DIFFERENTIATION OF PLURIPOTENT STEM CELLS

CROSS REFERENCE TO RELATED APPLICATION

[0001] The present application claims the benefit of U.S. Provisional Patent Application Serial No. 61/378,480, filed August 31, 2010, which is incorporated herein by reference in its entirety for all purpose.

FIELD OF THE INVENTION

[0002] The present invention provides methods to promote the differentiation of pluripotent stem cells into insulin producing cells. In particular, the present invention provides a method utilizing an agent that degrades retinoic acid to produce a population of pancreatic endocrine precursor cells.

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 β cells, appropriate for engraftment. One approach is the generation of functional β cells from pluripotent stem cells, such as, for example, 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, for example, 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. Definitive endoderm cells express a number of markers, such as, HNF3 beta, GATA4, MIXL1, CXCR4 and SOX17.

[0005] Formation of the pancreas arises from the differentiation of definitive endoderm into pancreatic endoderm. 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,

among other cell types, exocrine tissue and endocrine tissue. Exocrine and endocrine tissues arise from the differentiation of pancreatic endoderm.

[0006] Pancreatic development *in vivo* relies, at least in part, upon appropriate regulation of the signals that specify organ progenitor fields. Kinkel *et al* (PNAS May 12, 2009 vol. 106 no. 19 7864-7869) state "Pancreatic cell fates are specified by retinoic acid (RA), and proper size and localization of the pancreatic field are dependent on tight control of RA signaling. Here we show that the RA-degrading Cyp26 enzymes play a critical role in defining the normal anterior limit of the pancreatic field."

[0007] Cells bearing the features of islet cells have reportedly been derived from embryonic cells of the mouse. For example, Lumelsky *et al.* (Science 292:1389, 2001) report differentiation of mouse embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Soria *et al.* (Diabetes 49:157, 2000) report that insulin-secreting cells derived from mouse embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice.

[0008] In one example, Hori *et al.* (PNAS 99: 16105, 2002) disclose that treatment of mouse embryonic stem cells with inhibitors of phosphoinositide 3-kinase (LY294002) produced cells that resembled β cells.

[0009] In another example, Blyszczuk *et al.* (PNAS 100:998, 2003) reports the generation of insulin-producing cells from mouse embryonic stem cells constitutively expressing Pax4.

[00010] Micallef *et al.* reports that retinoic acid can regulate the commitment of embryonic stem cells to form PDX1 positive pancreatic endoderm. Retinoic acid is most effective at inducing Pdx1 expression when added to cultures at day 4 of embryonic stem cell differentiation during a period corresponding to the end of gastrulation in the embryo (Diabetes 54:301, 2005).

[0010] Miyazaki *et al.* reports a mouse embryonic stem cell line over-expressing Pdx1. Their results show that exogenous Pdx1 expression clearly enhanced the expression of insulin, somatostatin, glucokinase, neurogenin3, p48, Pax6, and Hnf6 genes in the resulting differentiated cells (Diabetes 53: 1030, 2004).

[0011] Skoudy *et al.* reports that activin A (a member of the TGF-β superfamily) upregulates the expression of exocrine pancreatic genes (p48 and amylase) and endocrine genes (Pdx1, insulin, and glucagon) in mouse embryonic stem cells. The maximal effect was observed using 1nM activin A. They also observed that the expression level of insulin and Pdx1 mRNA was not affected by retinoic acid; however, 3nM FGF7 treatment resulted in an increased level of the transcript for Pdx1 (Biochem. J. 379: 749, 2004).

[0012] Shiraki *et al.* studied the effects of growth factors that specifically enhance differentiation of embryonic stem cells into PDX1 positive cells. They observed that TGF-β2 reproducibly yielded a higher proportion of PDX1 positive cells (Genes Cells. 2005 Jun; 10(6): 503-16.).

[0013] Gordon *et al.* demonstrated the induction of brachyury [positive]/ HNF3 beta [positive] endoderm cells from mouse embryonic stem cells in the absence of serum and in the presence of activin along with an inhibitor of Wnt signaling (US 2006/0003446A1).

[0014] Gordon *et al.* (PNAS, Vol 103, page 16806, 2006) states "Wnt and TGF-beta/ nodal/ activin signaling simultaneously were required for the generation of the anterior primitive streak".

[0015] However, the mouse model of embryonic stem cell development may not exactly mimic the developmental program in higher mammals, such as, for example, humans.

[0016] Thomson *et al.* isolated embryonic stem cells from human blastocysts (Science 282:114, 1998). Concurrently, Gearhart and coworkers derived human embryonic germ (hEG) cell lines from fetal gonadal tissue (Shamblott *et al.*, Proc. Natl. Acad. Sci. USA 95:13726, 1998). Unlike mouse embryonic stem cells, which can be prevented from differentiating simply by culturing with Leukemia Inhibitory Factor (LIF), human embryonic stem cells must be maintained under very special conditions (U.S. Pat. No. 6,200,806; WO 99/20741; WO 01/51616).

[0017] D'Amour *et al.* describes 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). Transplanting these cells under

the kidney capsule of mice resulted in differentiation into more mature cells with characteristics of some endodermal organs. Human embryonic stem cell-derived definitive endoderm cells can be further differentiated into PDX1 positive cells after addition of FGF-10 (US 2005/0266554A1).

[0018] D'Amour *et al.* (Nature Biotechnology - 24, 1392 - 1401 (2006)) states: "We have developed a differentiation process that converts human embryonic stem (hES) cells to endocrine cells capable of synthesizing the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin. This process mimics *in vivo* pancreatic organogenesis by directing cells through stages resembling definitive endoderm, gut-tube endoderm, pancreatic endoderm and endocrine precursor en route to cells that express endocrine hormