, these cell cultures and differentiation media may be used in conjunction with differentiation at the air-liquid interface to provide previously unattained yields of cells expressing markers characteristic of pancreatic endocrine cells.

[0044] The culturing may occur at the air-liquid interface for all stages involved in the differentiation pathway from pluripotent stem cell to pancreatic endocrine cell, or it may involve culturing on a planar culture submersed in medium for the early stages of differentiation, and culturing at the air-liquid interface during the later stages of differentiation. Preferably, the process of the invention involves the combination of culturing pluripotent stem cells on a support surface submerged in medium through the early stages, and then culturing at the air-liquid interface for the later stages of differentiation. In such embodiments, the cells may initially be seeded on a solid surface for submerged culturing and then removed from the solid support and re-seeded on a porous support for culturing at the air-liquid interface. Alternatively, the cells

may be seeded initially on a porous support that is then submerged in media for the early stages of differentiation and subsequently positioned at the air-liquid interface for the later stages of differentiation. Culturing at the air-liquid interface for the later stages of differentiation significantly enhances the expression of endocrine markers in comparison to culturing the cells in a submerged state for the entire process, indicating that a greater percentage of the cells have differentiated into pancreatic endocrine cells.

[0045] In one embodiment, the present invention is directed to differentiating endoderm progenitor cells at the air-liquid interface of a culture vessel partially filled with media into pancreatic endoderm progenitor cells that are positive for NKX6.1, PDX1, and HB9. This invention is based, in part, on the discovery that culturing at the air-liquid interface significantly enhances expression of endocrine markers. Furthermore, it was discovered that pancreatic endocrine precursor cells can be readily generated at the air-liquid interface resulting in generation of predominantly single hormone insulin positive cells. Single-cell seeding at the air-liquid interface was found to improve consistency of insulin production.

Definitions

[0046] Stem cells are undifferentiated cells defined by their ability, at the single cell level, to both self-renew and differentiate. Stem cells may produce progeny cells, including self-renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate *in vitro* into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm, and ectoderm). Stem cells also give rise to tissues of multiple germ layers following transplantation and contribute substantially to most, if not all, tissues following injection into blastocysts.

[0047] Stem cells are classified by their developmental potential. Pluripotent stem cells are able to give rise to all embryonic cell types.

[0048] Differentiation is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell such as, for example, a nerve cell or a muscle cell. A differentiated cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term "committed", when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of

cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. "De-differentiation" refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the lineage of a cell defines the heredity of the cell, *i.e.*, which cells it came from and to what cells it can give rise. The lineage of a cell places the cell within a hereditary scheme of development and differentiation. A lineage-specific marker refers to a characteristic specifically associated with the phenotype of cells of a lineage of interest and can be used to assess the differentiation of an uncommitted cell to the lineage of interest.

[0049] "Markers", as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker as compared to an undifferentiated cell. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

[0050] As used herein, a cell is "positive for" a specific marker or "positive" when the specific marker is sufficiently detected in the cell. Similarly, the cell is "negative for" a specific marker, or "negative" when the specific marker is not sufficiently detected in the cell. In particular, positive by FACS is usually greater than 2%, whereas the negative threshold by FACS is usually less than 1%. Positive by PCR is usually less than 34 cycles (Cts); whereas negative by PCR is usually more than 34.5 cycles.

[0051] In attempts to replicate the differentiation of pluripotent stem cells into functional pancreatic endocrine cells in static *in vitro* cell cultures, the differentiation process is often viewed as progressing through a number of consecutive stages. In particular, the differentiation process is commonly viewed as progressing through six stages. In this step-wise progression, "Stage 1" refers to the first step in the differentiation process, the differentiation of pluripotent stem cells into cells expressing markers characteristic of definitive endoderm cells (hereinafter referred to alternatively as "Stage 1 cells"). "Stage 2" refers to the second step, the differentiation of cells expressing markers characteristic of definitive endoderm cells into cells expressing markers characteristic of gut tube cells (hereinafter referred to alternatively as "Stage 2 cells"). "Stage 3" refers to the third step, the differentiation of cells expressing markers

characteristic of gut tube cells into cells expressing markers characteristic of foregut endoderm cells (hereinafter referred to alternatively as "Stage 3 cells"). "Stage 4" refers to the fourth step, the differentiation of cells expressing markers characteristic of foregut endoderm cells into cells expressing markers characteristic of pancreatic foregut precursor cells (hereinafter referred to alternatively as "Stage 4 cells"). "Stage 5" refers to the fifth step, the differentiation of cells expressing markers characteristic of pancreatic foregut precursor cells into cells expressing markers characteristic of pancreatic endoderm cells and/or pancreatic endocrine precursor cells (hereinafter referred to collectively as "pancreatic endoderm/endocrine precursor cells" or alternatively as "Stage 5 cells"). "Stage 6" refers to the differentiation of cells expressing markers characteristic of pancreatic endoderm/endocrine precursor cells into cells expressing markers characteristic of pancreatic endocrine cells (hereinafter referred to alternatively as "Stage 6 cells").

[0052] However, it should be noted that not all cells in a particular population progress through these stages at the same rate. Consequently, it is not uncommon in *in vitro* cell cultures to detect the presence of cells that have progressed less, or more, down the differentiation pathway than the majority of cells present in the population, particularly at the later differentiation stages. For example, it is not uncommon to see the appearance of markers characteristic of pancreatic endocrine cells during the culture of cells at Stage 5. For purposes of illustrating the present invention, characteristics of the various cell types associated with the above-identified stages are described herein.

[0053] "Definitive endoderm cells," as used herein, refers to cells which bear the characteristics of cells arising from the epiblast during gastrulation and which form the gastrointestinal tract and its derivatives. Definitive endoderm cells express at least one of the following markers: FOXA2 (also known as hepatocyte nuclear factor 3β("HNF3β")), GATA4, SOX17, CXCR4, Brachyury, Cerberus, OTX2, goosecoid, C-Kit, CD99, and MIXL1. Markers characteristic of the definitive endoderm cells include CXCR4, FOXA2 and SOX17. Thus, definitive endoderm cells may be characterized by their expression of CXCR4, FOXA2 and SOX17. In addition, depending on the length of time cells are allowed to remain in Stage 1, an increase in HNF4α may be observed.

[0054] "Gut tube cells," as used herein, refers to cells derived from definitive endoderm that can give rise to all endodermal organs, such as lungs, liver, pancreas, stomach, and intestine. Gut tube cells may be characterized by their substantially increased expression of HNF4α over

that expressed by definitive endoderm cells. For example, ten to forty fold increase in mRNA expression of HNF4α may be observed during Stage 2.

[0055] "Foregut endoderm cells," as used herein, refers to endoderm cells that give rise to the esophagus, lungs, stomach, liver, pancreas, gall bladder, and a portion of the duodenum. Foregut endoderm cells express at least one of the following markers: PDX1, FOXA2, CDX2, SOX2, and HNF4α. Foregut endoderm cells may be characterized by an increase in expression of PDX1, compared to gut tube cells. For example, greater than fifty percent of the cells in Stage 3 cultures typically express PDX1.

[0056] "Pancreatic foregut precursor cells," as used herein, refers to cells that express at least one of the following markers: PDX1, NKX6.1, HNF6, NGN3, SOX9, PAX4, PAX6, ISL1, gastrin, FOXA2, PTF1a, PROX1 and HNF4α. Pancreatic foregut precursor cells may be characterized by being positive for the expression of PDX1, NKX6.1, and SOX9.

[0057] "Pancreatic endoderm cells," as used herein, refers to cells that express at least one of the following markers: PDX1, NKX6.1, HNF1 β, PTF1 α, HNF6, HNF4 α, SOX9, NGN3; gastrin; HB9, or PROX1. Pancreatic endoderm cells may be characterized by their lack of substantial expression of CDX2 or SOX2.

[0058] "Pancreatic endocrine precursor cells," as used herein, refers to pancreatic endoderm cells capable of becoming a pancreatic hormone expressing cell. Pancreatic endocrine precursor cells express at least one of the following markers: NGN3; NKX2.2; NeuroD1; ISL1; PAX4; PAX6; or ARX. Pancreatic endocrine precursor cells may be characterized by their expression of NKX2.2 and NeuroD1.

[0059] "Pancreatic endocrine cells," as used herein, refer to cells capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, ghrelin, and pancreatic polypeptide. In addition to these hormones, markers characteristic of pancreatic endocrine cells include one or more of NGN3, NeuroD1, ISL1, PDX1, NKX6.1, PAX4, ARX, NKX2.2, and PAX6. Pancreatic endocrine cells expressing markers characteristic of ß cells can be characterized by their expression of insulin and at least one of the following transcription factors: PDX1, NKX2.2, NKX6.1, NeuroD1, ISL1, HNF3β, MAFA and PAX6.

[0060] Used interchangeably herein are "d1", "1d", and "day 1"; "d2", "2d", and "day 2", and so on. These number letter combinations refer to a specific day of incubation in the different stages during the stepwise differentiation protocol of the instant application.

[0061] "Glucose" is used herein to refer to dextrose, a sugar commonly found in nature.

[0062] "NeuroD1" is used herein to identify a protein expressed in pancreatic endocrine progenitor cells and the gene encoding it.

[0063] "LDN-193189" refers to ((6-(4-(2-(piperidin-1-yl)ethoxy)phenyl)-3-(pyridin-4-yl)pyrazolo[1,5-a]pyrimidine, hydrochloride; DM-3189)) a BMP receptor inhibitor available under the trademark STEMOLECULE™ from Stemgent, Inc., Cambridge, MA, USA.

Characterization, Source, Expansion and Culture of Pluripotent Stem Cells

A. Characterization of Pluripotent Stem Cells

[0064] Pluripotent stem cells may express one or more of the designated TRA-1-60 and TRA-1-81 antibodies (Thomson *et al.* 1998, *Science* 282:1145-1147). Differentiation of pluripotent stem cells *in vitro* results in the loss of TRA-1-60 and TRA-1-81 expression. Undifferentiated pluripotent stem cells typically have alkaline phosphatase activity, which can be detected by fixing the cells with 4% paraformaldehyde, and then developing with an alkaline phosphatase substrate kit sold under the trademark VECTOR® Red as a substrate, as described by the manufacturer (Vector Laboratories, CA, USA). Undifferentiated pluripotent stem cells also typically express OCT4 and TERT, as detected by RT-PCR.

[0065] Another desirable phenotype of propagated pluripotent stem cells is a potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm. Pluripotency of stem cells may be confirmed, for example, by injecting cells into severe combined immunodeficiency (SCID) mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining histologically for evidence of cell types from these three germ layers. Alternatively, pluripotency may be determined by the creation of embryoid bodies and assessing the embryoid bodies for the presence of markers associated with the three germinal layers.

[0066] Propagated pluripotent stem cell lines may be karyotyped using a standard G-banding technique and compared to published karyotypes of the corresponding primate species. It is desirable to obtain cells that have a "normal karyotype," which means that the cells are euploid, wherein all human chromosomes are present and not noticeably altered.

B. Sources of Pluripotent Stem Cells

[0067] Exemplary types of pluripotent stem cells that may be used include established lines of pluripotent cells, including pre-embryonic tissue (such as, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily, before approximately 10 to 12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, the human embryonic stem cell lines H1, H7, and H9 (WiCell Research Institute, Madison, WI, USA). Cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells are also suitable. Induced pluripotent cells (IPS), or reprogrammed pluripotent cells, derived from adult somatic cells using forced expression of a number of pluripotent related transcription factors, such as OCT4, NANOG, SOX2, KLF4, and ZFP42 (*Annu Rev Genomics Hum Genet* 2011, 12:165-185; *see also* IPS, *Cell*, 126(4): 663-676) may also be used. The human embryonic stem cells used in the methods of the invention may also be prepared as described by Thomson *et al.* (U.S. Patent No. 5,843,780; *Science*, 1998, 282:1145-1147; *Curr Top Dev Biol* 1998, 38:133-165; *Proc Natl Acad Sci U.S.A.* 1995, 92:7844-7848). Mutant human embryonic stem cell lines, such as, BG01v (BresaGen, Athens, Ga.), or cells derived from adult human somatic cells, such as, cells disclosed in Takahashi *et al.*, *Cell* 131: 1-12 (2007) may also be used. In certain embodiments, pluripotent stem cells suitable for use in the present invention may be derived according to the methods described in: Li *et al.* (*Cell Stem Cell* 4: 16-19, 2009); Maherali *et al.* (*Cell Stem Cell* 1: 55-70, 2007); Stadtfeld *et al.* (*Cell Stem Cell* 2: 230-240); Nakagawa *et al.* (*Nature Biotechnol* 26: 101-106, 2008); Takahashi *et al.* (*Cell* 131: 861-872, 2007); and U.S. Patent App. Pub. No. 2011/0104805. In certain embodiments, the pluripotent stem cells may be of non-embryonic origins. All of these references, patents, and patent applications are herein incorporated by reference in their entirety, in particular, as they pertain to the isolation, culture, expansion and differentiation of pluripotent cells.

C. Expansion and Culture of Pluripotent Stem Cells

[0068] Pluripotent stem cells are typically cultured on a layer of feeder cells that support the pluripotent stem cells in various ways. Alternatively, pluripotent stem cells may be cultured in a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of

pluripotent stem cells without undergoing substantial differentiation. The growth of pluripotent stem cells in feeder-free culture without differentiation is often supported using a medium conditioned by culturing previously with another cell type. Alternatively, the growth of pluripotent stem cells in feeder-free culture without differentiation can be supported using a chemically defined medium.

[0069] Pluripotent cells may be readily expanded in culture using various feeder layers or by using matrix protein coated vessels. Alternatively, chemically defined surfaces in combination with defined media such as media sold under the trademark mTESR®1 (StemCell Technologies, Vancouver, Canada) may be used for routine expansion of the cells. Pluripotent cells may be readily removed from culture plates using enzymatic digestion, mechanical separation, or various calcium chelators such as ethylenediaminetetraacetic acid (EDTA). Alternatively, pluripotent cells may be expanded in suspension in the absence of any matrix proteins or feeder layer.

[0070] Many different methods of expanding and culturing pluripotent stem cells may be used in the claimed invention. For example, the methods of the invention may use the methods of Reubinoff *et al.*, Thompson *et al.*, Richard *et al.* and U.S. Patent App. Pub. No. 2002/0072117. Reubinoff *et al.* (*Nature Biotechnology* 18: 399-404 (2000)) and Thompson *et al.* (*Science* 282: 1145-1147 (1998)) disclose the culture of pluripotent stem cell lines from human blastocysts using a mouse embryonic fibroblast feeder cell layer. Richards *et al.* (*Stem Cells* 21: 546-556, 2003) evaluated a panel of eleven different human adult, fetal, and neonatal feeder cell layers for their ability to support human pluripotent stem cell culture, noting that human embryonic stem cell lines cultured on adult skin fibroblast feeders retain human embryonic stem cell morphology and remain pluripotent. U.S. Patent App. Pub. No. 2002/0072117 discloses cell lines that produce media that support the growth of primate pluripotent stem cells in feeder-free culture. The cell lines employed are mesenchymal and fibroblast-like cell lines obtained from embryonic tissue or differentiated from embryonic stem cells. U.S. Patent App. Pub. No. 2002/072117 also discloses the use of the cell lines as a primary feeder cell layer.

[0071] Other suitable methods of expanding and culturing pluripotent stem cells are disclosed, for example, in Wang *et al.*, Stojkovic *et al.*, Miyamoo *et al.* and Amit *et al.* Wang *et al.* (*Stem Cells* 23: 1221-1227, 2005) disclose methods for the long-term growth of human pluripotent stem cells on feeder cell layers derived from human embryonic stem cells. Stojkovic *et al.* (*Stem Cells* 2005 23: 306-314, 2005) disclose a feeder cell system derived from the spontaneous

differentiation of human embryonic stem cells. Miyamoto *et al.* (*Stem Cells* 22: 433-440, 2004) disclose a source of feeder cells obtained from human placenta. Amit *et al.* (*Biol. Reprod* 68: 2150-2156, 2003) disclose a feeder cell layer derived from human foreskin.

[0072] Other suitable methods of expanding and culturing pluripotent stem cells are disclosed, for example, in Inzunza *et al.*, U.S. Patent No. 6,642,048, WO 2005/014799, Xu *et al.* and U.S. Patent App. Pub. No. 2007/0010011. Inzunza *et al.* (*Stem Cells* 23: 544-549, 2005) disclose a feeder cell layer from human postnatal foreskin fibroblasts. U.S. Patent No. 6,642,048 discloses media that support the growth of primate pluripotent stem cells in feeder-free culture, and cell lines useful for production of such media. U.S. Patent No. 6,642,048 reports mesenchymal and fibroblast-like cell lines obtained from embryonic tissue or differentiated from embryonic stem cells; as well as methods for deriving such cell lines, processing media, and growing stem cells using such media. WO 2005/014799 discloses a conditioned medium for the maintenance, proliferation, and differentiation of mammalian cells. WO 2005/014799 reports that the culture medium produced via the disclosure is conditioned by the cell secretion activity of murine cells; in particular, those differentiated and immortalized transgenic hepatocytes, named MMH (Met Murine Hepatocyte). Xu *et al.* (*Stem Cells* 22: 972-980, 2004) discloses a conditioned medium obtained from human embryonic stem cell derivatives that have been genetically modified to over express human telomerase reverse transcriptase. U.S. Patent App. Pub. No. 2007/0010011 discloses a chemically defined culture medium for the maintenance of pluripotent stem cells.

[0073] An alternative culture system employs serum-free medium supplemented with growth factors capable of promoting the proliferation of embryonic stem cells. Examples of such culture systems include, but are not limited, to Cheon *et al.*, Levenstein *et al.* and U.S. Patent App. Pub. No. 2005/0148070. Cheon *et al.* (BioReprod DOI:10.1095/biolreprod.105.046870, October 19, 2005) disclose a feeder-free, serum-free culture system in which embryonic stem cells are maintained in unconditioned serum replacement (SR) medium supplemented with different growth factors capable of triggering embryonic stem cell self-renewal. Levenstein *et al.* (*Stem Cells* 24: 568-574, 2006) disclose methods for the long-term culture of human embryonic stem cells in the absence of fibroblasts or conditioned medium, using media supplemented with bFGF. U.S. Patent App. Pub. No. 2005/0148070 discloses a method of culturing human embryonic stem cells in defined media without serum and without fibroblast feeder cells, the method comprising: culturing the stem cells in a culture medium containing albumin, amino acids, vitamins, minerals,

at least one transferrin or transferrin substitute, at least one insulin or insulin substitute, the culture medium essentially free of mammalian fetal serum and containing at least about 100 ng/ml of a fibroblast growth factor capable of activating a fibroblast growth factor signaling receptor, wherein the growth factor is supplied from a source other than just a fibroblast feeder layer, the medium supported the proliferation of stem cells in an undifferentiated state without feeder cells or conditioned medium.

[0074] Other suitable methods of culturing and expanding pluripotent stem cells are disclosed in U.S. Patent App. Pub. No. 2005/0233446, U.S. Patent No. 6,800,480, U.S. Patent App. Pub. No. 2005/0244962 and WO 2005/065354. U.S. Patent App. Pub. No. 2005/0233446 discloses a defined media useful in culturing stem cells, including undifferentiated primate primordial stem cells. In solution, the media is substantially isotonic as compared to the stem cells being cultured. In a given culture, the particular medium comprises a base medium and an amount of each of bFGF, insulin, and ascorbic acid necessary to support substantially undifferentiated growth of the primordial stem cells. U.S. Patent No. 6,800,480 reports that a cell culture medium for growing primate-derived primordial stem cells in a substantially undifferentiated state is provided which includes a low osmotic pressure, low endotoxin basic medium that is effective to support the growth of primate-derived primordial stem cells. The disclosure of the 6,800,480 patent further reports that the basic medium is combined with a nutrient serum effective to support the growth of primate-derived primordial stem cells and a substrate selected from the group consisting of feeder cells and an extracellular matrix component derived from feeder cells. This medium is further noted to include non-essential amino acids, an anti-oxidant, and a first growth factor selected from the group consisting of nucleosides and a pyruvate salt. U.S. Patent App. Pub. No. 2005/0244962 reports that one aspect of the disclosure provides a method of culturing primate embryonic stem cells and that the stem cells in culture are essentially free of mammalian fetal serum (preferably also essentially free of any animal serum) and in the presence of fibroblast growth factor that is supplied from a source other than just a fibroblast feeder layer.

[0075] WO 2005/065354 discloses a defined, isotonic culture medium that is essentially feeder-free and serum-free, comprising: a basal medium, bFGF, insulin and ascorbic acid in amounts sufficient to support growth of substantially undifferentiated mammalian stem cells. Furthermore, WO 2005/086845 discloses a method for maintenance of an undifferentiated stem cell, said method comprising exposing a stem cell to a member of the transforming growth

factor-β (TGF-β) family of proteins, a member of the fibroblast growth factor (FGF) family of proteins, or nicotinamide (NIC) in an amount sufficient to maintain the cell in an undifferentiated state for a sufficient amount of time to achieve a desired result.

[0076] The pluripotent stem cells may be plated onto a suitable culture substrate. In one embodiment, the suitable culture substrate is an extracellular matrix component, such as those derived from basement membrane or that may form part of adhesion molecule receptor-ligand couplings. A suitable culture substrate is a reconstituted basement membrane sold under the trademark MATRIGEL™ (BD Biosciences, Franklin Lakes, NJ). MATRIGEL™ is a soluble preparation from Engelbreth-Holm Swarm tumor cells that gels at room temperature to form a reconstituted basement membrane.

[0077] Other extracellular matrix components and component mixtures known in the art are suitable as an alternative. Depending on the cell type being proliferated, this may include laminin, fibronectin, proteoglycan, entactin, heparan sulfate, and the like, alone or in various combinations.

[0078] The pluripotent stem cells may be plated onto the substrate in a suitable distribution and in the presence of a medium, which promotes cell survival, propagation, and retention of the desirable characteristics. All these characteristics benefit from careful attention to the seeding distribution and can readily be determined by one of skill in the art. Suitable culture media may be made from the following components, Dulbecco's modified Eagle's medium (DMEM) sold under the trademark GIBCO™ (Part #11965-092) by Life Technologies Corporation, Grand Island, NY; Knockout Dulbecco's modified Eagle's medium (KO DMEM) sold under the trademark GIBCO™ (Part #10829-018) by Life Technologies Corporation, Grand Island, NY; Ham's F12/50% DMEM basal medium; 200 mM L-glutamine sold under the trademark GIBCO™ (Part #15039-027) by Life Technologies Corporation, Grand Island, NY; non-essential amino acid solution sold under the trademark GIBCO™ (Part #11140-050) by Life Technologies Corporation, Grand Island, NY; β-mercaptoethanol, Sigma-Aldrich Company, LLC Saint Louis, MO, (Part #M7522); human recombinant basic fibroblast growth factor (bFGF) sold under the trademark GIBCO™ (Part #13256- 029) by Life Technologies Corporation, Grand Island, NY.

Differentiation of Pluripotent Stem Cells

[0079] As pluripotent cells differentiate towards β cells, they differentiate through various stages each of which may be characterized by the presence or absence of particular markers. Differentiation of the cells into these stages is achieved by the specific culturing conditions including the presence and lack of certain factors added to the culture media. In general, this differentiation may involve differentiation of pluripotent stem cells into definitive endoderm cells. These definitive endoderm cells may then be further differentiated into gut tube cells, which may, in turn, be differentiated into foregut endoderm cells. Foregut endoderm cells may be differentiated into pancreatic foregut precursor cells which can, in turn, be further differentiated into pancreatic endoderm cells, pancreatic endocrine precursor cells or both. These cells may then be differentiated into pancreatic hormone producing cells (such as β cells). This invention provides for staged differentiation of pluripotent stem cells toward pancreatic endocrine cells by culturing the cells at the air-liquid interface that exists within a culture vessel partially filled with medium, specifically by culturing Stage 4 to Stage 6 cells at the air-liquid interface.

Differentiation of Pluripotent Stem Cells into Cells Expressing Markers Characteristic of Pancreatic Endocrine Cells

[0080] Characteristics of pluripotent stem cells are well known to those skilled in the art, and additional characteristics of pluripotent stem cells continue to be identified. Pluripotent stem cell markers include, for example, the expression of one or more of the following: ABCG2, cripto, FOXD3, CONNEXIN43, CONNEXIN45, OCT4, SOX2, NANOG, hTERT, UTF1, ZFP42, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81.

[0081] Exemplary pluripotent stem cells include the human embryonic stem cell line H9 (NIH code: WA09), the human embryonic stem cell line H1 (NIH code: WA01), the human embryonic stem cell line H7 (NIH code: WA07), and the human embryonic stem cell line SA002 (Cellartis, Sweden). Also suitable are cells that express at least one of the following markers characteristic of pluripotent cells: ABCG2, cripto, CD9, FOXD3, CONNEXIN43, CONNEXIN45, OCT4, SOX2, NANOG, hTERT, UTF1, ZFP42, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81.

[0082] Also, suitable for use in the present invention is a cell that expresses at least one of the

markers characteristic of the definitive endoderm lineage. In one embodiment of the present invention, a cell expressing markers characteristic of the definitive endoderm lineage is a primitive streak precursor cell. In an alternate embodiment, a cell expressing markers characteristic of the definitive endoderm lineage is a mesendoderm cell. In an alternate embodiment, a cell expressing markers characteristic of the definitive endoderm lineage is a definitive endoderm cell.

[0083] Also suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endoderm lineage. In one embodiment of the present invention, a cell expressing markers characteristic of the pancreatic endoderm lineage is a pancreatic endoderm cell wherein the expression of PDX1 and NKX6.1 are substantially higher than the expression of CDX2 and SOX2. In certain embodiments, more than thirty percent of the cells express PDX1 and NKX6.1 and less than thirty percent of the cells express CDX2 or SOX2 as measured by FACS. Particularly useful are cells in which the expression of PDX1 and NKX6.1 is at least two-fold higher than the expression of CDX2 or SOX2.

[0084] Also suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endocrine lineage. In one embodiment of the invention, a cell expressing markers characteristic of the pancreatic endocrine lineage is a pancreatic endocrine cell. In one embodiment, the pancreatic endocrine cell is capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, or pancreatic polypeptide. In a preferred embodiment, the pancreatic endocrine cell is an insulin-producing β cell.

[0085] In certain embodiments of the invention, to arrive at cells expressing markers characteristic of pancreatic endocrine cells, a protocol starting with pluripotent stem cells or inducible pluripotent cells, preferably pluripotent stem cells, is employed. This protocol includes the following:

Stage 1: Pluripotent stem cells, such as embryonic stem cells obtained from cell culture lines, are treated with appropriate factors to induce differentiation into cells expressing markers characteristic of definitive endoderm cells.

Stage 2: Cells resulting from Stage 1 are treated with appropriate factors to induce further differentiation into cells expressing markers characteristic of gut tube cells.

Stage 3: Cells resulting from Stage 2 are treated with appropriate factors to induce further differentiation into cells expressing markers characteristic of foregut endoderm cells.

Stage 4: Cells resulting from Stage 3 are treated with appropriate factors to induce further differentiation into cells expressing markers characteristic of pancreatic foregut precursor cells. The cells are optionally cultured at the air-liquid interface at late Stage 4.

Stage 5: Cells resulting from Stage 4 are treated with appropriate factors and cultured at the air-liquid interface to induce further differentiation into cells expressing markers characteristic of pancreatic endoderm/endocrine precursor cells.

Stage 6: Cells resulting from Stage 5 are treated with appropriate factors and cultured at the air-liquid interface to induce further differentiation into cells expressing markers characteristic of pancreatic endocrine cells.

[0086] While the invention, in certain embodiments, encompasses differentiating pluripotent stem cells (*e.g.* pre-Stage 1 cells) to Stage 6 cells, the invention also encompasses differentiating cells at other intermediate stages towards Stage 6. In particular, the invention encompasses differentiation of Stage 4 to Stage 6 cells. Moreover, although the process is described in discrete stages, the treatment, as well as the progress of the cells through the differentiation process, may be sequential or continuous.

Stage 1: Differentiation of pluripotent stem cells into cells expressing markers characteristic of definitive endoderm cells

[0087] Pluripotent stem cells may be differentiated into cells expressing markers characteristic of definitive endoderm cells by any method known in the art or by any method proposed herein. Methods useful for differentiating pluripotent stem cells into cells expressing markers characteristic of definitive endoderm cells are disclosed in: U.S. Patent App. Pub. No. 2007/0254359; U.S. Patent App. Pub. No. 2009/0170198; U.S. Patent App. Pub. No. 2009/0170198; U.S. Patent App. Pub. No. 2011/0091971; U.S. Patent App. Pub. No.

2010/0015711; U.S. Patent App. Pub. No. 2010/0015711; U.S. Patent App. Pub. No. 2012/0190111; U.S. Patent App. Pub. No. 2012/0190112; U.S. Patent App. Pub. No. 2012/0196365; U.S. Patent App. Pub. No. 20100015711; U.S. Patent App. Pub. No. 2012/0190111; U.S. Patent App. Pub. No. 2012/0190112; U.S. Patent App. Pub. No. 2012/0196365; U.S. Patent App. Pub. No. 20100015711; U.S. Patent App. Pub. No. 2012/0190111; U.S. Patent App. Pub. No. 2012/0190112; U.S. Patent App. Pub. No. 2012/0196365; U.S. Provisional Patent Application No. 61/076,900; U.S. Provisional Patent Application No. 61/076,908; and U.S. Provisional Patent Application No. 61/076,915, which are incorporated by reference in their entireties as they relate to pluripotent stem cells and to the differentiation of pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage.

[0088] In one embodiment of the invention, pluripotent stem cells are treated with a medium supplemented with activin A and WNT3A to result in the generation of cells expressing markers characteristic of definitive endoderm cells. Treatment may involve contacting pluripotent stem cells with a medium containing about 50 ng/ml to about 150 ng/ml, alternatively about 75 ng/ml to about 125 ng/ml, alternatively about 100 ng/ml of activin A. The treatment may also involve contacting the cells with about 10 ng/ml to about 50 ng/ml, alternatively about 15 ng/ml to about 30 ng/ml, alternatively about 20 ng/ml of WNT3A. The pluripotent cells may be cultured for approximately two to five days, preferably about three days, to facilitate their differentiation into cells expressing markers characteristic of definitive endoderm cells. In one embodiment, the cells are cultured in the presence of activin A and WNT3A for one day, followed by culturing in the presence of activin A (without WNT3A being present) for the remainder.

[0089] In another embodiment of the invention, pluripotent stem cells are treated with a medium supplemented with growth differentiation factor 8 ("GDF8") and a glycogen synthase kinase-3 β ("GSK3β") inhibitor (such as the cyclic aniline-pyridinotriazine compounds disclosed in U.S. Patent App. Pub. No. 2010/0015711; incorporated herein by reference in its entirety) to induce differentiation into cells expressing markers characteristic of definitive endoderm cells. A preferred GSK3β inhibitor is 14-Prop-2-en-1-yl-3,5,7,14,17,23,27-heptaazatetracyclo [19.3.1.1~2,6~.1~8,12~]heptacosa-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one, referred to herein as ("MCX Compound"). Treatment may involve contacting pluripotent stem cells with a medium supplemented with about 50 ng/ml to about 150 ng/ml, alternatively about 75 ng/ml to

about 125 ng/ml, alternatively about 100 ng/ml of GDF8. The treatment may also involve contacting the cells with about 0.1 to 5 μM, alternatively about 0.5 to about 2.5 μM, preferable about 1 μM of MCX compound. The pluripotent cells may be cultured for approximately two to five days, preferably two to three days, to facilitate their differentiation into cells expressing markers characteristic of definitive endoderm cells.

[0090] In one embodiment, the cells are cultured in the presence of GDF8 and MCX compound for one day, followed by culturing in the presence of GDF8 and a lower concentration of MCX compound for one day, followed by culturing in the presence of GDF8 for one day in the absence of the MCX compound. In particular, the cells are cultured in the presence of GDF8 and about 1 μM of MCX compound for one day, followed by culturing in the presence of GDF8 and about 0.1 μM of MCX compound for one day, followed by culturing in the presence of GDF8 for one day in the absence of the MCX compound. In an alternate embodiment, the cells are cultured in the presence of GDF8 and about 1 μM of MCX compound for one day, followed by culturing in the presence of GDF8 and about 0.1 μM MCX compound for one day.

[0091] Generation of cells expressing markers characteristic of definitive endoderm cells may be determined by testing for the presence of the markers before and after following a particular protocol. Pluripotent stem cells typically do not express such markers. Thus, differentiation of pluripotent cells can be detected when the cells begin to express markers characteristic of definitive endoderm cells. Methods for assessing expression of protein and nucleic acid markers in cultured or isolated cells are standard in the art. These methods include RT-PCR, Northern blots, *in situ* hybridization (*see, e.g.*, Current Protocols in Molecular Biology (Ausubel *et al.*, eds. 2001 supplement)), and immunoassays (such as immunohistochemical analysis of sectioned material, Western blotting, and for markers that are accessible in intact cells, flow cytometry analysis (FACS) (*see, e.g.*, Harlow and Lane, Using Antibodies: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press (1998)).

[0092] Additionally, the efficiency of differentiation may be determined by exposing a treated cell population to an agent (such as an antibody) that specifically recognizes a protein marker expressed by the differentiated cells of interest.

[0093] The differentiated cells may also be further purified. For example, after treating pluripotent stem cells with the methods of the present invention, the differentiated cells may be purified by exposing a treated cell population to an agent (such as an antibody) that specifically

recognizes a protein marker characteristically expressed by the differentiated cells being purified.

Stage 2: Differentiation of cells expressing markers characteristic of definitive endoderm cells into cells expressing markers characteristic of gut tube cells

[0094] The cells expressing markers characteristic of definitive endoderm cells may be further differentiated into cells expressing markers characteristic of gut tube cells. In one embodiment, the formation of cells expressing markers characteristic of gut tube cells includes culturing the cells expressing markers characteristic of definitive endoderm cells with a medium containing fibroblast growth factor ("FGF")7 or FGF10 to differentiate these cells. For example, the culture medium may include from about 25 ng/ml to about 75 ng/ml, alternatively from about 30 ng/mL to about 60 ng/ml, alternatively about 50 ng/ml of FGF7 or FGF10, preferably FGF7. The cells may be cultured under these conditions for about two to three days, preferably about two days.

[0095] In another embodiment, differentiation into cells expressing markers characteristic of gut tube cells includes culturing cells expressing markers characteristic of definitive endoderm cells with FGF7 or FGF10 and ascorbic acid (Vitamin C). The culture medium may include from about 0.1 mM to about 0.5 mM ascorbic acid, alternatively from about 0.2 mM to about 0.4 mM, alternatively about 0.25 mM of ascorbic acid. The culture medium may also include from about 10 ng/ml to about 35 ng/ml, alternatively from about 15 ng/ml to about 30 ng/ml, alternatively about 25 ng/ml of FGF7 or FGF10, preferably FGF7. For example, the culture medium may include about 0.25 mM of ascorbic acid and about 25 ng/ml of FGF7. In one embodiment, the Stage 1 cells are treated for 2 days with FGF7 and ascorbic acid.

Stage 3: Differentiation of cells expressing markers characteristic of gut tube cells into cells expressing markers characteristic of foregut endoderm cells

[0096] Cells expressing markers characteristic of gut tube cells may be further differentiated into cells expressing markers characteristic of foregut endoderm cells. In one embodiment, Stage 2 cells are further differentiated into Stage 3 cells by culturing these cells in a culture medium supplemented with a Smoothened ("SMO") receptor inhibitor (such as "MRT10" (N-[[[3-benzoylamino)phenyl]amino]thioxomethyl]-3,4,5-trimethoxybenzamide)) or Cyclopamine)

or a Sonic Hedgehog ("SHH") signaling pathway antagonist (such as Smoothened Antagonist 1 ("SANT-1") ((E)-4-benzyl-N-((3,5-dimethyl-1-phenyl-1H-pyrazol-4-yl) methylene-piperazin-1-amine)), or Hedgehog Pathway Inhibitor 1 ("HPI-1") (2-methoxyethyl 1,4,5,6,7,8-hexahydro-4-(3-hydroxyphenyl)-7-(2-methoxyphenyl)-2-methyl-5-oxo-3-quinolinecarboxylate)), retinoic acid, and Noggin. Alternatively, Stage 2 cells may be differentiated into Stage 3 cells by culturing these cells in a culture medium supplemented with a SMO receptor inhibitor, SHH signaling pathway antagonist, retinoic acid, and Noggin. The cells may be cultured for approximately two to four days, preferably about two days. In one embodiment, the medium is supplemented with from about 0.1 μM to about 0.3 μM of SANT-1, from about 0.5 μM to about 3 μM of retinoic acid and from about 75 ng/ml to about 125 ng/ml of Noggin. In another embodiment, the medium is supplemented with about 0.25 μM of SANT-1, about 2 μM of retinoic acid and about 100 ng/ml of Noggin.

[0097] In an alternate embodiment, Stage 2 cells are further differentiated into Stage 3 cells by treating the Stage 2 cells with a medium supplemented with FGF7 or FGF10, retinoic acid, a SMO receptor inhibitor (such as MRT10 or Cyclopamine) or SHH signaling pathway antagonist (such as SANT-1 or HPI-1), a protein kinase C ("PKC") activator (such as ((2S,5S)-(E,E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam ("TPB")) EMD Chemicals, Inc., Gibbstown, NJ), phorbol-12,13-dibutyrute ("PDBu"), phorbol-12-myristate-13-acetate ("PMA") or indolactam V ("ILV")), a bone morphogenic protein ("BMP") inhibitor (such as LDN-193189, Noggin or Chordin), and ascorbic acid. In another embodiment, the medium may be supplemented with FGF7 or FGF10, retinoic acid, an SMO receptor inhibitor, an SHH signaling pathway antagonist (such as SANT-1), a PKC activator (such as TPB), a BMP inhibitor (such as LDN-193189), and ascorbic acid. The cells may be cultured in the presence of these growth factors, small molecule agonists, and antagonists for about two to four days, preferably about two to three days.

[0098] In a further embodiment, the medium is supplemented with from about 15 ng/ml to about 35 ng/ml of FGF7, from about 0.5 μM to about 2 μM of retinoic acid, from about 0.1 μM to about 0.4 μM of SANT-1, from about 100 to about 300 nM of TPB, from about 50 nM to about 200 nM of LDN-193189, and from about 0.15 mM to about 0.35 mM of ascorbic acid. In another embodiment, the medium is supplemented with about 25 ng/ml of FGF7, about 1 μM of

retinoic acid, about 0.25 μM of SANT-1, about 200 nM of TPB, about 100 nM of LDN-193189, and from about 0.25 mM of ascorbic acid.

Generation of Stage 4 to Stage 6 cells by culturing at the air-liquid interface

[0099] Although the present invention contemplates culturing at the air-liquid interface for all stages in the path from pluripotent cell to pancreatic endocrine cell, the invention preferably provides for the formation of Stage 1 to Stage 3 cells in submerged culture, and Stage 4 to Stage 6 cells by culturing cells at the air-liquid interface. Accordingly, in certain embodiments, the present invention provides a stepwise method of differentiating pluripotent cells comprising culturing during Stages 4 to 6 at the air-liquid interface. In certain embodiments, cells may be cultured at the air-liquid interface during the entirely of Stages 4 through 6. In other embodiments, only late Stage 4 to Stage 6, or only Stages 5 and 6, or only Stages 4 and 5, or only Stages 4 and 6 include culturing at the air-liquid interface.

[0100] When cells are cultured at the air-liquid interface (air-liquid interface), the cells may be cultured on a porous substrate such that the cells are in contact with air on the top side and with cell culture media at the bottom side. For example, a sufficient volume of media may be added to the bottom of a culture vessel containing the porous substrate (*e.g.* a filter insert) such that the media contacts the bottom surface of cells residing on the substrate but does not encapsulate or submerge them. Suitable porous substrates can be formed of any material that will not adversely affect the growth and differentiation of the cells. Exemplary porous substrates are made of polymers such as polyethylene terephthalate (PET), polyester, or polycarbonate. Suitable porous substrates may be coated or uncoated. In one embodiment, the porous substrate may be coated with MATRIGEL™. In one embodiment of the invention, the porous substrate is a porous filter insert, which may be coated with MATRIGEL™. Preferably, however, the porous substrate is an uncoated filter insert. The porosity of the substrate should be sufficient to maintain cell viability and promote differentiation of the cells. Suitable substrates include filter inserts having a pore size of from about 0.3 to about 3.0 μm, about 0.3 to about 2.0 μm, about 0.3 to about 1.0 μm, about 0.3 to about 0.8 μm, about 0.3 to about 0.6 μm, about 0.3 to about 0.5 μm, about 0.5 to about 3.0 μm, about 0.6 to about 3.0 μm, about 0.8 to about 3.0 μm, about 1.0 to about 3.0 μm, about 2.0 μm to about 3.0 μm, preferably about 0.4 μm, and a pore density of from about 50 to about 120 million pores/cm^2, about 60 to about 110 million pores/cm^2, about 70 to about

100 million pores/cm^2, preferably about 80 to about 100 million pores/cm^2, about 90 to about 100 million pores/cm^2, more preferably about 100 million pores/cm^2

[0101] The media may advantageously be exchanged or refreshed daily or every other day. The cells grown on top of the porous substrate are generally not single cells, rather they are in the form of a sheet or exist as an aggregate cluster of cells. Cells cultured at the air-liquid interface may experience much higher oxygen tension as compared to cells submerged in media.

[0102] The present invention thus encompasses the generation of Stage 4 to Stage 6 cells, preferably Stage 5 and Stage 6 cells, at the air-liquid interface. Stage 4 cells may be cultured entirely in planar cultures, entirely at the air-liquid interface, or the cells may be cultured in submerged planar culture during the early portion of Stage 4 and then cultured at the air-liquid interface for the latter portion of Stage 4. These cells may be produced by differentiating pluripotent stem cells or by further differentiating Stage 3, 4 or 5 cells derived from other means.

[0103] In one embodiment, the present invention provides a method for producing cells expressing markers characteristic of pancreatic endocrine cells, perferably β cells, from pluripotent stem cells, comprising culturing pluripotent stem cells, differentiating the pluripotent stem cells into cells expressing markers characteristic of foregut endoderm cells; and differentiating the cells expressing markers characteristic of foregut endoderm cells into cells expressing markers characteristic of pancreatic endocrine/β cells by culturing at the air-liquid interface.

[0104] In another embodiment, the present invention provides a method for producing cells expressing markers characteristic of pancreatic endocrine cells, preferably β cells, from pluripotent stem cells, comprising culturing pluripotent stem cells, differentiating the pluripotent stem cells into cells expressing markers characteristic of pancreatic foregut precursor cells, and differentiating the cells expressing markers characteristic of pancreatic foregut precursor cells into cells expressing markers characteristic of pancreatic endocrine cells by culturing at the air-liquid interface.

[0105] The method may include treatment with a medium supplemented with triiodothyronine (T3), thyroxine (T4), analogues of T3 or T4, or mixtures thereof (collectively referred to hereafter as "T3/T4"), or an activin receptor-like kinase ("ALK") 5 inhibitor, or both T3/T4 and an ALK5 inhibitor. Suitable thyroid hormone analogues may include: GC-1 (Sobertirome) available from R & D Systems, Inc. Catalogue # 4554; DITPA (3,5-diiodothyropropionic acid);

KB-141, discussed in *J. Steroid Biochem. Mol. Biol.* 2008, 111: 262-267 and *Proc. Natl. Acad. Sci. US* 2003, 100: 10067-10072; MB07344, discussed in *Proc. Natl. Acad. Sci. US* 2007, 104: 15490-15495; T0681, discussed in *PLoS One*, 2010, 5e8722 and *J. Lipid Res.* 2009, 50: 938-944; and GC-24, discussed in *PLoS One*, 2010 e8722 and *Endocr. Pract.* 2012, 18(6): 954-964, the disclosures of which are incorporated herein in their entirety. Useful ALK5 inhibitors include: ALK5 inhibitor II (Enzo, Farmingdale, NY); ALK5i (Axxora, San Diego, CA); SD208 (R & D systems (MN)); TGF-B inhibitor SB431542 (Xcess Biosciences (San Diego, CA)); ITD-1 (Xcess Biosciences (San Diego, CA)); LY2109761 (Xcess Biosciences (San Diego, CA)); A83-01 (Xcess Biosciences (San Diego, CA)); LY2157299 (Xcess Biosciences (San Diego, CA)); TGF-β receptor inh V (EMD Chemicals, Gibstown, NJ); TGF-β receptor inh I (EMD Chemicals, Gibstown, NJ); TGF-β receptor inh IV (EMD Chemicals, Gibstown, NJ); TGF-β receptor inh VII (EMD Chemicals, Gibstown, NJ); TGF-β receptor inh VIII (EMD Chemicals, Gibstown, NJ); TGF-β receptor inh II (EMD Chemicals, Gibstown, NJ); TGF-β receptor inh VI (EMD Chemicals, Gibstown, NJ); TGF-β receptor inh III (EMD Chemicals, Gibstown, NJ). The method may include differentiating the cells expressing markers characteristic of foregut endoderm cells into cells expressing markers characteristic of pancreatic foregut precursor cells by treatment with a medium supplemented with T3/T4 or ALK5 inhibitor and culturing in a planar culture. The method may also include differentiating cells expressing markers characteristic of pancreatic foregut precursor cells into cells expressing markers characteristic of β cells by treatment with media supplemented with T3/T4, or an ALK5 inhibitor, or both, and culturing at the air-liquid interface.

[0106] In one embodiment, the method includes treatment with a medium supplemented with T3/T4 and an ALK5 inhibitor. In other embodiments, the method includes treating Stage 3 cells with a medium supplemented with T3/T4 or an ALK5 inhibitor. The method may also include treating cells expressing markers characteristic of pancreatic endoderm/endocrine precursor cells with a medium supplemented with T3/T4 and an ALK5 inhibitor.

[0107] One embodiment of the invention is a method of forming cells expressing markers characteristic of β cells comprising differentiating cells expressing markers characteristic of foregut endoderm cells into cells expressing markers characteristic of β cells by culturing at the air-liquid interface. A cell expressing markers characteristic of β cells expresses insulin and at least one of the following transcription factors: PDX1, NKX2.2, NKX6.1, NeuroD1, 1SL1,

HNF3β, MAFA, PAX4, and PAX6. In one embodiment, the methods of the invention result in the formation of cells, which are positive for NKX6.1, PDX1, and HB9. Accordingly, the invention provides a method of inducing the expression of PDX1, NKX6.1 and HB9 in human cells by culturing pancreatic endoderm cells at the air-liquid interface under conditions sufficient to induce such expression. The invention also provides a method for inducing the expression of PDX1, NKX6.1 and NGN3 in human cells by culturing pancreatic endoderm cells at the air-liquid interface. The method may include treatment with a medium supplemented with T3, an ALK5 inhibitor, or both. Thus, in one embodiment, the medium may be supplemented with T3, while in another embodiment, the medium may be supplemented with an ALK5 inhibitor. In another embodiment, the medium may be supplemented with both T3 and an ALK5 inhibitor. The Stage 6 cells may be cells that are positive for NKX6.1, PDX1, and HB9. In other embodiments, the Stage 6 cells are single hormone positive cells. For example, the Stage 6 cells may be cells that (a) co-express NKX6.1 and chromogranin-A or (b) co-express NKX6.1 and insulin.

[0108] Culturing of the cells at the air-liquid interface includes seeding the cells on a porous substrate such as a porous filter insert. In certain embodiments, the substrate pore size may range from about 0.4 to about 3 microns, or any of the pore sizes mentioned herein. Seeding may be accomplished by releasing cells as single cells or clusters of cells from monolayer cultures into a suspension and subsequently aliquoting the cell suspension onto a porous substrate positioned at the air-liquid interface. The cells may be seeded onto the porous substrate from a suspension comprising about 1000 cells/µl to about 100,000 cells/µl, about 1000 cells/µl to about 90,000 cells/µl , about 1000 cells/µl to about 80,000 cells/µl , about 1000 cells/µl to about 70,000 cells/µl , about 1000 cells/µl to about 60,000 cells/µl , about 1000 cells/µl to about 50,000 cells/µl , about 1000 cells/µl to about 40,000 cells/µl , about 1000 cells/µl to about 30,000 cells/µl , about 1000 cells/µl to about 20,000 cells/µl , about 1000 cells/µl to about 10,000 cells/µl , about 1000 cells/µl to about 5000 cells/µl , about 5000 cells/µl to about 100,000 cells/µl , about 10,000 cells/µl to about 100,000 cells/µl , about 20,000 cells/µl to about 100,000 cells/µl , about 30,000 cells/µl to about 100,000 cells/µl, about 40,000 cells/µl to about 100,000 cells/µl , about 50,000 cells/µl to about 100,000 cells/µl , about 60,000 cells/µl to about 100,000 cells/µl, about 20,000 cells/µl to about 80,000 cells/µl, about 30,000 cells/µl to about 70,000 cells/µl, about 40,000 cells/µl to about 60,000 cells/µl, preferably about 50,000

cells/μl. The cells may be seeded as droplets of the cell suspension containing individual cells or clumps of cells. The resulting cell deposit may contain from about 5 x 10^6 to about 5 x 10^7 cells/cm^2, about 6 x 10^6 to about 5 x 10^7 cells/cm^2, about 7 x 10^6 to about 5 x 10^7 cells/cm^2, about 8 x 10^6 to about 5 x 10^7 cells/cm^2, about 9 x 10^6 to about 5 x 10^7 cells/cm^2, about 1 x 10^7 to about 5 x 10^7 cells/cm^2, about 2 x 10^7 to about 5 x 10^7 cells/cm^2, about 2 x 10^7 to about 5 x 10^7 cells/cm^2, about 3 x 10^7 to about 5 x 10^7 cells/cm^2, about 3 x 10^7 to about 5 x 10^7 cells/cm^2, about 4 x 10^7 to about 5 x 10^7 cells/cm^2, about 5 x 10^6 to about 4 x 10^7 cells/cm^2, about 5 x 10^6 to about 3 x 10^7 cells/cm^2, about 5 x 10^6 to about 2 x 10^7 cells/cm^2, about 5 x 10^6 to about 1 x 10^7 cells/cm^2, about 5 x 10^6 to about 9 x 10^6 cells/cm^2, about 5 x 10^6 to about 8 x 10^6 cells/cm^2, about 5 x 10^6 to about 7 x 10^6 cells/cm^2, about 5 x 10^6 to about 6 x 10^6 cells/cm^2, about 7 x 10^6 to about 4 x 10^7 cells/cm^2, about 8 x 10^6 to about 3 x 10^7cells/cm^2, about 9 x 10^6 to about 2 x 10^7 cells/cm^2, preferably on the order of or about1 x 10^7 cells/cm^2.

[0109] In one embodiment, the invention relates to a method of enhancing expression of HB9 protein by culturing and differentiating a population of PDX1 and NKX6.1 co-positive pancreatic endoderm precursor cells into PDX1 and NKX6.1 co-positive pancreatic endocrine cells at the air-liquid interface on a porous substrate. Alternatively, HB9 protein expression can be induced by culturing and differentiating a population of foregut endoderm cells, consisting primarily of PDX1 positive cells, at the air-liquid interface. In some embodiments, the population of pancreatic endoderm cells is obtained by a stepwise differentiation of pluripotent cells at least, in part, at the air-liquid interface.

[0110] In another embodiment, the invention provides a method of enhancing the number of single hormone positive cells (*e.g.* cells that co-express NKX6.1 and insulin or cells that produce NKX6.1 and chromogranin-A) by culturing and differentiating a population of PDX1 and NKX6.1 co-expressing cells at an air-liquid interface. In another embodiment, pancreatic endoderm cells cultured at the air-liquid interface are further differentiated to pancreatic endocrine cells by treatment with a compound selected from the following: ALK5 inhibitor, BMP inhibitor, gamma-secretase inhibitor, Ephrin ligands, EphB inhibitor, PKC inhibitor, EGFr inhibitor, retinoic acid, vitamin C, T3/T4, glucose, cell cycle regulators, WNT regulators, SHH inhibitor, or combinations thereof.

[0111] In some embodiments, pancreatic endoderm cells cultured at the air-liquid interface are further differentiated into pancreatic endocrine precursor cells and to pancreatic hormone

expressing cells. In an alternate embodiment, the invention encompasses cells prepared by the methods of the invention that express insulin but not NKX6.1. In some embodiments, a pancreatic endoderm population generated at the air-liquid interface is transplanted into diabetic animals for further *in vivo* maturation to functional pancreatic endocrine cells.

Stage 4: Differentiation of cells expressing markers characteristic of foregut endoderm cells into cells expressing markers characteristic of pancreatic foregut precursor cells

[0112] In one embodiment, the methods of the invention include treating Stage 3 cells with a differentiation medium comprising a growth medium supplemented with one or more of the following: (a) an ALK5 inhibitor selected from the group consisting of: TGF-β receptor inh V, TGF-β receptor inh I, TGF-β receptor inh IV, TGF-β receptor inh VII, TGF-β receptor inh VIII, TGF-β receptor inh II, TGF-β receptor inh VI, TGF-β receptor inh III, TGF-B inhibitor SB431542, SD208, ITD-1, LY2109761, A83-01, LY2157299, ALK5i and ALK5 inhibitor II; (b) a thyroid hormone selected from the group consisting of T3, T4, analogues of T3, analogues of T4 and mixtures thereof; (c) a smoothened receptor inhibitor selected from MRT10 or cyclopamine; (d) a SHH signaling pathway antagonist selected from SANT-1 or HPI-1; (e) a BMP Receptor Inhibitor selected from LDN-193189, Noggin or Chordin; (f) a PKC activator selected from TPB, PDBu, PMA, and ILV; (g) a fibroblast growth factor selected from FGF7 or FGF10; (h) retinoic acid; (i) ascorbic acid; (j) heparin; and (k) zinc sulfate. For example, a growth medium such as MCDB131 or BLAR may be supplemented with a SMO inhibitor (such as MRT10 or Cyclopamine) or SHH signaling pathway antagonist (such as SANT-1 or HPI-1), a BMP inhibitor (such as LDN-193189, Noggin or Chordin), ascorbic acid, and a PKC activator (such as TPB, PDBu, PMA or ILV), to provide a useful differentiation media. Culturing Stage 3 cells in such medium for about two to four days, preferably about three days, usually is sufficient to differentiate the Stage 3 cells into Stage 4 cells. In another embodiment, the medium may be supplemented with a SMO inhibitor and SHH signaling pathway antagonist. In a preferred embodiment, the Stage 3 cells may be treated with a medium supplemented with about 0.25 μM SANT-1; about 100 nM retinoic acid; about 2 ng/ml FGF7; about 100 nM LDN-193189; about 0.25 mM ascorbic acid; and about 100 nM TPB for three days. In another embodiment, the

medium is further supplemented with T3, such as from about 5 nM to about 25 nM, alternatively about 10 nM of T3.

[0113] In Stage 4, the cells may be cultured on a planar culture or at the air-liquid interface. Specifically, the present invention provides an *in vitro* cell culture for differentiating cells derived from pluripotent stem cells at the air-liquid interface comprising: (a) a culture vessel; (b) a volume of growth medium within said vessel sufficient to fill only a portion of the volume of said vessel; (c) air within said vessel that fills a portion of said vessel adjoining said medium; (d) a porous substrate located at the interface between said medium and said air; and (e) cells derived from pluripotent stem cells disposed upon the surface of said substrate such that said medium contacts only a portion of the surface of said cells. Alternatively, cells expressing markers characteristic of foregut endoderm cells can be differentiated into cells expressing markers characteristic of pancreatic foregut precursor cells by treatment with a medium supplemented as described above in a planar culture.

[0114] In a further embodiment, the cells at the end of Stage 4 may be treated with a Rho-associated kinase ("ROCK") inhibitor such as Y27632 ((1R,4r)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide), GSK269962 (N-[3-[[2-(4-Amino-1,2,5-oxadiazol-3?-yl)-1-ethyl-1H-imidazo[4,5-c]pyridin-6-yl]oxy]phenyl]-4-[2-(4-morpholinyl)ethoxy]benzamide), H1152 ((S)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine, 2HCl,) and, SR3677 (*N*-[2-[2-(Dimethylamino)ethoxy]-4-(1*H*-pyrazol-4-yl)phenyl-2,3-dihydro-1,4-benzodioxin-2-carboxamide dihydrochloride). In certain embodiments about 10 μM of the ROCK inhibitor may be used.

[0115] In certain embodiments, only late in Stage 4 are cells cultured at the air-liquid interface. In one embodiment, only late Stage 4 cells that were treated with a ROCK inhibitor are cultured at the air-liquid interface. In certain embodiments, the cells may be treated with a cell detachment solution, such as a solution containing proteolytic and collagenolytic enzymes prior to culturing at the air-liquid interface.

[0116] In an alternate embodiment, Stage 3 cells may be treated with a differentiation medium comprising a growth medium supplemented with an ALK5 inhibitor, Noggin, and a PKC activator (such as TPB). In certain embodiments, the medium may be supplemented with about 0.1 μM ALK5 inhibitor, about 100 ng/mL of Noggin, and about 500 nM TPB. The cell culture may be in a monolayer format. The treatment may last for a total of about three days. In certain

embodiments, the cells may be treated for two days and then on the last day the cells may be treated with proteolytic enzymes, collagenolytic enzymes or both, such as dispase, and broken into cell clusters having a diameter of less than about 100 microns followed by culturing in the presence of an ALK5 inhibitor and LDN-193189. In certain embodiments, the cell clusters having a diameter of less than about 100 microns may be cultured in a medium supplemented with about 200 nM ALK5 inhibitor and about 100 nM LDN-193189.

Stage 5: Differentiation of cells expressing markers characteristic of pancreatic foregut precursor cells into cells expressing markers characteristic of pancreatic endoderm/endocrine precursor cells

[0117] In one embodiment, the methods of the invention include treating Stage 4 cells with a differentiation medium comprising a growth medium supplemented with one or more of the following: (a) an ALK5 inhibitor selected from the group consisting of: TGF-β receptor inh V, TGF-β receptor inh I, TGF-β receptor inh IV, TGF-β receptor inh VII, TGF-β receptor inh VIII, TGF-β receptor inh II, TGF-β receptor inh VI, TGF-β receptor inh III, TGF-B inhibitor SB431542, SD208, ITD-1, LY2109761, A83-01, LY2157299, ALK5i and ALK5 inhibitor II; (b) a thyroid hormone selected from the group consisting of T3, T4, analogues of T3, analogues of T4 and mixtures thereof; (c) a smoothened receptor inhibitor selected from MRT10 or cyclopamine; (d) a SHH signaling pathway antagonist selected from SANT-1 or HPI-1; (e) a BMP Receptor Inhibitor selected from LDN-193189, Noggin or Chordin; (f) a PKC activator selected from TPB, PDBu, PMA, and ILV; (g) a fibroblast growth factor selected from FGF7 or FGF10; (h) retinoic acid; (i) ascorbic acid; (j) heparin; and (k) zinc sulfate, and culturing the cells at the air-liquid interface for about two to four days, preferably about three days, to differentiate the cells into Stage 5 cells. In another embodiment, the growth medium is supplemented with a SMO inhibitor (such as MRT10 or cyclopamine) or SHH signaling pathway antagonist (such as SANT-1 or HPI-1), retinoic acid, T3, ascorbic acid, a BMP Receptor Inhibitor (such as LDN-193189, Noggin, or Chordin) and an ALK5 inhibitor. In another embodiment, the methods of the invention include treating Stage 4 cells with a medium supplemented with a SMO inhibitor, SHH signaling pathway antagonist, retinoic acid, T3, ascorbic acid, a BMP Receptor Inhibitor and an ALK5 inhibitor and culturing the cells at the air-liquid interface for about two to four days, preferably about three days, to differentiate the cells into Stage 5 cells. In one embodiment, the Stage 4 cells are differentiated into Stage 5 cells by

treating the cells with a medium supplemented with about 0.25 μM SANT-1, about 50 nM retinoic acid, about 0.25 mM ascorbic acid, about 50 nM LDN-193189, about 10 nM of T3 and about 1000 nM ALK5 inhibitor. In certain embodiments, the ALK5 inhibitor is SD208 ((2-(5-Chloro-2-fluorophenyl)pteridin-4-yl]pyridin-4-yl-amine). In one embodiment, the medium is supplemented with about 1000 nM of SD208.

[0118] In yet another embodiment, the methods of the invention include treating Stage 4 cells with a medium supplemented with heparin, a SMO inhibitor or SHH signaling pathway antagonist, retinoic acid, a BMP Receptor Inhibitor and an ALK5 inhibitor and culturing the cells at the air-liquid interface for about two to four days, preferably about three days, to differentiate the cells into Stage 5 cells. In an alternate embodiment, the medium may be supplemented with both a SMO inhibitor and SHH signaling pathway antagonist, along with retinoic acid, a BMP Receptor Inhibitor and an ALK5 inhibitor.

[0119] The medium may further be supplemented with $ZnSO_4$. For example, about 10 μM $ZnSO_4$ may be added. Thus, in one embodiment, the Stage 4 cells may be differentiated into Stage 5 cells by treating the Stage 4 cells with a medium supplemented with heparin, $ZnSO_4$, a SMO inhibitor or SHH signaling pathway antagonist, retinoic acid, LDN-193189 and ALK5 inhibitor II. In an alternate embodiment, the medium may be supplemented with both a SMO inhibitor and SHH signaling pathway antagonist. In one embodiment, the Stage 4 cells are differentiated into Stage 5 cells by treating the cells with a medium supplemented with about 10 μg/ml of heparin, about 0.25 μM SANT-1, about 50 nM retinoic acid, about 50 nM LDN-193189, about 10 nM of T3 and about 1000 nM ALK5 inhibitor. Suitable ALK5 inhibitors include but are not limited to SD208, ALK5 inhibitor II, TGF-β receptor inh V, TGF-β receptor inh I, TGF-β receptor inh IV, TGF-β receptor inh VII, TGF-β receptor inh VIII, TGF-β receptor inh II, TGF-β receptor inh VI, TGF-β receptor inh III and combinations thereof.

[0120] In one embodiment, the ALK5 inhibitor is ALK5 inhibitor II. In another embodiment, about 1000 nM of ALK5 inhibitor II is used. In an alternate embodiment, the Stage 4 cells are treated with a medium supplemented with about 10 μg/ml of heparin, about 0.25 μM SANT-1, about 50 nM retinoic acid, about 100 nM LDN-193189, and about 10000 nM of ALK5 inhibitor II.

[0121] In yet another alternate embodiment, the methods of the invention include treating Stage 4 cells with a medium supplemented with a SMO inhibitor or SHH signaling pathway antagonist,

retinoic acid, and an ALK5 inhibitor and culturing the cells at the air-liquid interface for about 2 days to differentiate the cells into Stage 5 cells. In an alternate embodiment, the medium may be supplemented with both a SMO inhibitor and SHH signaling pathway antagonist. In one embodiment, the Stage 4 cells are differentiated into Stage 5 cells by treating the cells with a medium supplemented with about 0.25 μM SANT-1, about 50 nM retinoic acid, about 50 nM LDN-193189, and about 1000 nM of an ALK5 inhibitor (such as SD208 or ALK5 inhibitor II). In certain embodiments, the medium may be MCDB-131 (Life Technologies Corporation, Grand Island, NY).

[0122] The amount of cells seeded for culturing at the air-liquid interface may vary. For example, to culture the cells at the air-liquid interface, droplets of a cell suspension containing from about 2 x 10^5 cells/μl to about 6 x 10^5 cells/μl, 3 x 10^5 cells/μl to about 6 x 10^5 cells/μl, 4 x 10^5 cells/μl to about 6 x 10^5 cells/μl, 5 x 10^5 cells/μl to about 6 x 10^5 cells/μl, 2 x 10^5 cells/μl to about 5 x 10^5 cells/μl, 2 x 10^5 cells/μl to about 4 x 10^5 cells/μl, or about 3 x 10^5 cells/μl may be seeded onto a porous substrate such as a filter located at the air-liquid interface. In some embodiments, droplets of a cell suspension containing from about 0.5 x 10^5 cells/μl to about 0.75 x 10^5 cells/μl, about 0.6 x 10^5 cells/μl to about 0.75 x 10^5 cells/μl, or about 0.5 x 10^5 cells/μl to about 0.6 x 10^5 cells/μl are seeded onto a porous support to be cultured at the air-liquid interface.

[0123] In another embodiment, the methods of the invention include treating Stage 4 cells with a medium supplemented with a BMP Receptor Inhibitor (*e.g.*, LDN-193189, Noggin or Chordin) and an ALK5 inhibitor for about 1 day to differentiate Stage 4 cells into Stage 5 cells. For example, the medium may be supplemented with about 100 nM of LDN-193189 and with about 200 nM of ALK5 inhibitor. Preferably, this embodiment also includes pre-treating the cells with dispase. The cells may be in the form of clusters. In certain embodiments, the cells may be treated with a cell detachment solution, such as a solution containing proteolytic and collagenolytic enzymes prior to culturing at the air-liquid interface. In one embodiment, Stage 4 cells cultured according to embodiments of the invention are utilized and differentiated into Stage 5 cells, while in other embodiments Stage 4 cells cultured according to other protocols may be utilized.

[0124] In accordance with the foregoing method, the invention further provides a cell culture for differentiating cells expressing markers characteristic of pancreatic foregut precursor cells into cells expressing markers characteristic of pancreatic endoderm/pancreatic endocrine

precursor cells comprising: (a) a culture vessel; (b) a volume of growth medium within said vessel sufficient to fill only a portion of the volume of said vessel; (c) air within said vessel that fills a portion of said vessel adjoining said medium; (d) a porous substrate located at the interface between said medium and said air; and (e) cells expressing markers characteristic of pancreatic foregut precursor cells derived from pluripotent stem cells disposed upon the surface of said substrate such that said medium contacts only a portion of the surface of said cells.

[0125] In certain embodiments, culturing cells in Stage 5 at the air-liquid interface may enhance expression of pancreatic hormones. Accordingly, the invention also provides for methods of enhancing expression of pancreatic hormones by culturing cells at the air-liquid interface. In some embodiments, the cells in Stage 5 may be treated as described herein and in the Tables VIII to XIII below. In certain embodiments, the method may also reduce expression of PTF1a, SOX9, CDX2 (intestine marker), ZIC1 (ectoderm marker), and SOX2 (anterior endoderm marker).

[0126] In one embodiment, the method includes differentiating cells expressing markers characteristic of pancreatic foregut precursor cells into cells expressing markers characteristic of pancreatic endocrine cells by treatment with a medium supplemented with T3/T4, or an ALK5 inhibitor or both T3/T4 and an ALK5 inhibitor and culturing at the air-liquid interface

Stage 6: Differentiation of cells expressing markers characteristic of pancreatic endoderm/pancreatic endocrine precursor cells into cells expressing markers characteristic of pancreatic endocrine cells

[0127] In one embodiment of the invention, the methods include treating Stage 5 cells with a differentiation medium comprising a growth medium supplemented with one or more of the following: (a) an ALK5 inhibitor selected from the group consisting of: TGF-β receptor inh V, TGF-β receptor inh I, TGF-β receptor inh IV, TGF-β receptor inh VII, TGF-β receptor inh VIII, TGF-β receptor inh II, TGF-β receptor inh VI, TGF-β receptor inh III, TGF-B inhibitor SB431542, SD208, ITD-1, LY2109761, A83-01, LY2157299, ALK5i and ALK5 inhibitor II; (b) a thyroid hormone selected from the group consisting of T3, T4, analogues of T3, analogues of T4 and mixtures thereof; (c) a smoothened receptor inhibitor selected from MRT10 or cyclopamine; (d) a SHH signaling pathway antagonist selected from SANT-1 or HPI-1; (e) a BMP Receptor Inhibitor selected from LDN-193189, Noggin or Chordin; (f) a PKC activator selected from TPB, PDBu, PMA, and ILV; (g) a fibroblast growth factor selected from FGF7 or

FGF10; (h) retinoic acid; (i) ascorbic acid; (j) heparin; and (k) zinc sulfate and culturing at the air-liquid interface for about two to four days, preferably about three days, to differentiate the Stage 5 cells into Stage 6 cells. In one embodiment, the growth medium is supplemented with a SMO inhibitor (such as MRT10 or Cyclopamine) or SHH signaling pathway antagonist (such as SANT-1 or HPI-1), retinoic acid, ascorbic acid, T3/T4, and an ALK5 inhibitor. In an alternate embodiment, the medium may be supplemented with both a SMO inhibitor and SHH signling pathway antagonist. The Stage 5 cells may be differentiated into Stage 6 cells by treatment with a medium supplemented with about 0.25 μM SANT-1, about 50 nM RA, about 0.25 mM ascorbic acid, about 500 mM of ALK5 inhibitor, and about 0.1 nM of T3 for about three days. Alternatively, Stage 5 cells may be differentiated into Stage 6 cells by treatment with a medium supplemented with about 0.25 μM SANT-1, about 50 nM retinoic acid, about 0.25 mM ascorbic acid, about 500 nM ALK5 inhibitor and 10 nM T3 for about three days. The cells may be cultured in such media for an additional two days, or more, if desired.

[0128] Alternatively, Stage 5 cells may be differentiated into Stage 6 cells by treatment with a medium supplemented with heparin, a SMO inhibitor or SHH signaling pathway antagonist, a BMP inhibitor, T3/T4, and an ALK5 inhibitor and culturing at the air-liquid interface for about six to fourteen days, alternatively about 6 days, alternatively about 7 days, alternatively about 8 days, alternatively about 9 days, alternatively about 10 days, alternatively about 11 days, alternatively about 12 days, alternatively about 13 days, and alternatively about 14 days. In an alternate embodiment, the medium may be supplemented with both a SMO inhibitor and SHH signaling pathway antagonist. For example, the cells may be cultured in the medium supplemented with about 10 μg/ml of heparin, about 0.25 μM SANT-1, about 100 nM LDN-193189, about 1000 nM of T3 and about 500 to about 10,000 nM, about 1000 to about 10,000 nM, about 5000 to about 10,000 nM, about 600 to about 5000 nM, about 700 to about 5000 nM, about 800 to about 5000 nM, about 900 to about 5000 nM, about 1000 nM to about 5000 nM, about 600 to about 1000 nM, about 700 to about 1000 nM, about 800 to about 1000 nM, about 600 to about 1200 nM, about 700 to about 1200 nM, about 800 to about 1200 nM, about 900 to about 1200 nM, alternatively about 500 nM, alternatively about 1000 mM, and alternatively about 10,000 nM of an ALK5 inhibitor.

[0129] Suitable ALK5 inhibitors include but are not limited to SD208, ALK5 inhibitor II, TGF-β receptor inh V, TGF-β receptor inh I, TGF-β receptor inh IV, TGF-β receptor inh VII, TGF-β

receptor inh VIII, TGF-β receptor inh II, TGF-β receptor inh VI, TGF-β receptor inh III and combinations thereof.

[0130] In one embodiment, the ALK5 inhibitor is ALK5 inhibitor II. In another embodiment, about 1000 nM of ALK5 inhibitor II is used. Accordingly, in one embodiment, Stage 5 cells may be differentiated into Stage 6 cells by treatment with a medium supplemented with heparin, SMO inhibitor or SHH signaling pathway antagonist, a BMP inhibitor, T3/T4, and ALK5 inhibitor and culturing at the air-liquid interface for about six days. In an alternate embodiment, the medium may be supplemented with both a SMO inhibitor and SHH signaling pathway antagonist. In certain embodiments, the cells may be treated with a cell detachment solution, such as a solution containing proteolytic and collagenolytic enzymes prior to culturing at the air-liquid interface.

[0131] In another embodiment, Stage 5 cells may be differentiated into Stage 6 cells by treatment with a medium supplemented with heparin, a SMO inhibitor or SHH signaling pathway antagonist, a BMP inhibitor, T3, and ALK5 inhibitor II and culturing at the air-liquid interface for about 5 days to about 15 days, about 6 days to about 14 days, about 7 days to about 13 days, about 8 days to about 12 days, about 9 days to about 11 days, about 5 days to about 10 days, about 10 days to about 15 days, alternatively about 5 days, alternatively about 6 days, alternatively about 7 days, alternatively about 8 days, alternatively about 9 days, alternatively about 10 days, alternatively about 11 days, alternatively about 12 days, alternatively about 13 days, alternatively about 14 days, alternatively about 15 days. In one embodiment, the cells are cultured at the air-liquid interface for 5 days or more, 6 days or more, 7 days or more, 8 days or more, 9 days or more, 10 days or more, 11 days or more, 12 days or more, 13 days or more, 14 days or more, 15 days or more. In one embodiment, the cells are cultured at the air-liquid interface for 15 days or less, 14 days or less, 13 days or less, 12 days or less, 11 days or less, 10 days or less, 9 days or less, 8 days or less,7 days or less, 6 days or less, 5 days or less. In one embodiment, the cells are cultured at the air-liquid interface for about 10 days. In another embodiment, the cells are cultured at the air-liquid interface for about 11 days. In an alternate embodiment, the cells are cultured at the air-liquid interface for about 12 days. In yet another embodiment, the cells are cultured at the air-liquid interface for about 15 days. In these embodiments, the medium may be supplemented with about 10 µg/ml of heparin, about 0.25 µM SANT-1, about 100 nM LDN-193189, about 1000 nM of T3 and about 10,000 nM of ALK5

inhibitor II. In certain embodiments, the medium may be further supplemented with Zinc sulfate ($ZnSO_4$). For example, the medium may be further supplemented with about 10 µM $ZnSO_4$. In an alternate embodiment, the medium may be supplemented with both a SMO inhibitor and SHH signaling pathway antagonist

[0132] In accordance with the foregoing method, the invention further provides a cell culture for differentiating cells expressing markers characteristic of pancreatic endoderm/pancreatic endocrine precursor cells into cells expressing markers characteristic of pancreatic endocrine cells, comprising: (a) a culture vessel; (b) a volume of growth medium within said vessel sufficient to fill only a portion of the volume of said vessel; (c) air within said vessel that fills a portion of said vessel adjoining said medium; (d) a porous substrate located at the interface between said medium and said air; and (d) cells expressing markers characteristic of pancreatic endoderm/pancreatic endocrine precursor cells derived from pluripotent stem cells disposed upon the surface of said substrate such that said medium contacts only a portion of the surface of said cells.

[0133] In one embodiment, Stage 5 cells cultured according to embodiments of the invention are utilized and differentiated into Stage 6 cells, while in other embodiments Stage 5 cells cultured according to other protocols may be utilized.

[0134] In another embodiment, the methods of the invention result in the generation of Stage 6 cells, which are single-hormone positive. Thus, in one embodiment, the methods of the invention result in Stage 6 cells which co-express NKX6.1 and chromogranin-A. In another embodiment, the methods of the invention result in Stage 6 cells which co-express NKX6.1 and insulin.

[0135] In certain embodiments of the invention, the method employs BLAR a custom medium (see Table II) at Stages 4 to 6. The medium may preferably be exchanged every day or alternatively every other day. In certain embodiments of the invention, the methods include treating the Stage 4 to Stage 6 cells with the specified components in the amounts recited in Tables VIII to XIII, herein.

[0136] In another embodiment, the invention relates to a method of producing Stage 6 cells co-expressing NKX6.1 and chromogranin-A comprising culturing at the air-liquid interface in Stages 4 to 6, preferably Stages 5 and 6. In yet another embodiment, the invention relates to a

method of producing single hormone insulin positive cells expressing NKX6.1 cells by culturing at the air-liquid interface in Stages 4 to 6, preferably Stages 5 and 6.

[0137] Culturing cells at the air-liquid interface during, or after, Stage 4 may significantly enhance expression of pancreatic endoderm markers along with endocrine-related markers. Accordingly, the invention provides for methods of enhancing expression of pancreatic endoderm and endocrine-related markers by culturing cells during, or after Stage 4 at the air-liquid interface.

[0138] In another embodiment, the invention also provides for methods of increasing the yield of NKX6.1 positive cells co-expressing insulin, chromogranin-A or chromagranin-A and insulin by culturing Stage 4 and subsequent cells at the air-liquid interface in the presence of an ALK5 inhibitor. In one embodiment, the ALK5 inhibitor is ALK5 inhibitor II. Other suitable ALK 5 inhibitors include but are not limited to, TGF-β receptor inh V, TGF-β receptor inh I, TGF-β receptor inh IV, TGF-β receptor inh VII, TGF-β receptor inh VIII, TGF-β receptor inh II, TGF-β receptor inh VI, TGF-β receptor inh III and combinations thereof. In some embodiments, in addition to the ALK5 inhibitor, the cells may be treated as described in the Tables VIII to XIII below.

[0139] In one embodiment, the invention provides for methods of increasing the NKX6.1 positive cells co-expressing insulin, chromogranin-A or chromagranin-A and insulin by culturing cells during Stage 5 at the air-liquid interface in the presence of ALK5 inhibitor II. In one embodiment, the method further comprises culturing cells during Stage 5 in the presence of ALK5 inhibitor II and T3.

In vivo maturation of Stage 6 cells

[0140] In certain embodiments of the invention, Stage 6 cells prepared in accordance with the methods of the invention may be further matured *in vivo*. In one embodiment, these cells may be matured further by *in vivo* transplantation into a mammal. For example, the cells may be transplanted under the kidney capsule of a mouse. In one embodiment, the Stage 6 cells that are further matured *in vivo* are cells that co-express NXK6.1 and insulin. In another embodiment, the Stage 6 cells that are further matured *in vivo* are cells that co-express NXK6.1 and chromagranin. In an alternate embodiment, *in vivo* maturation of (a) cells that co-express NXK6.1 and insulin or (b) cells that co-express NXK6.1 and chromagranin results in early C-

peptide production. In certain embodiments, the level of C-peptide production from transplanting approximately 3 million Stage 6 cells is similar to the amount of C-peptide produced by transplanting approximately 3,000 human islets.

[0141] Culturing at the air-liquid interface according to the methods described herein is also well-suited for use in screening compounds for their effect on the secretion of pancreatic hormones and endocrine markers. In particular, Stage 4 to Stage 6 cells cultured at the air-liquid interface can be used in various culture formats, including from 384 to 6-well formats, to evaluate the effect that the inclusion of a variety of small molecules or biologics, at various doses and time intervals, have on subsequent expression of pancreatic endoderm, pancreatic endocrine precursor, pancreatic endocrine, and pancreatic β cell markers. Such an evaluation may be accomplished by measuring gene expression by PCR, protein expression by FACS, immune staining, or by ELISA for secretion of factors by cells affected by the addition of the small molecules or biologics.

Cells obtainable by the methods of the invention

[0142] The invention also provides a cell or population of cells obtainable by a method of the invention. The invention also provides a cell or population of cells obtained by a method of the invention.

[0143] The invention also provides a cell or population of cells, preferably expressing markers characteristic of pancreatic endocrine cells, characterized by significant co-expression of NKX6.1 and chromogranin-A. The invention also provides an insulin positive cell or a population of insulin positive cells, preferably expressing markers characteristic of pancreatic endocrine cells, characterized by NKX6.1 expression (optionally >30%). These are previously undescribed cell populations as explained in Example 10.

Methods for treatment

[0144] The invention provides methods of treatment. In particular, the invention provides methods for treating a patient suffering from, or at risk of developing, diabetes.

[0145] The invention also provides a cell or population of cells obtainable or obtained by a method of the invention for use in a method of treatment. In particular, the invention provides a cell or population of cells obtainable or obtained by a method of the invention for use in a method of treating a patient suffering from, or at risk of developing, diabetes.

[0146] The diabetes may be Type 1 or Type 2 diabetes.

[0147] In one embodiment, the method of treatment comprises implanting cells obtained or obtainable by a method of the invention into a patient.

[0148] In one embodiment, the method of treatment comprises

differentiating pluripotent stem cells *in vitro* into Stage 1, Stage 2, Stage 3, Stage 4, Stage 5 or Stage 6 cells, for example as described herein,

and implanting the differentiated cells into a patient.

[0149] In one embodiment, the method further comprises the step of culturing pluripotent stem cells, for example as described herein, prior to the step of differentiating the pluripotent stem cells.

[0150] In one embodiment, the method further comprises the step of differentiating the cells *in vivo,* after the step of implantation.

[0151] In one embodiment, the patient is a mammal, preferably a human.

[0152] In one embodiment, the cells may be implanted as dispersed cells or formed into clusters that may be infused into the hepatic portal vein. Alternatively, cells may be provided in biocompatible degradable polymeric supports, porous non-degradable devices or encapsulated to protect from host immune response. Cells may be implanted into an appropriate site in a recipient. The implantation sites include, for example, the liver, natural pancreas, renal subcapsular space, omentum, peritoneum, subserosal space, intestine, stomach, or a subcutaneous pocket.

[0153] To enhance further differentiation, survival or activity of the implanted cells *in vivo*, additional factors, such as growth factors, antioxidants or anti-inflammatory agents, can be administered before, simultaneously with, or after the administration of the cells. These factors can be secreted by endogenous cells and exposed to the administered cells *in situ*. Implanted cells can be induced to differentiate by any combination of endogenous and exogenously administered growth factors known in the art.

[0154] The amount of cells used in implantation depends on a number of various factors including the patient's condition and response to the therapy, and can be determined by one skilled in the art.

[0155] In one embodiment, the method of treatment further comprises incorporating the cells into a three-dimensional support prior to implantation. The cells can be maintained *in vitro* on this support prior to implantation into the patient. Alternatively, the support containing the cells can be directly implanted in the patient without additional *in vitro* culturing. The support can optionally be incorporated with at least one pharmaceutical agent that facilitates the survival and function of the transplanted cells.

EXAMPLES

Example 1

Culturing pancreatic endocrine precursor cells at the air-liquid interface

[0156] This example examines and demonstrates that pancreatic endocrine precursor cells (Stage 5 cells) can be further matured upon culturing at the air-liquid interface. To culture pancreatic endocrine precursor cells at the air-liquid interface, embryonic stem cells were differentiated into pancreatic endocrine precursor cells based on the protocol discussed below.

[0157] Cells of the human embryonic stem cell line H1 were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, Franklin Lakes, NJ)-coated dishes in mTESR®1 media (StemCell Technologies, Vancouver, Canada) supplemented with 10 μM of Y27632 (Rock inhibitor, Catalog No. Y0503, Sigma-Aldrich, St. Louis, MO). Forty-eight hours

post-seeding, the cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a) Stage 1: (3 days): 60-70% confluent adherent cultures of undifferentiated H1 cells plated on 1:30 MATRIGEL™ coated surfaces were exposed to GIBCO® RPMI 1640 medium (Life Technologies Corporation, Grand Island, NY) supplemented with 0.2% fetal bovine serum (FBS) (Hyclone, Utah), 100 ng/ml activin-A (AA; Pepro-tech; Rocky Hill, NJ), and 20 ng/ml of Wnt3A (R&D Systems, Inc., Minneapolis, MN) for day one only. For the next two days, the cells were cultured in GIBCO® RPMI with 0.5% FBS and 100 ng/ml AA.

b) Stage 2: (3 days): The Stage 1 cells were then exposed to Dulbecco's modified eagle's medium (DMEM-F12) (Life Technologies Corporation, NY) supplemented with 2% FBS and 50 ng/ml of FGF7 (Pepro-tech) for three days.

c) Stage 3: (4 days): The Stage 2 cells were then cultured for four days in DMEM-HG medium (Life Technologies Corporation, Grand Island, NY) supplemented with 0.25 μM SANT-1 (Sigma-Aldrich; St. Louis, MO), 2 μM retinoic acid (Sigma-Aldrich), 100 ng/ml of Noggin (R&D Systems), and 1% (v/v) of a supplement sold under the trademark B27® by Life Technologies Corporation, Grand Island, NY (Catalogue#: 17504044).

d) Stage 4: (3 days): The Stage 3 cells were then cultured for three days in DMEM-HG medium supplemented with 0.1 μM ALK5 inhibitor (ALK5i; Axxora, San Diego, CA), 100 ng/ml of Noggin, 500 nM TPB ((2S,5S)-(E,E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam; EMD Chemicals Inc, Gibbstown NJ) and 1% B27 in monolayer format. For the last day of culture, the cells were treated with 5 mg/ml Dispase (Becton Dickinson, Bedford, MA, #354235) for 5 minutes, followed by gentle pipetting to mix and break into cell clusters (< 100 micron). The cell clusters were transferred into a disposable polystyrene 125 ml Spinner Flask (Corning), and spun at 80 to 100 rpm overnight in suspension with DMEM-HG supplemented with 200 nM ALK5 inhibitor, 100 nM LDN-193189 (Stemgent, CA), and 1% B27.

e) Stage 5: (1 day): The Stage 4 cells were then treated with 5 mg/ml Dispase for 5 minutes, followed by gentle pipetting to mix and break into cell clusters (< 100 micron). The cell clusters were transferred into a disposable polystyrene 125 ml Spinner Flask (Corning, NY), and spun at 80 to 100 rpm overnight in suspension with DMEM-HG supplemented with 200 nM ALK5 inhibitor, 100 nM LDN-193189 (Stemgent, CA), and 1% B27.

[0158] Stage 5 day 1 clusters were seeded on 0.4 micron porous cell culture filter inserts (BD Biosciences, PET membranes, #353493) in 6-well plates (in 10 microliter aliquots containing ~1 million cells) and cultured for 3 weeks at the air-liquid interface by adding 1.5 ml of DMEM-HG + 1% B27 at the bottom of the insert and no media above the insert. FIGS. 1 A to H depict phase contrast images of the clusters at various time points post-seeding at the air-liquid interface. FIGS. 2 A to K show immunostaining results for the following proteins at 1 week post-seeding of the cell clusters on the filters: DAPI (FIG. 2A); insulin (FIG. 2B); HB9 (Fig. 2C); DAPI (FIG. 2D); glucagon(FIG. 2E); insulin (FIG. 2F); DAPI (FIG. 2G); insulin (FIG. 2H); somatostatin (FIG. 2I); NKX6.1 (FIG. 2J); and insulin (FIG. 2K). While FIGS. 3 A to H depict immunostaining results for the following proteins at 2 weeks post-seeding on the filters: insulin (FIG. 3A); glucagon (FIG. 3B); insulin (FIG. 3C); somatostatin (FIG. 3D); insulin (FIG. 3E); NKX6.1 (FIG. 3F); HB9 (FIG. 3G); and NKX6.1 (FIG. 3H). In FIG. 2, panels A-C, D-F, G-I and J-K were taken from the same fields. In FIG. 3, panels A-B, C-D, E-F, and G-H, respectively, were taken from the same fields. FIGS. 4 A to D depict the results of immunostaining for the following proteins at 3 weeks post-seeding on the filters: insulin (FIG. 4A); glugacon (FIG. 4B); insulin (FIG. 4C); and somatostatin (FIG. 4D). In FIG. 4, panels A-B and C-D, respectively, were taken from the same fields.

[0159] At Stage 4 and subsequent cultures, mRNA was collected for PCR analysis of relevant pancreatic endoderm/endocrine genes. Total RNA was extracted with the RNeasy® Mini Kit (Qiagen; Valencia, CA) and reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturers' instructions. cDNA was amplified using Taqman Universal Master Mix and Taqman Gene Expression Assays which were pre-loaded onto custom Taqman Arrays (Applied Biosystems). The data were analyzed using Sequence Detection Software (Applied Biosystems) and normalied to undifferentiated human embryonic stem (hES) cells using the ΔΔCt method ((*i.e.* qPCR results

corrected with internal controls ($\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$)). All primers were purchased from Applied Biosystems. FACS and immunofluorescence analysis was done as previously described (*Diabetes*, 61, 20126, 2012).

[0160] FIGS. 5A to R depict data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 1: PDX1 (FIG.5A); NKX6.1 (FIG. 5B); Pax4 (FIG. 5C); Pax6 (FIG. 5D); NGN3 (FIG. 5E); NKX2.2 (FIG. 5F); ABCC8 (FIG. 5G); chromogranin-A (FIG. 5H); PCSK1 (FIG. 5I); IAPP (FIG. 5J); insulin (FIG. 5K); glucagon (FIG. 5L); somatostatin (FIG. 5M); ghrelin (FIG. 5N); Ptf1a (FIG. 5O); Zic1 (FIG. 5P); CDX2 (FIG. 5Q); and SOX9 (FIG. 5R). Following a 3-week culture at the air-liquid interface, there was a significant time-dependent increase in the expression of markers associated with maturation of endocrine cells, such as ABCC8, IAPP (Amylin), and PCSK1. There was a significant drop in PTF1a and SOX9 expression and very low expression of CDX2 (intestine marker), ZIC1 (ectoderm marker), and SOX2 (anterior endoderm marker), while expression of NKX6.1 and PDX1 were maintained at a very high level. Expression of all of the pancreatic hormones was significantly enhanced through the 3-week culture period at the air-liquid interface.

Example 2

Culturing pancreatic endocrine precursor cells at the air-liquid interface using various filter inserts

[0161] This example examines the type and porosity of filter inserts in differentiation of pancreatic endoderm cells at the air-liquid interface. To examine the effects of type and porosity of the filter inserts, embryonic stem cells were differentiated using the protocol discussed below.

[0162] Cells of the human embryonic stem cell line H1 (passage 40) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes or MATRIGEL™-coated filter inserts (Millipore PIHT 30R 48) in a media comprising DMEM-F12 (Invitrogen, Ca), GlutaMax™ (1:100 dilution, Invitrogen), 0.25 mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 μM of Y27632 (Rock inhibitor, Catalog No. Y0503, Sigma-Aldrich, St. Louis, MO). Forty-eight hours post-seeding, the cultures were washed in incomplete PBS

(phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a. Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant, Catalog No. 68700), 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich, Catalog No. S3187), 1X GlutaMax™ (Invitrogen, Catalog No. 35050-079), 4.5 mM D-Glucose (Sigma-Aldrich, Catalog No. G8769), 100 ng/ml GDF8 (R&D Systems) and 1 μM MCX compound. The cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, 100 ng/ml GDF8, and 0.1 μM MCX compound. Subsequently, the cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-Glucose, and 100 ng/ml GDF8.

b. Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

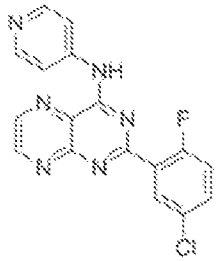
c. Stage 3 (2 days): The Stage 2 cells were then treated with MCDB-131 medium supplemented with a 1:200 dilution of ITS-X (Invitrogen, CA); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1 (Sigma, MO); 1 μM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days.

d. Stage 4 (3 days): The Stage 3 cells were then treated with MCDB-131 medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 100

nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; 10 nM T3 (T6397, Sigma) and 100 nM TPB for three days.

e. Stage 5 (3 days): The Stage 4 cells were then treated with MCDB-131 medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 50 nM RA; 0.25 mM ascorbic acid; 10 nM T3, 50 nM LDN-193189; 1000 nM ALK5 inhibitor SD208, for three days. SD208 (2-(5-Chloro-2-fluorophenyl)pteridin-4-yl]pyridin-4-yl-amine) is a 2,4-disubstituted pteridine, ATP-competitive inhibitor of the TGF-βR I kinase, disclosed in *Molecular Pharmacology* 2007, 72:152-161, and having the structure shown in Formula I.

Formula 1:



f. Stage 6 (5 days): The Stage 5 cells were then treated with MCDB-131 medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 50 nM RA; 0.025 mM ascorbic acid; 500 nM ALK5 inhibitor; 0.1 nM T3 for three days.

[0163] In some cultures, at Stage 3 to Stage 6, cells cultured on planar dishes were treated with 1X ACCUTASE™ (StemCell Tech, Vancouver) for 1-3 minutes at room temperature followed by removal of the enzyme and scraping of the cells by a cell scraper. The resulting suspension of cells was seeded at a density of about 2-6 x 10^6 cells onto 0.4 micron porous cell culture filter inserts having a surface area of approximately 4.2 cm^2 in 6-well plates. The various filter inserts used are identified in Table I. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter. In some cultures, the filters were coated for 1 hour at room temperature with MATRIGEL™ (1:30 dilution) and the cells were seeded on top of the coated inserts at the air-liquid interface or with the media on top of the insert. The media was replaced every other day for the duration of the study.

[0164] FIG. 6 depicts data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 2: PDX1 (FIG.6A); NKX6.1 (FIG. 6B); PAX4 (FIG. 6C); PAX6 (FIG. 6D); NGN3 (FIG. 6E); NKX2.2 (FIG. 6F); ABCC8 (FIG. 6G); chromogranin-A (FIG. 6H); PCSK1 (FIG. 6I); IAPP (FIG. 6J); insulin (FIG. 6K); and glucagon (FIG. 5L). Culturing Stage 4 cells on filter inserts (Corning, 3412, BD 353493, and Millipore PIHT 30R 48) significantly enhanced pancreatic endoderm markers along with endocrine-related markers. Coating of the filters with MATRIGEL™ significantly diminished the benefits of culturing on the filter at the air-liquid

interface. Furthermore, cells cultured on low pore density filter inserts (BD 353090, Corning 3452) showed less survival and differentiation.

Table I. List of filter inserts evaluated in Example 2

Filter insert	Polymer composition	Pore size (microns)	Pore density (#/cm^2)	Coating
BD, #353493	PET	0.4	100 +/- 10 x 10^6	No
BD, #353090	PET	0.4	2 +/- 0.2 x 10^6	No
Corning, #3452	Polyester	0.4	4 x 10^6	+/- MATRIGEL™ coating (1:30 dilution)
Corning, #3412	Polycarbonate	0.4	100 x 10^6	No
Millipore, #PIHT 30R 48	PET	0.4	100 x 10^6	+/- MATRIGEL™ coating (1:30 dilution)

Example 3

Pancreatic endoderm cells cultured at the air-liquid interface showed enhanced expression of endocrine markers as compared to planar cultures or cultures maintained on filters at liquid-liquid interface

[0165] This example is directed to differences in the propensity of differentiation of pancreatic endoderm cells cultured on planar substrates as compared to those cultured at the air-liquid interface. In addition, the effect of the air-liquid interface was further highlighted by differentiating cells on inserts but with media added to both the top and bottom of the inserts.

[0166] Cells of the human embryonic stem cell line H1 (passage 40) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes or MATRIGEL™-coated filter inserts (Millipore PIHT 30R 48) in a media comprising of DMEM-F12 (Invitrogen, Ca), GlutaMax™ (1:100 dilution, Invitrogen), 0.25 mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 μM of Y27632 (Rock inhibitor, Catalog No. Y0503, Sigma-Aldrich, St. Louis, MO). Forty-eight hours post-seeding, the cultures were washed in incomplete

PBS (phosphate buffered saline without Mg or Ca). For cells cultured on filter inserts, at the beginning of Stage 1, in some cultures media was added only to the bottom of the insert and the top of the insert was kept at the air-liquid interface, while in other cultures media was also added to the top of the filter insert as well as to the bottom of the insert. The cells were then differentiated according to the following protocol:

a) Stage 1 (3 days): The cells were cultured for one day in MCDB-131 medium (Invitrogen, Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant, Catalog No. 68700), 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich, Catalog No. S3187), 1X GlutaMax™ (Invitrogen, Catalog No. 35050-079), 4.5 mM D-glucose (Sigma-Aldrich, Catalog No. G8769), 100 ng/ml GDF8 (R&D Systems) and 1 μM MCX compound. The cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, 100 ng/ml GDF8, and 0.1 μM MCX compound. Subsequently the cells were cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, and 100 ng/ml GDF8.

b) Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

c) Stage 3 (2 days): The Stage 2 cells were then treated with BLAR custom medium (manufactured by Invitrogen, *see* Table II for the components of BLAR media) supplemented with a 1:200 dilution of ITS-X (Invitrogen, CA); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1 (Sigma, MO); 1 μM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days.

d) Stage 4 (3 days): The Stage 3 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; 10 nM T3 (T6397, Sigma) and 100 nM TPB for three days.

e) Stage 5 (3 days): The Stage 4 cells were treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 50 nM RA; 0.25 mM ascorbic acid; 10 nM T3; 50 nM LDN-193189; 1000 nM ALK5 inhibitor (SD208) for three days.

f) Stage 6 (5 days): The Stage 5 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 50 nM RA; 0.025 mM ascorbic acid; 500 nM ALK5 inhibitor; 0.1 nM T3 for three days.

[0167] In some cultures, at the end of Stage 3, cells cultured on planar dishes were treated with 1X ACCUTASE™ (StemCell Tech, Vancouver) for 1-3 minutes at room temperature followed by removal of the enzyme and scraping of the cells by a cell scraper. The resulting suspension of cells were seeded at a density of 2-6 x 10^6 cells (in 50-100 μl aliquots) on 0.4 micron porous cell culture filter inserts (BD 353493) in 6-well plates. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter. The media was replaced every other day for the duration of the study.

[0168] FIG. 7 depicts data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 3: PDX1 (FIG.7A); NKX6.1 (FIG. 7B); PAX4 (FIG. 7C); PAX6 (FIG. 7D); NGN3 (FIG. 7E); NKX2.2 (FIG. 7F); ABCC8 (FIG. 7G); chromogranin-A (FIG. 7H); PCSK1 (FIG. 7I); IAPP (FIG. 7J); insulin (FIG. 7K); and glucagon (FIG. 7L).

[0169] Culturing cells on filter inserts at the air-liquid interface in Stages 4 through 6, significantly enhanced pancreatic endocrine-related markers. Furthermore, cells cultured and differentiated on MATRIGEL™-coated filter inserts from the start of Stage 1 with media on top and bottom of the filter inserts did show lower levels of pancreatic endoderm and endocrine

expression as compared to cells cultured on planar cultures or at the air-liquid interface. Furthermore, pancreatic endoderm cells cultured on filter inserts at the air-liquid interface showed the highest expression of pancreatic endocrine cells as compared to all the tested configurations.

Table II. List of components of BLAR media

Component	Concentration (mM)
Amino Acids	
Glycine	3.0E-02
Alanine	3.0E-02
Arginine	3.0E-01
Aspargine	1.0E-01
Aspartic Acid	1.0E-01
Cysteine	2.0E-01
Glutamic acid	3.0E-02
Histidine	1.1E-01
Isoleucine	1.0E-02
Leucine	9.0E-02
Lysine hydrochloride	1.5E-01
Methiane	3.0E-02
Phenylalanine	3.0E-02
Proline	1.0E-01
Serine	1.0E-01
Theronine	3.0E-02
Tryptophan	2.0E-03
Tyrosinedisodium	1.0E-02
Vair-liquid interfacene	3.0E-02
Vitamins	
Biotin	3.0E-05
Choline chloride	5.0E-03
D-Calcium pantothenate	1.5E-03
Folinic Acid Calcium salt	2.3E-03
Niacinamide	4.9E-03
Pyridoxine hydrochloride	9.7E-04
Riboflavin	1.0E-05

Table II. List of components of BLAR media	
Component	**Concentration (mM)**
Thiamine hydrochloride	3.0E-03
Vitamin B12	3.7E-06
i-Inositol	2.8E-03
Minerals/other	
Calcium Chloride ($CaCl_2$-$2H_2O$)	3.0E-01
Cupric sulfate ($CuSO_4$-5H2O)	4.8E-06
Ferric sulfate ($FeSO_4$-$7H_2O$)	1.0E-03
Magnesium Sulfate (MgSO4-$7H_2O$)	4.1E-01
Potassium Chloride (KCl)	3.8E+00
Selenious Acid H_2SeO_3	
Sodium Bicarbonate ($NaHCO_3$)	1.4E+01
Sodium Chloride (NaCl)	1.1E+02
Sodium Phosphate dibasic (Na2HPO4-$7H_2O$)	5.0E-01
Zinc Sulfate ($ZnSO_4$-H_2O)	1.0E-04
Adenine	1.0E-03
D-Glucose (Dextrose)	5.0E+00
Lipoic Acid	1.2E+05
Phenol Red	1.0E-02
Sodium Pyruvate	1.0E+00
Thymidine	9.8E-05

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Example 4

Pancreatic endoderm cells cultured at the air-liquid interface and treated with ALK5 inhibitor II showed a significantly larger number of NKX6.1 positive cells co-expressing chromogranin-A and insulin

[0170] This example was carried out to show that ALK5 inhibitor II was unique in generating a significant population of cells at the air-liquid interface that expressed NKX6.1 and insulin or chromogranin-A. Furthermore, this observation was unique to cultures at the air-liquid interface. Submerged cultures in monolayer planar cultures failed to show a significant number of NKX6.1 cells expressing insulin or chromogranin-A.

[0171] Cells of the human embryonic stem cell line H1 (passage 40) were seeded as single cells at 1 X 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes or Matrigel-coated filter inserts (Millipore PIHT 30R 48) in a media comprising of DMEM-F12 (Invitrogen, Ca), Glutamax (1:100 dilution, Invitrogen), 0.25 mM ascorbic acid (Sigma, Mo), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-B (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 µM of Y27632 (Rock inhibitor, Catalog No. Y0503, SigmaAldrich, St. Louis, MO). Forty-eight hours post seeding, cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a) Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant Catalog No. 68700), 0.0012 g/ml sodium bicarbonate (SigmaAldrich Catalog No. S3187), 1X GlutaMax™ (Invitrogen Catalog No. 35050-079), 4.5 mM D-Glucose (SigmaAldrich Catalog No. G8769), 100 ng/ml GDF8 (R&D Systems) and 1 µM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-Glucose, 100 ng/ml GDF8, and 0.1 µM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-Glucose, and 100 ng/ml GDF8.

b) Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-Glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

c) Stage 3 (2 days): The Stage 2 cells were then treated with BLAR custom medium (Invitrogen) supplemented with a 1:200 dilution of ITS-X (Invitrogen, Ca); 4.5 mM Glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 µM SANT-1 (Sigma, MO); 1 µM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days.

d) Stage 4 (3 days): The Stage 3 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM Glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 µM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; and 100 nM TPB for three days.

e) Stage 5 (- 3 days): The Stage 4 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM Glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 µM SANT-1; 50 nM RA; 50 nM LDN; 500-1000 nM of various ALK5 inhibitors (*see* Table III for the list of inhibitors used) for three days.

f) Stage 6 (7 days): The Stage 5 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM Glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 µM SANT-1; 50 nM RA; 500-1000 nM ALK5 inhibitor (see Table III for list of inhibitors tested).

[0172] In some cultures, at the end of day one of Stage 4, cells cultured on planar dishes were treated with 1X Accutase (StemCell Tech, Vancouver) for 1-3 min at room temperature followed by removal of the enzyme and scraping of cells by a cell scraper. The resulting suspension of

cells were seeded at a density of 2-4 X 10^6 cells (in 25-50 µl aliquots) on 0.4 micron porous cell culture filter inserts (BD 353493) in 6-well plates. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter. Media was replaced every other day for the duration of the study.

[0173] Figure 8 depicts data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated and cultured at the air-liquid interface as outlined in Example 4 after day 1 of Stage 4, day 3 of Stage 5 and day 6 of Stage 6 at the air-liquid interface: PDX1 (FIG 8A), NKX6.1 (FIG. 8B), NGN3 (FIG. 8C), ABCC8 (FIG. 8D), PCSK1 (FIG. 8E), Ghrelin (Fig. 8F), glucagon (FIG. 8G), and insulin (FIG. 8H).

[0174] Figure 9 depicts data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 4 and cultured in planar monolayer cultures at day 3 of Stage 5 and day 4 of Stage 6 for PDX1 (FIG 9A), NKX6.1 (FIG. 9B), NGN3 (FIG. 9C), ABCC8 (FIG. 9D), PCSK1 (FIG. 9E), Ghrelin (Fig. 9F), glucagon (FIG. 9G), and insulin (FIG. 9H). Comparison of Figs 8 and 9 reveal that treatment of planar cultures at Stage 5 and Stage 6 with ALK5 inhibitor II resulted in a drop in insulin expression at Stage 6 as compared to Stage 5. However, treatment of cultures at the air-liquid interface with ALK5 inhibitor resulted in enhancement of insulin expression at Stage 6 as compared to Stage 5. The same pattern also applied to NGN3 and NKX6.1 expression in the air-liquid interface cultures as compared to monolayer cultures.

[0175] Figure 10 shows immunostaining results for Stage 6 cells cultured at the air-liquid interface in media treated either with 1 micro molar SD208 inhibitor (Panel 9A) or 1 micro molar ALK5 inhibitor II (Panel 10B) and stained for chromogranin-A (pan-endocrine marker) and NKX6.1 (Pancreatic precursor marker and β cell specific marker). Cultures treated with ALK5 inhibitor II resulted in co-expression of NKX6.1 and chromogranin-A. However, cultures treated with SD208 showed very low co-expression of NKX6.1 and chromogranin-A.

[0176] Culturing pancreatic foregut precursor cells on filter inserts at the air-liquid interface in combination with ALK5 inhibitor II significantly enhanced the number of NKX6.1 positive cells co-expressing insulin or chromogranin-A. Furthermore, the same protocol applied to cells cultured on traditional monolayer cultures failed to show significant numbers of NKX6.1 positive cells co-expressing insulin or chromogranin-A. Lastly, treatment of cells cultured at the

air-liquid interface with ALK5 inhibitors other than ALK5 inhibitor II failed to show a significant number of NKX6.1 positive cells co-expressing insulin or chromogranin-A. These results indicate that a unique combination of culturing at the air-liquid interface with a medium supplemented with ALK5 inhibitor II resulted in co-expression of insulin/chromogranin-A and NKX6.1.

Table III- ALK5 inhibitors tested in Example 4

Compound	Vendor	Catalogue Number
TGF-B inhibitor SB431542	Xcess Biosciences (San Diego, Ca)	M60015-2Ss
SD208	R & D systems (MN)	3269
ITD-1	Xcess Biosciences (San Diego, Ca)	M600060-2S
LY2109761	Xcess Biosciences (San Diego, Ca)	M60035-2S
A83-01	Xcess Biosciences (San Diego, Ca)	M60021-2S
LY2157299	Xcess Biosciences (San Diego, Ca)	M60064-2S
ALK5 inhibitor II	Enzo (Farmingdale, NY)	ALX-270-445

Example 5

Comparison of various ALK5 inhibitors in Stages 5 and 6 for cells cultured at the air-liquid interface

[0177] This example shows that ALK5 inhibitor II was unique in generating a significant population of cells at the air-liquid interface that expressed NKX6.1 and insulin or chromogranin-A. Additional TGF-β inhibitors tested are listed in Table IV.

[0178] Cells of the human embryonic stem cell line H1 (passage 40) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes in a media comprising DMEM-F12 (Invitrogen, Ca), GlutaMax™ (1:100 dilution, Invitrogen), 0.25

mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 μM of Y27632 (Rock inhibitor, Catalog No. Y0503, Sigma-Aldrich). Forty-eight hours post-seeding, cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a. Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant, Catalog No. 68700); 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich, Catalog No. S3187); 1X GlutaMax™ (Invitrogen, Catalog No. 35050-079); 4.5 mM D-glucose (Sigma-Aldrich, Catalog No. G8769); 100 ng/ml GDF8 (R&D Systems); and 1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with: 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-Glucose; 100 ng/ml GDF8; and 0.1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, and 100 ng/ml GDF8.

b. Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

c. Stage 3 (2 days): The Stage 2 cells were then treated with BLAR custom medium (Invitrogen) supplemented with a 1:200 dilution of ITS-X (Invitrogen, CA); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1 (Sigma, MO); 1 μM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days.

d. Stage 4 (2 days): The Stage 3 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; and 100 nM TPB for two days, then at the end of stage 4, cells cultured on planar dishes were treated for 4 hours with 10 μM of Y27632, rinsed with PBS and treated for 5 minutes at room temperature with 1X TrypLE™ (Invitrogen) followed by removal of the enzyme, rinsing with basal media and scraping of cells by a cell scraper. The resulting suspension of cells was seeded at a density of 0.5-0.75 x 10^5 cells (in 10 μl aliquots) onto 0.4 micron porous cell culture filter inserts (BD 353493) in 6-well plates, or onto 10 cm filter inserts (Corning, #3419) in 10 cm dishes. 1.5 ml of media was added to the bottom of each insert in the 6-well plates and 7.5 ml of media was added to the bottom of each 10 cm insert.). No further media was added to the apical side of the filter. Media was replaced every day for the duration of the study.

e. Stage 5 (3 days): The Stage 4 cells were then cultured at the air-liquid interface in BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 μg/ml of heparin (Sigma, #H3149), 0.25 μM SANT-1; 50 nM RA; 100 nM LDN-193189; 1000 nM of various ALK5 inhibitor (*see* Table IV for the list of inhibitors used) for three days.

f. Stage 6 (6 days): The Stage 5 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 μg/ml of heparin (Sigma, #H3149), 0.25 μM SANT-1; 100 nM LDN-193189, 1000 nM T3, 1000 nM ALK5 inhibitor (*see* Table IV for list of inhibitors tested) for six days.

[0179] Figure 23 depicts data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated and cultured at the air-liquid interface as outlined in Example 5 after day 4 of Stage 5, and day 6 of Stage 6: PDX1 (FIG 23A), NKX6.1 (FIG. 23B), NGN3 (FIG. 23C), ABCC8 (FIG. 23D), glucagon (FIG. 23E),

and insulin (FIG. 23F). Similar to the results of Example 4, culturing pancreatic foregut precursor cells on filter inserts at the air-liquid interface and treatment with ALK5 inhibitor II significantly enhanced the expression of insulin and glucagon. Treatment of pancreatic foregut precursor cells with other ALK5 inhibitors did not result in significant expression of either insulin or glucagon.

Table IV- ALK5 (also referred to as TGF-β receptor) inhibitors tested in Example 5

Compound	Vendor	Catalogue Number
TGF-β receptor inh V	EMD	616456
TGF-β receptor inh I	EMD	616451
TGF-β receptor inh IV	EMD	616454
TGF-β receptor inh VII	EMD	616458
TGF-β receptor inh VIII	EMD	616459
TGF-β receptor inh II	EMD	616452
TGF-β receptor inh VI	EMD	616464
TGF-β receptor inh III	EMD	616453

Example 6

Effect of seeding cell density at the air-liquid interface on subsequent differentiation into endocrine cells

[0180] This example identifies a range of seeding densities at the air-liquid interface and the resulting expression of endocrine markers. To conduct the studies in this example, embryonic stem cells were differentiated using the protocol discussed below.

[0181] Cells of the human embryonic stem cell line H1 (passage 40) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes in a media comprising DMEM-F12 (Invitrogen, CA), GlutaMax™ (1:100 dilution, Invitrogen), 0.25 mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 μM of Y27632 (Rock inhibitor, Catalog

No. Y0503, Sigma). Forty-eight hours post-seeding, cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a) Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen, Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant, Catalog No. 68700); 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich, Catalog No. S3187); 1X GlutaMax™ (Invitrogen, Catalog No. 35050-079); 4.5 mM D-glucose (Sigma-Aldrich, Catalog No. G8769); 100 ng/ml GDF8 (R&D Systems); and 1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, 100 ng/ml GDF8, and 0.1 μM MCX compound. Subsequently, the cells were cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-Glucose, and 100 ng/ml GDF8.

b) Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO); and 25 ng/ml FGF7 (R & D Systems, MN).

c) Stage 3 (2 days): The Stage 2 cells were then treated with BLAR custom medium (Invitrogen) supplemented with a 1:200 dilution of ITS-X (Invitrogen, CA); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1 (Sigma, MO); 1 μM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days. Then, at the end of Stage 3, cells cultured on planar dishes were treated for 4 hours with 10 μM of Y27632, rinsed with PBS and treated for 5 minutes at room temperature with 1X TrypLE™ (Invitrogen) followed by removal of the enzyme, rinsing with basal media and scraping of cells by a cell scraper. The resulting suspension of cells were seeded at a density of 0.1, 0.5, 1 and 5 x 10^6 cells (in 10 μl aliquots) onto 0.4 micron

porous cell culture filter inserts (BD 353493) in 6-well plates. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter. Media was replaced every day for the duration of the study.

d) Stage 4 (2 days): The Stage 3 cells were then cultured at the air-liquid interface in BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 0.25 µM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; and 100 nM TPB for two days.

e) Stage 5 (3 days): The Stage 4 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 0.25 µM SANT-1; 50 nM RA; 100 nM LDN-193189; 10000 nM of ALK5 inhibitor II for three days.

f) Stage 6 (14 days): The Stage 5 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 0.25 µM SANT-1; 10000 nM ALK5 inhibitor, 100 nM LDN-193189, and 1000 nM T3 for fourteen days.

[0182] RNA samples were collected at Stages 4, 5, and 6, and analyzed by real-time PCR. Figure 11 depicts data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 6 and cultured at the air-liquid interface: ABCC8 (FIG. 11A); glucagon (FIG. 11B); amylin (FIG. 11C); insulin (FIG. 11D); NGN3 (FIG. 11E); NKX2.2 (Fig. 11F); NKX6.1 (FIG. 11G); and PDX1 (FIG. 11H). Seeding densities in the range of 0.1-1 x 10^6 cells/10 µl resulted in similar expression of pancreatic endoderm and endocrine markers at Stages 5 and 6. At the highest tested seeding density (5 x 10^6 cells/10 µl) at the air-liquid interface, there was a drop in expression of endocrine markers.

Example 7

Comparison of 0.4, 1, and 3 micron pore size filter inserts

[0183] This example compares the effect of filter pore size on subsequent differentiation at the air-liquid interface. To conduct the studies in this example, embryonic stem cells were differentiated using the protocol discussed below.

[0184] Cells of the human embryonic stem cell line H1 (passage 40) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes in a media comprising of DMEM-F12 (Invitrogen, CA), GlutaMax™ (1:100 dilution, Invitrogen), 0.25 mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 µM of Y27632 (Rock inhibitor, Catalog No. Y0503, Sigma). Forty-eight hours post-seeding, cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a) Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen, Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant, Catalog No. 68700); 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich, Catalog No. S3187), 1X GlutaMax™ (Invitrogen Catalog No. 35050-079); 4.5 mM D-glucose (Sigma-Aldrich, Catalog No. G8769), 100 ng/ml GDF8 (R&D Systems); and 1 µM MCX. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, 100 ng/ml GDF8, and 0.1 µM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-Glucose, and 100 ng/ml GDF8.

b) Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

c) Stage 3 (2 days): The Stage 2 cells were then treated with BLAR custom medium (Invitrogen) supplemented with a 1:200 dilution of ITS-X (Invitrogen, CA); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 µM SANT-1 (Sigma, MO); 1 µM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days.

d) Stage 4 (2 days): The Stage 3 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 µM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; and 100 nM TPB for two days. Then, at the end of stage 4, cells cultured on planar dishes were treated for 4 hours with 10 µM of Y27632, rinsed with PBS and treated for 5 minutes at room temperature with 1X TrypLE™ (Invitrogen) followed by removal of the enzyme, rinsing with basal media and scraping of cells by a cell scraper. The resulting suspension of cells were seeded at a density of 0.5-0.75 x 10^6 cells (in 10 µl aliquots) onto 0.4, 1, or 3 micron porous cell culture filter inserts in 6-well plates. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter. Media was replaced every day for the duration of the study.

e) Stage 5 (3 days): The Stage 4 cells were then cultured at the air-liquid interface in BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 0.25 µM SANT-1; 50 nM RA; 100 nM LDN-193189; 10000 nM of ALK5 inhibitor II for three days.

f) Stage 6 (15 days): The Stage 5 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 0.25 µM SANT-1; 100 nM LDN-193189, 1000 nM T3, 10000 nM ALK5 inhibitor II for fifteen days.

[0185] RNA samples were collected at Stage 6 and analyzed by real-time PCR. Figure 12 depicts data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in this Example and cultured at the air-liquid interface: ABCC8 (FIG. 12A); glucagon (FIG. 12B); amylin (FIG. 12C); insulin (FIG. 12D); NGN3 (FIG. 12E); NKX2.2 (Fig. 12F); NKX6.1 (FIG. 12G); and PDX1 (FIG. 12H). Filter inserts pore sizes ranging from 0.4 to 3 micron did not significantly impact expression of pancreatic endoderm or endocrine markers at the air-liquid interface.

Example 8

Comparison of differentiating pancreatic foregut precursor cells at the air-liquid interface to liquid-liquid (L/L) interface on filter inserts

[0186] This example compares the impact of culturing at the air-liquid interface to culturing at the liquid-liquid interface on differentiation of pancreatic foregut precursor cells on filter inserts. To conduct the studies in this example, embryonic stem cells were differentiated using the protocol discussed below.

[0187] Cells of the human embryonic stem cell line H1 (passage 40) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes in a media comprising of DMEM-F12 (Invitrogen, CA), GlutaMax™ (1:100 dilution, Invitrogen), 0.25 mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 μM of Y27632 (Rock inhibitor, Catalog No. Y0503, Sigma-Aldrich). Forty-eight hours post-seeding, cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a) Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant, Catalog No. 68700); 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich Catalog No. S3187); 1X GlutaMax™ (Invitrogen, Catalog No. 35050-079), 4.5 mM D-glucose (Sigma-Aldrich, Catalog No. G8769); 100 ng/ml GDF8 (R&D Systems); and 1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with

2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, 100 ng/ml GDF8, and 0.1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, and 100 ng/ml GDF8.

b) Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

c) Stage 3 (2 days): The Stage 2 cells were then treated with BLAR custom medium (Invitrogen) supplemented with a 1:200 dilution of ITS-X (Invitrogen, CA); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1 (Sigma, MO); 1 μM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days.

d) Stage 4 (2 days): The Stage 3 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; and 100 nM TPB for two days, then at the end of Stage 4, cells cultured on planar dishes were treated for 4 hours with 10 μM of Y27632, rinsed with PBS and treated for 5 minutes at room temperature with 1X TrypLE™ (Invitrogen) followed by removal of the enzyme, rinsing with basal media and scraping of cells by a cell scraper. The resulting suspension of cells were seeded at a density of 0.5-0.75 x 10^6 cells (in 10 μl aliquots) on MATRIGEL™-coated 0.4 micron porous cell culture filter inserts in 6-well plates. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter. For L/L condition, media was also added on top of the filter inserts resulting in liquid-liquid interface. Media was replaced every day for the duration of the study.

e) Stage 5 (3 days): The Stage 4 cells were then cultured at the air-liquid interface in BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 0.25 µM SANT-1; 50 nM RA; 100 nM LDN-193189; 10000 nM of various ALK5 inhibitor II for three days.

f) Stage 6 (10 days): The Stage 5 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 0.25 µM SANT-1; 100 nM LDN-193189, 1000 nM T3, 10000 nM ALK5 inhibitor II for ten days.

[0188] RNA samples were collected at Stages 5 and 6 and analyzed by real-time PCR. Figure 13 depicts data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 8 and cultured at the air-liquid interface: ABCC8 (FIG. 13A); glucagon (FIG. 13B); amylin (FIG. 13C); insulin (FIG. 13D); NGN3 (FIG. 13E); NKX2.2 (Fig. 13F); NKX6.1 (FIG. 13G); and PDX1 (FIG. 13H). The most dramatic difference was seen with significant up-regulation (7X) of glucagon in L/L condition as compared to the air-liquid interface.

Example 9

Pancreatic endoderm/endocrine precursor cells cultured at the air-liquid interface can be used to screen a library of compounds

[0189] This example examines the use of air-liquid interface cultures to screen libraries of compounds. To do so embryonic stem cells were differentiated using the protocol discussed below.

[0190] Cells of the human embryonic stem cell line H1 (passage 40) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes in a media comprising of DMEM-F12 (Invitrogen, Ca), GlutaMax™ (1:100 dilution, Invitrogen), 0.25 mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 µM of Y27632 (Rock inhibitor, Catalog

No. Y0503, Sigma). Forty-eight hours post-seeding, cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a) Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant, Catalog No. 68700), 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich, Catalog No. S3187), 1X GlutaMax™ (Invitrogen, Catalog No. 35050-079), 4.5 mM D-glucose (Sigma-Aldrich, Catalog No. G8769), 100 ng/ml GDF8 (R&D Systems) and 1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, 100 ng/ml GDF8, and 0.1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, and 100 ng/ml GDF8.

b) Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

c) Stage 3 (2 days): The Stage 2 cells were then treated with BLAR custom medium (Invitrogen) supplemented with a 1:200 dilution of ITS-X (Invitrogen, CA); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1 (Sigma, MO); 1 μM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days.

d) Stage 4 (2 days): The Stage 3 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; and 200 nM TPB for two days, then at the end

of Stage 4, cells cultured on planar dishes were treated for 4 hours with 10 µM of Y27632, rinsed with PBS and treated for 5 minutes at room temperature with 1X TrypLE™ (Invitrogen) followed by removal of the enzyme, rinsing with basal media and scraping of cells by a cell scraper. The resulting suspension of cells were seeded at a density of 0.5-0.75 x 10^6 cells (in 10 µl aliquots) on MATRIGEL™-coated 0.4 micron porous cell culture filter inserts in 6-well plates. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter.

e) Stage 5 (3 days): The Stage 4 cells were then cultured at the air-liquid interface in BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 10 µM $ZnSO_4$ (Sigma, Z0251), 0.25 µM SANT-1; 50 nM RA; 100 nM LDN-193189; 10000 nM of ALK5 inhibitor II for three days.

f) Stage 6 (12 days): The Stage 5 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 10 µM $ZnSO_4$ (Sigma, Z0251), 0.25 µM SANT-1; 100 nM LDN-193189; 1000 nM T3; 10000 nM ALK5 inhibitor II for twelve days. At this stage, compounds listed in Table V were screened to identify potential compounds that affect endoderm and endocrine markers.

Table V. List of compounds tested at Stage 6

Catalogue#/Vendor	Inhibitor	Target Kinase	Tested concentration
328007-EMD	ERK Inhibitor II	ERK1, ERK2	2 µM
420119-EMD	JNK Inhibitor II	JNK	2 µM
420136-EMD	JNK Inhibitor IX	JNK2, JNK3	2 µM
444939-EMD	MEK1/2 Inhibitor	MEK1/2	2 µM
454861-EMD	MNK1 Inhibitor	MNK1	2 µM
475863-EMD	MK2a Inhibitor	MK2a	2 µM

Table V. List of compounds tested at Stage 6			
Catalogue#/Vendor	Inhibitor	Target Kinase	Tested concentration
506156-EMD	p38 MAP Kinase Inhibitor V	p38, CK1	2 μM
513000-EMD	PD 98059	MEK	2 μM
553014-EMD	Raf Kinase Inhibitor IV	B-Raf	2 μM
559389-EMD	SB 203580	p38 MAPK	2 μM
616373-EMD	Tpl2 Kinase Inhibitor	Tpl2 Kinase	2 μM
692000-EMD	ZM 336372	c-Raf 1	2 μM
M60043-25/XcessBio	IDH1	Isocitrate dehydrogenase	2 μM
M60668-25/XcessBio	AGI5198	Isocitrate dehydrogenase	2 μM

[0191] RNA samples were collected at Stage 6 and analyzed by real-time PCR. Figure 14 depicts data from real-time PCR analyses of the expression of the following genes in cells of the human embryonic stem cell line H1 differentiated as outlined in Example 9 and cultured at the air-liquid interface: ABCC8 (FIG. 14A); glucagon (FIG. 14B); amylin (FIG. 14C); insulin (FIG. 14D); ISL-1 (FIG. 14E); MNX1 (FIG. 14F); NKX6.1 (FIG. 14G); and SLC30A8 (FIG. 14H). This example shows that the potential of cells cultured at the air-liquid interface as a screening tool.

Example 10

FACS profile of Stage 5 and Stage 6 cells cultured at the air-liquid interface

[0192] This example studies the composition of Stage 5 and Stage 6 cultures at the air-liquid interface. To conduct the studies in this example, embryonic stem cells were differentiated into Stage 5 and Stage 6 cultures using the protocol described below.

[0193] Cells of the human embryonic stem cell line H1 (passage 40) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes in a media comprising of DMEM-F12 (Invitrogen, Ca), GlutaMax™ (1:100 dilution, Invitrogen),

0.25 mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 μM of Y27632 (Rock inhibitor, Catalog No. Y0503, Sigma). Forty-eight hours post-seeding, cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a) Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen, Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant, Catalog No. 68700), 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich Catalog No. S3187); 1X GlutaMax™ (Invitrogen, Catalog No. 35050-079); 4.5 mM D-glucose (Sigma-Aldrich, Catalog No. G8769); 100 ng/ml GDF8 (R&D Systems); and 1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, 100 ng/ml GDF8, and 0.1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, and 100 ng/ml GDF8.

b) Stage 2 (2 days): the Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

c) Stage 3 (2 days): The Stage 2 cells were then treated with BLAR custom medium (Invitrogen) supplemented with a 1:200 dilution of ITS-X (Invitrogen, Ca); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1 (Sigma, MO); 1 μM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days.

d) Stage 4 (3 days): The Stage 3 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; and 200 nM TPB for three days, then at the end of Stage 4, cells cultured on planar dishes were treated for 4 hours with 10 μM of Y27632, rinsed with PBS and treated for 5 minutes at room temperature with 1X TrypLE™ (Invitrogen) followed by removal of the enzyme, rinsing with basal media and scraping of cells by a cell scraper. The resulting suspension of cells were seeded at a density of 0.5-0.75 x 10^6 cells (in 10 μl aliquots) on MATRIGEL™-coated 0.4 micron porous cell culture filter inserts in 6-well plates. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter.

e) Stage 5 (3 days): The Stage 4 cells were then cultured at the air-liquid interface in BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 μg/ml of heparin (Sigma, #H3149), 10 μM $ZnSO_4$ (Sigma, Z0251), 0.25 μM SANT-1; 50 nM RA; 100 nM LDN-193189; 10000 nM of various ALK5 inhibitor II for three days.

f) Stage 6 (15 days): The Stage 5 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM Glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 μg/ml of heparin (Sigma, #H3149), 10 μM $ZnSO_4$ (Sigma, Z0251), 0.25 μM SANT-1; 100 nM LDN-193189, 1000 nM T3, 10000 nM ALK5 inhibitor II for 5-15 days.

[0194] Cells were harvested at Stage 5 and various time points at Stage 6 and analyzed by FACS. FACS staining was conducted as previously described (*Diabetes*, 61, 2016, 2012) and using antibodies listed in Table VI. Figure 15 depicts FACS profile of cells collected at Stage 5. Figure 16 shows FACS profile of Stage 6 day 5 cells cultured at the air-liquid interface. Lastly, Figure 17 shows profile of Stage 6 day 15 of cells cultured at the air-liquid interface. As shown in Figure 15, at Stage 5, there were few cells co-expressing insulin and NKX6.1 (~1%) and a significant portion of PDX1 positive cells were in active cell cycle as measured by co-expression of PDX1 and KI-67 (~23%; KI-67 is indicative of cells that are in active cell cycle). However,

by Stage 6 day 5 (Figure 16), there was a significant drop in proliferating PDX1+ cells (8%) while there was a significant increase in the number of NKX6.1+ cells co-expressing chromogranin-A (51%; chromogranin-A is a pan endocrine marker) or insulin (14%). Moreover, there was a significant rise in cells expressing endocrine precursor markers ISL-1, NeuroD, and NKX2.2. This indicates that unique cultures of Stage 6 allowed for rapid maturation of cells away from a proliferating progenitor fate to early maturing endocrine cells. In addition, an increase in the percentage of cells co-expressing insulin and NXK6.1 (33%) was observed by prolonging Stage 6 to 15 days (Figure 17). Moreover, there was further decrease in the percentage of PDX1 positive cells which were in cell cycle (1%) and a further increase in the percentage of ISL-1 and NeuroD. Lastly, the majority of hormone positive cells were single hormone insulin positive cells (34% single hormone insulin positive cells, 7% single hormone glucagon positive cells, and 8% poly hormone cells). Significant co-expression of NKX6.1 and chromogranin-A and single hormone insulin positive cells expressing NKX6.1 (>30%) highlights a previously undescribed cell population.

Table VI. List of Antibodies used for FACS analysis of cells generated in Example 10

Antigen	Species	Source/Catalogue#	Dilution
Glucagon	Mouse	Sigma-Aldrich, G2654	1:250
Insulin	Rabbit	Cell Signaling, 3014B	1:10
NKX6.1	Mouse	Developmental Studies Hybridoma Bank; F55A12	1:50
NKX2.2	Mouse	Developmental Studies Hybridoma Bank;	1:100
PDX1	Mouse	BD BioSciences, 562161	1:50
Ki67	Mouse	BD Biosciences, 558595	1:20
Pax6	Mouse	BD Biosciences, 561552	1:20
Chromogranin-A	Rabbit	Dako, A0430	1:40
ISL-1	Mouse	BD Biosciences, 562547	1:20
NeuroD	Mouse	BD Bioscience, 563001	1:40
FOXA2	Mouse	BD Bioscience, 561589	1:80

Example 11

In vivo maturation of NKX6.1+ Chromogranin-A+ insulin+ cells, NKX6.1 + Chromogranin-A- insulin- and pancreatic progenitors co-expressing PDX1 and NKX6.1 versus human islets in SCID mice

[0195] This example highlights the *in vitro* composition of differentiated cells and the affect on *in vivo* cell performance. In particular, 5 million Stage 4 day 4 (PDX1+ NKX6.1+) pancreatic foregut precursor cells prepared according to Example 1 on planar cultures, 5 million NKX6.1+ chromogranin-A negative cells cultured at the air-liquid interface, and 3 million NKX6.1+ chromogranin-A positive cells prepared according to Example 10 at the air-liquid interface were transplanted into the kidney capsule of non-diabetic SCID mice as described in (Diabetes 2012, 61(8):2016-29) . The mice were tracked for circulating human C-peptide as a measure of the

maturation state of cells as a function of time. In addition, in separate cohorts of mice, 1500-4000 cadaveric human islet (PRODO labs, Irvine, CA) equivalents were transplanted as a positive control.

[0196] The NKX6.1+ chromogranin-A negative population was prepared as follows:

[0197] Cells of the human embryonic stem cell line H1 (passage 40) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes in a media comprising of DMEM-F12 (Invitrogen, Ca), GlutaMax™ (1:100 dilution, Invitrogen), 0.25 mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 μM of Y27632 (Rock inhibitor, Catalog No. Y0503, Sigma). Forty-eight hours post-seeding, cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a. Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen, Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant, Catalog No. 68700); 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich, Catalog No. S3187); 1X GlutaMax™ (Invitrogen, Catalog No. 35050-079); 4.5 mM D-glucose (Sigma-Aldrich Catalog No. G8769); 100 ng/ml GDF8 (R&D Systems); and 1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, 100 ng/ml GDF8, and 0.1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, and 100 ng/ml GDF8.

b. Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

c. Stage 3 (2 days): The Stage 2 cells were then treated with MCDB-131 medium supplemented with a 1:200 dilution of ITS-X (Invitrogen, CA); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1 (Sigma, MO); 1 μM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days. Then the Stage 3 cells were treated with 1X ACCUTASE™ for 1-3 minutes at room temperature followed by removal of the enzyme and scraping of cells by a cell scraper. The resulting suspension of cells were seeded at a density of ~2 x 10^6 cells/10 μl on 0.4 micron porous cell culture filter inserts. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter.

d. Stage 4 (2 days): The Stage 3 cells were then cultured at the air-liquid interface in MCDB-131 medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; 100 nM T3 (T6397, Sigma) and 100 nM TPB for two days.

e. Stage 5 (2 days): The Stage 4 cells were then treated with MCDB-131 medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 50 nM RA; 50 nM LDN-193189; 500 nM ALK5 inhibitor (SD208) for two days.

f. Stage 6 (6 days): The Stage 5 cells were then treated with MCDB-131 medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 50 nM RA; 500 nM ALK5 inhibitor; for six days

[0198] Table VII below highlights the expression level of various pancreatic endoderm and endocrine markers for the three human embryonic stem cell derived populations. Specifically,

Table VII compares the results of the following: (1) Stage 4 day 4 population generated according to Example 1 (Stage 4 day 4); (2) Pancreatic endoderm/endocrine precursor population generated according to Example 11 (Pancreatic endoderm/endocrine); and (3) NKX6.1+ chromogranin-A+ insulin+ population generated according to Example 10 (NKX6.1+ chromogranin-A+ insulin+). FACS profile information for each is depicted in FIGS. 17-19. Specifically, FIG. 17 depicts the FACS profile of the NKX6.1 + chromogranin-A + population; FIG. 18 depicts the FACS profile of the Stage 4 day 4 cells; and FIG. 19 depicts the FACS profile of Stage 6 day 6 pancreatic endocrine cells generated according to Example 11.

Table VII- Comparison of expression profile of three transplanted population as measured by FACS

	Population		
Marker	S4 day 4[a]	Pancreatic endoderm / endocrine[b]	NKX6.1+ chromogranin-A+ insulin+[c]
% PDX1+ Ki-67+	25	17	1
% NKX6.1+ Chromogranin-A+	10	10	57
% NKX6.1- Chromograin+	19	53	31
% Insulin+ NKX6.1+	1	1	33

[a] S4 day 4 population was generated according to Example 1.(See Figure 18)

[b] Pancreatic endoderm/endocrine population was generated according to Example 11. (See Figure 19)

[c] NKX6.1+ chromogranin-A+ insulin+ population was generated according to Example 10. (See Figure 17)

[0199] After implementation, the mice were periodically tested for the concentration of circulating human c-peptide. Circulating human C-peptide was tested by collecting blood via saphenous vein. Plasma was stored at -20°C and later assayed using a human C-peptide by ELISA kit (Alpco Diagnostics, Salem, NH). The results are shown graphically in FIG 20.

[01100] Figure 20 shows the kinetics of C-peptide production from the three ES-derived populations as compared to various doses of human islets. The population of cells expressing substantial co-expression of NKX6.1 and chromogranin-A and NKX6.1 and insulin resulted in significant early production of C-peptide. The level of C-peptide production was similar to transplanting approximately 4000 human islets at 12 weeks. However, by 16 weeks post-

transplant, the levels of human C-peptide had almost doubled the magnitude of C-peptide seen with transplanting 4000 human islets.

[01101] However, transplanting progenitor cells expressing PDX1 and NKX6.1, or a mixed population of pancreatic precursor cells and polyhormonal cells (chromogranin-A+ NKX6.1-) required significantly longer periods of time to secrete equivalent levels of C-peptide as 4000 human islets.

Example 12

Addition of gamma secretase inhibitor XX further augments maturation markers of Stage 6 cells cultured at the air-liquid interface

[01102] This example highlights that NOTCH inhibitors, such as gamma secretase inhibitors, further enhance maturation markers of β cells while retaining expression of NKX6.1. Cells of the human embryonic stem cell line H1 (passage 42) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes in a media comprising of DMEM-F12 (Invitrogen, Ca), GlutaMax™ (1:100 dilution, Invitrogen), 0.25 mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 μM of Y27632 (Rock inhibitor, Catalog No. Y0503, Sigma). Forty-eight hours post-seeding, cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a) Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant Catalog No. 68700), 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich Catalog No. S3187); 1X GlutaMax™ (Invitrogen Catalog No. 35050-079); 4.5 mM D-glucose (Sigma-Aldrich Catalog No. G8769); 100 ng/ml GDF8 (R&D Systems); and 1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, 100 ng/ml GDF8, and 0.1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA,

0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, and 100 ng/ml GDF8.

b) Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

c) Stage 3 (2 days): The Stage 2 cells were then treated with BLAR custom medium (Invitrogen) supplemented with a 1:200 dilution of ITS-X (Invitrogen, Ca); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 µM SANT-1 (Sigma, MO); 1 µM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days.

d) Stage 4 (3 days): The Stage 3 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 µM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; and 200 nM TPB for three days, then at end of Stage 4, cells cultured on planar dishes were treated for 4 hours with 10 µM of Y27632, rinsed with PBS and treated for 5 minutes at room temperature with 1X TrypLE™ (Invitrogen) followed by removal of the enzyme, rinsing with basal media and scraping of cells by a cell scraper. The resulting suspension of cells were seeded at a density of 0.5-0.75 x 10^6 cells (in 10 µl aliquots) on MATRIGEL™-coated 0.4 micron porous cell culture filter inserts in 6-well plates. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter.

e) Stage 5 (3 days): The Stage 4 cells were then cultured at the air-liquid interface in BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 10 µM $ZnSO_4$ (Sigma, Z0251), 0.25 µM SANT-1; 50 nM RA; 100 nM LDN-193189; 10000 nM of various ALK5 inhibitor II for three days.

f) Stage 6 (14 days): The Stage 5 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM Glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 10 µM $ZnSO_4$ (Sigma, Z0251), 0.25 µM SANT-1; 100 nM LDN-193189, 1000 nM T3, 10000 nM ALK5 inhibitor II for 14 days.

[01103] At Stage 6, various doses (100 nM to 5000 nM) of gamma Secretase inhibitor XX (EMD, #565789) were tested. mRNA was collected at Stage 6 day 4 and Stage 6 day 8. FIG. 21 depicts the PCR data for key β cell maturation markers along with pancreatic progenitor markers. As shown in FIG. 21, maturation markers, such as Amylin (Panel 21A), insulin (Panel 21B), and MAFA (Panel 21C) were significantly upregulated while NKX6.1 (Panel 21D) expression was not significantly affected . However, pancreatic precursor markers, such as PTF1a (Panel 21 E) and SOX9 (Panel 21 F) were significantly down regulated.

Example 13

Presence of ALK5 inhibitor is essential for upregulation of MAFA and further addition of T3 further enhances MAFA expression

[01104] This example highlights the ability of ALK5 inhibitor II addition to upregulate MAFA expression, and that addition of T3 with ALK5 inhibitor and LDN-193189 further enhances expression of MAFA.

[01105] Cells of the human embryonic stem cell line H1 (passage 42) were seeded as single cells at 1 x 10^5 cells/cm^2 on MATRIGEL™ (1:30 dilution; BD Biosciences, NJ)-coated dishes in a media comprising of DMEM-F12 (Invitrogen, Ca), GlutaMax™ (1:100 dilution, Invitrogen), 0.25 mM ascorbic acid (Sigma, MO), 100 ng/ml of FGF2 (R & D systems, MN), 1 ng/ml of TGF-β (R & D systems), ITS-X (1:100 dilution), 2% fatty-acid free BSA (Lampire, PA), and 20 ng/ml of IGF-1 (R & D systems), supplemented with 10 µM of Y27632 (Rock inhibitor, Catalog No. Y0503, Sigma). Forty-eight hours post-seeding, cultures were washed in incomplete PBS (phosphate buffered saline without Mg or Ca). The cells were then differentiated according to the following protocol:

a) Stage 1 (3 days): Cells were cultured for one day in MCDB-131 medium (Invitrogen Catalog No.10372-019) supplemented with 2% fatty acid-free BSA (Proliant Catalog No. 68700), 0.0012 g/ml sodium bicarbonate (Sigma-Aldrich Catalog No. S3187); 1X GlutaMax™ (Invitrogen Catalog No. 35050-079); 4.5 mM D-glucose (Sigma-Aldrich Catalog No. G8769); 100 ng/ml GDF8 (R&D Systems); and 1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, 100 ng/ml GDF8, and 0.1 μM MCX compound. Cells were then cultured for an additional day in MCDB-131 medium supplemented with 2% fatty acid-free BSA, 0.0012 g/ml sodium bicarbonate, 1X GlutaMax™, 4.5 mM D-glucose, and 100 ng/ml GDF8.

b) Stage 2 (2 days): The Stage 1 cells were then treated for two days with MCDB-131 medium supplemented with 2% fatty acid-free BSA; 0.0012 g/ml sodium bicarbonate; 1X GlutaMax™; 4.5 mM D-glucose; 0.25 mM ascorbic acid (Sigma, MO) and 25 ng/ml FGF7 (R & D Systems, MN).

c) Stage 3 (2 days): The Stage 2 cells were then treated with BLAR custom medium (Invitrogen) supplemented with a 1:200 dilution of ITS-X (Invitrogen, Ca); 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1 (Sigma, MO); 1 μM RA (Sigma, MO); 25 ng/ml FGF7; 0.25 mM ascorbic acid; 200 nM TPB (PKC activator; Catalog No. 565740; EMD Chemicals, Gibbstown, NJ); and 100 nM LDN-193189 (BMP receptor inhibitor; Catalog No. 04-0019; Stemgent) for two days.

d) Stage 4 (3 days): The Stage 3 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 4.5 mM glucose; 1X GlutaMax™; 0.0017 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 0.25 μM SANT-1; 100 nM RA; 2 ng/ml FGF7; 100 nM LDN-193189; 0.25 mM ascorbic acid; and 200 nM TPB for three days, then at the end of Stage 4, cells cultured on planar dishes were treated for 4 hours with 10 μM of Y27632, rinsed with PBS and treated for 5 minutes at room temperature with 1X TrypLE™ (Invitrogen) followed by

removal of the enzyme, rinsing with basal media and scraping of cells by a cell scraper. The resulting suspension of cells were seeded at a density of 0.5-0.75 x 10^6 cells (in 10 µl aliquots) on MATRIGEL™-coated 0.4 micron porous cell culture filter inserts in 6-well plates. 1.5 ml of media was added to the bottom of each insert and no further media was added to the apical side of the filter.

e) Stage 5 (3 days): The Stage 4 cells were then cultured at the air-liquid interface in BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 10 µM $ZnSO_4$ (Sigma, Z0251), 0.25 µM SANT-1; 50 nM RA; 100 nM LDN-193189; 10000 nM of ALK5 inhibitor II for three days.

f) Stage 6 (8 days): The Stage 5 cells were then treated with BLAR medium supplemented with a 1:200 dilution of ITS-X; 20 mM Glucose; 1X GlutaMax™; 0.0015 g/ml sodium bicarbonate; 2% fatty acid-free BSA; 10 µg/ml of heparin (Sigma, #H3149), 10 µM $ZnSO_4$ (Sigma, Z0251), 0.25 µM SANT-1; 100 nM LDN-193189, 1000 nM T3, 10000 nM ALK5 inhibitor II for 8 days.

[01106] At Stage 6, ALK5 inhibitor, T3, or LDN were removed in various combinations to test for the impact of each factor on expression of NKX6.1, insulin, and MAFA. mRNA was collected at Stage 6 day 5 and Stage 6 day 8. FIG. 22 depicts the PCR data for key β cell maturation markers along with pancreatic progenitor markers. As shown in FIG. 22, removal of ALK5 inhibitor at Stage 6 resulted in a dramatic drop in expression of MAFA. Whereas the combination of ALK5 inhibitor, LDN-183189 (BMP receptor inhibitor) and T3 significantly enhanced expression of MAFA (FIG. 22A), insulin (FIG. 22B), Amylin (FIG. 22C), and moderately improved expression of NKX6.1 (FIG. 22D).

Example 14

Additional protocol for culturing Stage 6 cells at the Air-Liquid Interface

[01107] This example discloses additional materials and methods for culturing Stage 6 cells at the air-liquid interface.

[01108] Materials used include the following: 10 cm filter inserts from Corning (catalog number 3419, 0.4 micron polycarbonate membrane); MCDB-131 medium (Invitrogen, Catalog No.10372-019) or BLAR custom medium (manufactured by Invitrogen); ITS-X (Invitrogen, Ca); thyroid hormone (T3): Sigma ALK5 inhibitor II- ENZO (Catalog number- ALX-27-445); LDN-193189- StemGent (#04-0074); heparin (Sigma, H3149); and BSA- Fatty acid-free (Proliant/Lampire, 7500804).

[01109] Preparation of Stage 6 basal media: Add 1.5 grams/liter of sodium bicarbonate to MCDB 131 media, plus 2% BSA, plus 1:200X ITS-X, plus additional 15 mM glucose.

[01110] Preparation of Stage 6 differentiation media: To the Stage 6 basal media, add 10 microMolar ALK5 inhibitor II, 100 nM LDN-193189, and 1 microMolar T3.

Methods

[01111] Add 7.5 ml of Stage 6 differentiation media to the bottom of a 10 cm filter insert. Add clusters of cells in small volumes (20-30 µl) to the top of the filter inserts. Typically, approximately 50 cell clusters are placed per 10 cm of insert. At seeding, each cell cluster contains approximately 0.5 M cells.

[01112] The media is preferably changed every day. Cells can be removed from the filter insert by removing cell aggregates individually, such as by using a wide mouth pipette tip, or all of the aggregates can be removed at once by rinsing the top of the filter with basal media. The cell aggregates are loosely attached to the inserts.

[01113] In addition to the specific culture conditions described in the foregoing Examples, other suitable culture conditions for differentiating pluripotent cells, or their progeny, into pancreatic endocring cells are set forth in Tables VIII to XIII. As used in these tables, "ALK5 inh." is ALK5 inhibitor, "RA" is retinoic acid, "Vit. C" is ascorbic acid, "inh." is inhibitor, "act." is activator, $ZnSO_4$ is Zinc Sulfate, "MCX" is MCX compound, and "AA" is activin A. In certain embodiments, any one of the treatments at one stage (*e.g.* Stage 4) may be combined with any one of the treatments at another stage (*e.g.*, Stage 5).

Table VIII: Protocol for differentiating cells including culturing at the air-liquid interface

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5-Filter	Stage 6-Filter
Basal media	MCDB131 Intermediate Bicarbonate (Total: 2.35 g/l)	MCDB131 Intermediate Bicarbonate (Total: 2.35 g/l)	BLAR High Bicarbonate (Total: 2.93 g/l)	BLAR High Bicarbonate (Total: 2.93 g/l)	BLAR Intermediate Bicarbonate (Total: 2.35 g/l)	BLAR Intermediate Bicarbonate (Total: 2.35 g/l)
Supplement	0.5% FAF-BSA, 10 mM glucose	0.5% FAF-BSA, 10 mM glucose	2% FAF-BSA, 1:200 ITS-X, 10 mM glucose	2% FAF-BSA, 1:200 ITS-X, 10 mM glucose	2% FAF-BSA, 1:200 ITS-X, 20 mM glucose	2% FAF-BSA, 1:200 ITS-X, 20 mM glucose
Growth factors	100 ng/ml GDF8	25 ng/ml FGF7	25 ng/ml FGF7	2 ng/ml FGF7	10 µg/ml heparin	10 µg/ml heparin
Small molecule agonist/ antagonist	1.0 µM MCX Day 1 100 nM MCX at Day 2	0.25 mM Vit C	1 µM RA 0.25 µM SANT 200 nM TPB 100 nM LDN-193189 0.25 mM Vit C	100 nM LDN 0.25 µM SANT 100 nM TPB 100 nM RA 0.25 mM Vit C	0.25 µM SANT 50 nM RA 10000 nM ALK5 inh 100 nM LDN-193189 10 mM $ZnSO_4$	10000 nM ALK5 inh 1 µM T3 100 nM LDN-193189 10 mM $ZnSO_4$
Duration (days)	Approximately 2 to 5 days, preferably about 3 days	Approximately 2 to 3 days, preferably about 2 days	Approximately 2 to 4 days, preferably about 2 days	Approximately 2 to 4 days, preferably about 3 days	Approximately 2 to 4 days, preferably about 3 days	Approximately 3-15 days
Type of Culture	Planar	Planar	Planar	Planar	Air-liquid interface	Air-liquid interface

Table IX: Reagents used in differentiation protocol described in Table VII			
Reagent	Concentration	Vendor	Catalogue #
MCDB131 BLAR (Custom media)	(Add 1:100 X Glutamax) Intermediate bicarbonate: Add 1.5 g of sodium bicarbonate/1000 ml media. High-Bicarbonate: Add 2.5 g of bicarbonate/1000 ml of media	Invitrogen	10372019
FAF-BSA	0.5% at S1-S2 2% at S3-S6	Proliant	68700
ITS-X	1:200 dilution	Invitrogen	51500056
Glucose	10 mM for S1-S4 20 mM at S5-S6	Sigma	G8769
GDF8	100 ng/ml	Peprotech	120-00
MCX	1.0 µM for day 1 and 100 nM for day 2		
FGF7	25 ng/ml at S2, 25 ng/ml at S3, 2 ng/ml at S4	R & D Systems	251-KG
RA	1 µM at S3 100 nM at S4 50 nM at S5	Sigma	R2625
SANT-1 (Shh inhibitor)	0.25 µM	Sigma	S4572
LDN-193189 (BMP r antagonist)	100 nM at S3-S6	Stemgent	04-0019
TPB (PKC activator)	200 nM At S3, 100 nM at S4	ChemPartner	Custom
Ascorbic acid (Vit C)	0.25 mM at S2-S4	Sigma	A4544
ALK5 inh II	10000 nM at S5-S6	ENZO	ALX-270-

			445
T3	1 μM at S6	Sigma	T6397
Heparin	10 μg/ml at S5-S6	Sigma	H3149
ZnSulfate	10 μM at S5-S6	Sigma	Z0251
Filter inserts for 6-well plates	0.4 micron filters from BD or Millipore	BD Millipore	353493 PIHT15R48

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Table X: Exemplary culture conditions suitable for use in the methods of the invention

	Stage 4	Stage 5	Stage 6
Treatment of	Stage 3 cells	Stage 4 cells	Stage 5
with at least	ALK5 inh.; Noggin		
	RA; FGF7; Vit. C.		
	T3		
		Alk 5 inh.	
		T3 & ALK5 inh. (*e.g.* ALK5 inh. II)	
		T3 & ALK5 inh. (*e.g.* ALK5 inh. II); RA; LDN-193189	
		ALK5 inh. (*e.g.* ALK5 inh. II); RA; LDN-193189	
			T3 & ALK5 inh. (*e.g.* ALK5 inh. II)
			T3; ALK5 inh. (*e.g.* ALK5 inh. II) & LDN-193189
			T3; ALK5 inh. (*e.g.* ALK5 inh. II); LDN-193189 & heparin
Other optional components[a] (at least one of)	PKC act. (*e.g.* TPB); SANT-1; ROCK inh. (*e.g.* Y27632)	RA; Vit. C.; SANT-1; $ZnSO_4$; BMP inh. (*e.g.* LDN-193189); heparin	RA; Vit. C.; SANT-1; $ZnSO_4$; BMP inh. (*e.g.* LDN-193189); heparin
Culture at	Planar (the air-liquid interface optional for late Stage 4)	the air-liquid interface	the air-liquid interface
Duration of Treatment	Approximately 2 to 4 days; preferably 2 to 3 days	Approximately 2 to 4 days; preferably 2 to 3 days	Approximately 3 to 15 days
[a] excluded from list if mentioned in "with at least" category.			

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[0200] Exemplary ranges of the components recited in Table X as used in the methods of the invention are shown below:

Table XI: Exemplary amounts of culture components suitable for use in the methods of the invention

Component	Exemplary Suitable Amount	Alternatively
T3	about 0-1500 nM	about 10 nM about 1000 nM
ALK5 inhibitor	about to 75 nM to about 15000 nM	about 100 nM about 200 nM about 1000 nM about 2000 nM about 10000nM
SANT-1	from about 0.1 μM to about 0.3 μM	about 0.25 μM
Retinoic Acid	from about 25 nM to about 150 nM	about 50 nM about 100 nM
Ascorbic Acid	from about 0.1 to about 0.4 mM	about 0.25 mM
FGF7	from about 2 to about 35 ng/ml	about 2 ng/ml about 25 ng/ml
BMP Receptor Inhibitor (*e.g.* LDN-193189)	from about 50 to about 150 mM	about 100 mM
PKC activator (*e.g.* TPB)	From about 50 to about 150 mM	about 200 mM
Noggin	from about 50 ng/ml to about 150 ng/ml	about 100 ng/ml
Heparin	from about 5 μg/ml to about 15 μg/ml	about 10 μg/ml
ROCK inhibitor (*e.g.* Y27632)	from about 5 μM to about 15 μM	about 10 μM
Zn Sulfate	from about 5 μM to about 15 μM	about 10 μM

[0201] Table XI shown below, illustrates alternate exemplary culture conditions suitable for use in embodiment of methods of the invention.

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Table XII: Exemplary culture conditions suitable for use in embodiments of the methods of the invention

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
Treatment of	Pluripotent stem cells	Stage 1 Cells	Stage 2 Cells	Stage 3 cells	Stage 4 cells	Stage 5
With at least	GDF8 & MCX					
	AA & Wnt3A					
		FGF7 & Vit C				
			FGF7; RA; SANT; TPB; LDN & Vit C			
				ALK5 inh., Noggin		
				RA, FGF7, Vit. C.		
				T3		
				FGF7; LDN-193189; SANT; TPB; RA & Vit C		
					Alk 5 inh.	
					T3 & ALK5 inh.	
					T3 & ALK5 inh., RA	
					ALK5 inh., RA, LDN	
					heparin; SANT; RA; ALK5 inh; LDN; $ZnSO_4$	
						T3 & ALK5 inh.
						T3, ALK5 inh. & LDN
						T3, ALK5 inh., LDN & heparin
						heparin; ALK5 inh.; T3; LDN; $ZnSO_4$
Other optional components [a] (at least one of)				PKC act. (*e.g.* TPB); SANT-1; ROCK inh. (*e.g.* Y27632)	RA; Vit. C.; SANT-1; $ZnSO_4$; BMP inh. (*e.g.* LDN); heparin	RA; Vit. C.; SANT-1; $ZnSO_4$; BMP inh. (*e.g.* LDN); heparin
Duration (days)	Approximately 2 to 5 days, preferably about 3 days	Approximately 2 to 3 days, preferably about 2 days	Approximately 2 to 4 days, preferably about 2 days	Approximately 2 to 4 days, preferably about 3 days	Approximately 2 to 4 days, preferably about 3 days	Approximately 3 to 15 days, preferably about 7 to 15 days
Type of Culture	Planar	Planar	Planar	Planar (optional air-	Air Liquid Interface	Air Liquid Interface (Filter)

Table XII: Exemplary culture conditions suitable for use in embodiments of the methods of the invention						
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
				liquid interface late Stage 4)	(Filter)	
[a] excluded from list if mentioned in "with at least category"						

[0202] Table XII shown below, illustrates alternate exemplary culture conditions suitable for use in embodiment of methods of the invention.

Table XIII: Exemplary culture conditions suitable for use in embodiments of the methods of the invention						
	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
Treatment of	Pluripotent stem cells	Stage 1 Cells	Stage 2 Cells	Stage 3 cells	Stage 4 cells	Stage 5
With at least	GDF8 and MCX	FGF7 and Vit C	FGF7; RA; SANT; TPB; LDN and Vit C	FGF7; LDN; SANT; TPB; RA and Vit C	heparin; SANT; RA; ALK5 inh; LDN; $ZnSO_4$	heparin; ALK5 inh.; T3; LDN; $ZnSO_4$
Exemplary amounts	~100 ng/ml GDF8 and ~1.0 μM MCX Day 1 ~100 nM MCX at Day 2	~25 ng/ml FGF7; ~0.25 mM Vit C	~25 ng/ml FGF7; ~1 μM RA ~0.25 μM SANT ~200 nM TPB ~100 nM LDN ~0.25 mM Vit C	~2 ng/ml FGF7; ~100 nM LDN ~0.25 μM SANT ~100 nM TPB ~100 nM RA ~0.25 mM Vit C	~0.25 μM SANT ~50 nM RA ~10000 nM ALK5 inh ~100 nM LDN ~10 mM $ZnSO_4$	~10 μg/ml heparin; ~10000 nM ALK5 inh ~1 μM T3 ~100 nM LDN ~10 mM $ZnSO_4$
Duration (days)	Approximately 2 to 5 days, preferably about 3 days	Approximately 2 to 3 days, preferably about 2 days	Approximately 2 to 4 days, preferably about 2 days	Approximately 2 to 4 days, preferably about 3 days	Approximately 2 to 4 days, preferably about 3 days	Approximately 3 to 15 days, preferably about 7 to 15 days
Type of Culture	Planar	Planar	Planar	Planar	Air Liquid Interface (Filter)	Air Liquid Interface (Filter)

[0203] As detailed above, the present invention provides, *inter alia*, a method of forming cells expressing markers characteristic of β cells comprising differentiating cells expressing markers characteristic of foregut endoderm cells into cells expressing markers characteristic of β cells by treatment with a medium supplemented with T3/T4, or an ALK5 inhibitor, or both T3/T4 and an ALK5 inhibitor and culturing at the air-liquid interface. In one embodiment, only Stage 4 to Stage 6 cells are cultured at the air-liquid interface. The Stage 6 cells may be positive for

NKX6.1, PDX1, and HB9. Accordingly, the invention also provides a method of inducing PDX1, NKX6.1, and HB9 expression in cells derived from pluripotent stem cells comprising: (a) culturing pluripotent stem cells; (b) differentiating the pluripotent stem cells into cells expressing markers characteristic of the foregut endoderm cells; and (c) differentiating the cells expressing markers characteristic of the foregut endoderm cells into cells expressing PDX1, NKX6.1, and HB9 by treatment with a medium supplemented with T3/T4, or an ALK5 inhibitor, or both T3/T4 and an ALK5 inhibitor, and culturing at the air-liquid interface. Further, the resulting Stage 6 cells may be single hormone positive cells. In one embodiment, the Stage 6 cells co-express NKX6.1 and chromogranin-A. In another embodiment, the stage 6 cells co-express NKX6.1 and insulin.

[0204] In certain embodiments, the methods include treating Stage 5 cells with a medium supplemented with T3/T4 and an ALK5 inhibitor, such as ALK5 inhibitor II. In these embodiments, the medium may advantageously be supplemented further with one or more of retinoic acid, ascorbic acid, SANT-1 or LDN-139189.

[0205] While the invention has been described and illustrated herein by references to various specific materials, procedures and examples, it is understood that the invention is not restricted to the particular combinations of material and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art. It is intended that the specification and examples be considered as exemplary, only, with the true scope and spirit of the invention being indicated by the following claims. All references, patents, and patent applications referred to in this application are herein incorporated by reference in their entirety.

CLAIMS

What is claimed is:

1. A method for producing cells expressing markers characteristic of pancreatic endocrine cells from pluripotent stem cells, comprising the steps of:

    a. culturing pluripotent stem cells;

    b. differentiating the pluripotent stem cells into cells expressing markers characteristic of pancreatic foregut precursor cells; and

    c. differentiating the cells expressing markers characteristic of pancreatic foregut precursor cells into cells expressing markers characteristic of pancreatic endocrine cells by treatment with at least one medium supplemented with an ALK5 inhibitor, or a thyroid hormone selected from triiodothyronine, thyroxine, analogues of triiodothyronine, analogues of thyroxine and mixtures thereof, or both ALK5 inhibitor and thyroid hormone, and culturing at the air-liquid interface.

2. The method of claim 1, wherein the cells expressing markers characteristic of pancreatic endocrine cells are positive for NKX6.1, PDX1, and HB9.

3. The method of claim 1, wherein the cells expressing markers characteristic of pancreatic endocrine cells co-express NKX6.1 and chromogranin-A.

4. The method of claim 3, wherein at least thirty percent of the resulting cells co-express NKX6.1 and chromogranin-A.

5. The method of claim 1, wherein the cells expressing markers characteristic of pancreatic endocrine cells co-express NKX6.1 and insulin.

6. The method of claim 5, wherein at least thirty percent of the resulting cells co-express NKX6.1 and insulin.

7. The method of claim 1, wherein the method further comprises differentiating cells expressing markers characteristic of foregut endoderm cells into cells expressing markers characteristic of pancreatic foregut precursor cells by treatment with a medium supplemented with an ALK5 inhibitor, or a thyroid hormone selected from triiodothyronine, thyroxine, analogues of triiodothyronine, analogues of thyroxine and mixtures thereof, or both an ALK5 inhibitor and thyroid hormone in a planar culture.

8. The method of claim 7, wherein the method further comprises differentiating the cells expressing markers characteristic of pancreatic foregut precursor cells into cells expressing markers characteristic of pancreatic endoderm/endocrine precursor cells by treatment with a medium supplemented with an ALK5 inhibitor, or a thyroid hormone selected from triiodothyronine, thyroxine, analogues of triiodothyronine, analogues of thyroxine and mixtures thereof, or both an ALK5 inhibitor and thyroid hormone, and culturing at the air-liquid interface.

9. The method of claim 1, wherein the method comprises treatment with a medium supplemented with triiodothyronine and an ALK5 inhibitor.

10. The method of claim 1, wherein the method further comprises culturing the cells on a porous substrate.

11. The method of claim 10, wherein the porous substrate is uncoated.

12. The method of claim 1, wherein said ALK5 inhibitor is selected from the group consisting of: ALK5 inhibitor II, ALK5i, SD208, TGF-B inhibitor SB431542, ITD-1, LY2109761, A83-01, LY2157299, TGF-β receptor inh V, TGF-β receptor inh I, TGF-β receptor inh I TGF-β receptor inh IV, TGF-β receptor inh VII, TGF-β receptor inh VIII, TGF-β receptor inh II, TGF-β receptor inh VI, TGF-β receptor inh III.

13. The method of claim 12, wherein said ALK5 inhibitor is ALK5 inhibitor II.

14. The method of claim 1, wherein the method further comprises treating cells expressing markers characteristic of pancreatic endoderm/pancreatic endocrine precursor cells with a medium supplemented with an ALK5 inhibitor, or a thyroid hormone selected from

triiodothyronine, thyroxine, analogues of triiodothyronine, analogues of thyroxine and mixtures thereof, or both ALK5 inhibitor and thyroid hormone.

15. The method of claim 14, wherein the thyroid hormone is triiodothyronine and the ALK5 inhibitor is ALK5 inhibitor II.

16. The method of claim 15, wherein the medium is further supplemented with one or more of retinoic acid, ascorbic acid, SANT-1 or LDN-193189.

17. The method of claim 1, wherein the method increases the expression of pancreatic hormones.

18. The method of claim 16, wherein said the method reduces expression of PTF1a, SOX9, CDX2, ZIC1 and SOX2.

19. The method of claim 1, wherein the method increases the number of NXK6.1 positive cells that co-express insulin, chromogranin-A or both chromogranin-A and insulin.

20. The method of claim 1, wherein the pluripotent stem cells are of non-embryonic origins.

21. The method of claim 1, wherein the cells expressing markers characteristic of pancreatic endocrine cells express markers characteristic of β cells.

22. A method of producing cells expressing markers characteristic of pancreatic endocrine cells comprising differentiating cells expressing markers characteristic of foregut endoderm cells into cells expressing markers characteristic of pancreatic endocrine cells by treatment with at least one medium supplemented with an ALK5 inhibitor, or a thyroid hormone selected from triiodothyronine, thyroxine, analogues of triiodothyronine, analogues of thyroxine and mixtures thereof, or both ALK5 inhibitor and thyroid hormone, while culturing at the air-liquid interface.

23. The method of claim 22, wherein only cells expressing markers characteristic of pancreatic foregut precursor cells, pancreatic endoderm cells and pancreatic endocrine precursor cells are cultured at the air-liquid interface.

24. The method of claim 22, wherein the cells expressing markers characteristic of pancreatic endocrine cells are positive for NKX6.1, PDX1, and HB9.

25. The method of claim 22, wherein the cells expressing markers characteristic of pancreatic endocrine cells are single hormone positive cells.

26. The method of claim 25, wherein the cells expressing markers characteristic of pancreatic endocrine cells co-express NKX6.1 and chromogranin-A.

27. The method of claim 25, wherein the cells expressing markers characteristic of pancreatic endocrine cells co-express NKX6.1 and insulin.

28. The method of claim 22, wherein the method further comprises culturing the cells on a porous substrate.

29. The method of claim 28, wherein the cells are in a sheet or aggregate clusters on top of the porous substrate.

30. The method of claim 22, wherein the method comprises treating cells expressing markers characteristic of pancreatic endoderm/pancreatic endocrine precursor cells with a medium supplemented with an ALK5 inhibitor, or a thyroid hormone selected from triiodothyronine, thyroxine, analogues of triiodothyronine, analogues of thyroxine and mixtures thereof, or both ALK5 inhibitor and thyroid hormone.

31. The method of claim 30, wherein the thyroid hormone is triiodothyronine and the ALK5 inhibitor is ALK5 inhibitor II.

32. The method of claim 31, wherein the medium is further supplemented with one or more of retinoic acid, ascorbic acid, SANT-1 or LDN-193189.

33. The method of claim 22, wherein the cells expressing markers characteristic of pancreatic endocrine cells express markers characteristic of β cells.

34. A method of inducing PDX1, NKX6.1, and HB9 expression in cells derived from pluripotent stem cells comprising:

a. culturing pluripotent stem cells;

b. differentiating the pluripotent stem cells into cells expressing markers characteristic of pancreatic foregut precursor cells; and

c. differentiating the cells expressing markers characteristic of pancreatic foregut precursor cells into cells expressing PDX1, NKX6.1, and HB9 by treatment with a medium supplemented with an ALK5 inhibitor, or a thyroid hormone selected from triiodothyronine, thyroxine, analogues of triiodothyronine, analogues of thyroxine and mixtures thereof, or both ALK5 inhibitor and thyroid hormone, and culturing at the air-liquid interface.

35. The method of claim 34, wherein the pluripotent stem cells are of non-embryonic origins.

36. The method of claim 34, wherein the method further comprises culturing cells expressing markers characteristic of pancreatic endoderm cells or pancreatic endocrine precursor cells in a medium supplemented with triiodothyronine and an ALK5 inhibitor at the air-liquid interface.

37. The method of claim 36, wherein the ALK5 inhibitor is ALK5 inhibitor II.

38. The method of claim 34, wherein the medium is further supplemented with one or more of retinoic acid, ascorbic acid, SANT-1 or LDN-193189.

39. An in vitro cell culture for differentiating cells at an air-liquid interface comprising:

a. a culture vessel;

b. a volume of differentiation medium within said vessel sufficient to fill only a portion of the volume of said vessel;

c. air within said vessel that fills a portion of said vessel adjoining said medium;

d. a porous substrate located at the interface between said medium and said air; and

e. cells derived from pluripotent stem cells disposed upon the surface of said substrate such that said medium contacts only a portion of the surface of said cells; and

wherein said cells derived from pluripotent stem cells express markers characteristic of foregut endoderm cells.

40. An in vitro cell culture for differentiating cells at an air-liquid interface comprising:

a. a culture vessel;

b. a volume of differentiation medium within said vessel sufficient to fill only a portion of the volume of said vessel;

c. air within said vessel that fills a portion of said vessel adjoining said medium;

d. a porous substrate located at the interface between said medium and said air; and

e. cells derived from pluripotent stem cells disposed upon the surface of said substrate such that said medium contacts only a portion of the surface of said cells; and

wherein said cells derived from pluripotent stem cells express markers characteristic of pancreatic foregut precursor cells.

41. An in vitro cell culture for differentiating cells at an air-liquid interface comprising:

a. a culture vessel;

b. a volume of differentiation medium within said vessel sufficient to fill only a portion of the volume of said vessel;

c. air within said vessel that fills a portion of said vessel adjoining said medium;

d. a porous substrate located at the interface between said medium and said air; and

e. cells derived from pluripotent stem cells disposed upon the surface of said substrate such that said medium contacts only a portion of the surface of said cells; and

wherein said differentiation medium comprises a growth medium supplemented with an ALK5 inhibitor, or a thyroid hormone selected from the group consisting of triiodothyronine, thyroxine, triiodothyronine analogues, thyroxine analogues and mixtures thereof, or both an ALK5 inhibitor and thyroid hormone; and

wherein said growth medium is selected from MCDB131 and BLAR media.

42. The cell culture of claim 41, wherein the growth medium is BLAR medium.

43. The cell culture of claim 42, wherein said growth medium is supplemented with triiodothyronine and ALK5 inhibitor II.

44. The cell culture of claim 39, wherein said differentiation medium comprises a growth medium supplemented with a growth factor selected from FGF7, FGF10 and mixtures thereof, a PKC activator selected from TPB, PDBu, PMA and ILV, and a BMP Receptor Inhibitor selected from LDN-193189, Noggin and Chordin.

45. The cell culture of claim 44, wherein said growth medium is selected from MCDB131 and BLAR media.

46. The cell culture of claim 45, wherein the growth medium is BLAR medium.

47. The cell culture of claim 46, wherein said growth medium is supplemented with FGF7, TPB and LDN-193189.

48. The cell culture of claim 40, wherein said differentiation medium comprises a growth medium supplemented with an ALK5 inhibitor, and a BMP inhibitor selected from the group consisting of LDN-193189, Noggin or Chordin.

49. The cell culture of claim 48, wherein said growth medium is selected from MCDB131 and BLAR media.

50. The cell culture of claim 49, wherein the growth medium is BLAR medium.

51. The cell culture of claim 50, wherein said growth medium is supplemented with LDN-193189 and ALK5 inhibitor II.

52. An in vitro cell culture for differentiating cells at an air-liquid interface comprising:

a. a culture vessel;

b. a volume of differentiation medium within said vessel sufficient to fill only a portion of the volume of said vessel;

c. air within said vessel that fills a portion of said vessel adjoining said medium;

d. a porous substrate located at the interface between said medium and said air; and

e. cells derived from pluripotent stem cells disposed upon the surface of said substrate such that said medium contacts only a portion of the surface of said cells; and

wherein said cells derived from pluripotent stem cells express markers characteristic of pancreatic endoderm cells;

wherein said differentiation medium comprises a growth medium supplemented with an ALK5 inhibitor, or a thyroid hormone selected from the group consisting of triiodothyronine, thyroxine, triiodothyronine analogues, thyroxine analogues and mixtures thereof, or both an ALK5 inhibitor and thyroid hormone; and

wherein said growth medium is selected from MCDB131 and BLAR media.

53. The cell culture of claim 52, wherein the growth medium is BLAR medium.

54. The cell culture of claim 53, wherein said growth medium is supplemented with triiodothyronine and ALK5 inhibitor II.

55. An in vitro cell culture for differentiating cells at an air-liquid interface comprising:

a. a culture vessel;

b. a volume of differentiation medium within said vessel sufficient to fill only a portion of the volume of said vessel;

c. air within said vessel that fills a portion of said vessel adjoining said medium;

d. a porous substrate located at the interface between said medium and said air; and

e. cells derived from pluripotent stem cells disposed upon the surface of said substrate such that said medium contacts only a portion of the surface of said cells; and

wherein said cells derived from pluripotent stem cells express markers characteristic of pancreatic endocrine precursor cells;

wherein said differentiation medium comprises a growth medium supplemented with an ALK5 inhibitor,or a thyroid hormone selected from the group consisting of triiodothyronine, thyroxine, triiodothyronine analogues, thyroxine analogues and mixtures thereof, or both an ALK5 inhibitor and thyroid hormone; and

wherein said growth medium is selected from MCDB131 and BLAR media.

56. The cell culture of claim 55, wherein the growth medium is BLAR medium.

57. The cell culture of claim 56, wherein said growth medium is supplemented with triiodothyronine and ALK5 inhibitor II.

58. The cell culture of claim 43, wherein the growth medium is further supplemented with one or more of:

a. a smoothened receptor inhibitor selected from MRT10 or cyclopamine;

b. a SHH signaling pathway antagonist selected from SANT-1 or HPI-1;

c. a BMP Receptor Inhibitor selected from LDN-193189, Noggin or Chordin;

d. a PKC activator selected from TPB, PDBu, PMA, and ILV;

e. a fibroblast growth factor selected from FGF7 or FGF10;

f. retinoic acid;

g. ascorbic acid;

h. heparin; and

i. zinc sulfate.

59. The cell culture of claim 51 wherein, the media is further supplemented with one or more of:

a. a smoothened receptor inhibitor selected from MRT10 or cyclopamine;

b. a SHH signaling pathway antagonist selected from SANT-1 or HPI-1;

c. a thyroid hormone selected from the group consisting of triiodothyronine, thyroxine, triiodothyronine analogues, thyroxine analogues and mixtures thereof;

d. a PKC activator selected from TPB, PDBu, PMA, and ILV;

e. a fibroblast growth factor selected from FGF7 or FGF10;

f. retinoic acid;

g. ascorbic acid;

h. heparin; and

i. zinc sulfate.

60. The cell culture of claim 54, wherein the media is further supplemented with one or more of:

    a. a smoothened receptor inhibitor selected from MRT10 or cyclopamine;

    b. a SHH signaling pathway antagonist selected from SANT-1 or HPI-1;

    c. a BMP Receptor Inhibitor selected from LDN-193189, Noggin or Chordin;

    d. a PKC activator selected from TPB, PDBu, PMA, and ILV;

    e. a fibroblast growth factor selected from FGF7 or FGF10;

    f. retinoic acid;

    g. ascorbic acid;

    h. heparin; and

    i. zinc sulfate.

61. The cell culture of claim 57, wherein the media is further supplemented with one or more of:

    a. a smoothened receptor inhibitor selected from MRT10 or cyclopamine;

    b. a SHH signaling pathway antagonist selected from SANT-1 or HPI-1;

    c. a BMP Receptor Inhibitor selected from LDN-193189, Noggin or Chordin;

    d. a PKC activator selected from TPB, PDBu, PMA, and ILV;

    e. a fibroblast growth factor selected from FGF7 or FGF10;

    f. retinoic acid;

    g. ascorbic acid;

    h. heparin; and

i. zinc sulfate.

62. The cell culture of claim 58, wherein said medium is further supplemented with retinoic acid, ascorbic acid and SANT-1.

63. The cell culture of claim 59, wherein said medium is further supplemented with retinoic acid, heparin, SANT-1 and zinc sulfate.

64. The cell culture of claim 60, wherein said medium is further supplemented with LDN-193189, retinoic acid, ascorbic acid, and SANT-1.

65. The cell culture of claim 61, wherein said medium is further supplemented with LDN-193189, SANT-1, and heparin.

66. The cell culture of claim 61 wherein, at least about thirty percent of the resulting cells express NKX6.1 and insulin.

67. The cell culture of claim 61 wherein, at least about thirty percent of the resulting cells express NKX6.1 and chromogranin-A.

68. A medium useful for inducing differentiation in cells derived from pluripotent stem cells comprising a growth medium supplemented with:

a. an ALK5 inhibitor selected from the group consisting of: ALK5 inhibitor II, ALK5i, SD208, TGF-B inhibitor SB431542, ITD-1, LY2109761, A83-01, LY2157299, TGF-β receptor inh V, TGF-β receptor inh I, TGF-β receptor inh I TGF-β receptor inh IV, TGF-β receptor inh VII, TGF-β receptor inh VIII, TGF-β receptor inh II, TGF-β receptor inh VI, TGF-β receptor inh III; and

b. a BMP Receptor Inhibitor selected from LDN-193189, Noggin or Chordin; and

further comprising an SHH signaling pathway antagonist selected form SANT-1 or HPI-1.

69. The medium of claim 68, further comprising retinoic acid.

70. The medium of claim 69, comprising ALK5 inhibitor II, SANT-1, LDN-193189 and retinoic acid.

71. The medium of claim 70, further comprising one or more supplements selected from:

a. a smoothened receptor inhibitor selected from MRT10 or cyclopamine;

b. a thyroid hormone selected from the group consisting of triiodothyronine, thyroxine, analogues of triiodothyronine, analogues of thyroxine and mixtures thereof;

c. ascorbic acid;

d. heparin; and

e. zinc sulfate.

72. The differentiation medium of claim 70, wherein said growth medium is selected from MCDB-131 and BLAR media.

73. The differentiation media of claim 71, wherein said growth medium is supplemented with ALK5 inhibitor II, SANT-1, LDN-193189, heparin, zinc sulfate and retinoic acid.

74. A medium useful for inducing differentiation in cells derived from pluripotent stem cells comprising a growth medium supplemented with:

a. an ALK5 inhibitor selected from the group consisting of: ALK5 inhibitor II, ALK5i, SD208, TGF-B inhibitor SB431542, ITD-1, LY2109761, A83-01, LY2157299, TGF-β receptor inh V, TGF-β receptor inh I, TGF-β receptor inh I TGF-β receptor inh IV, TGF-β receptor inh VII, TGF-β receptor inh VIII, TGF-β receptor inh II, TGF-β receptor inh VI, TGF-β receptor inh III;

b. a thyroid hormone selected from the group consisting of triiodothyronine, thyroxine, analogues of triiodothyronine, analogues of thyroxine and mixtures thereof; and

c. a SHH signaling pathway antagonist selected from SANT-1 or HPI-1.

75. The medium of claim 74, further comprising retinoic acid.

76. The medium of claim 75, further comprising ascorbic acid.

77. The medium of claim 74, further comprising one or more supplements selected from:

    a. a BMP Receptor Inhibitor selected from LDN-193189, Noggin or Chordin;

    b. heparin; and

    c. zinc sulfate.

78. The differentiation medium of claim 77, wherein said growth medium is selected from MCDB-131 and BLAR media.

79. The differentiation medium of claim 78, wherein said growth medium is supplemented with ALK5 inhibitor II, LDN-139189, zinc sulfate, triiodothyronine, SANT-1 and heparin.

80. An *in vitro* cell culture comprising a population of differentiated pluripotent stem cells expressing markers characteristic of pancreatic endocrine cells wherein at least thirty percent of said differentiated cells express NKX6.1 and chromagranin.

81. A method of screening compounds for effect on pancreatic hormone production comprising culturing cells according to the method of claim 1, in the presence of a test compound and measuring the effect of said test compound on the production of pancreatic hormones.

82. The screening method of claim 81, wherein said pancreatic hormone is insulin.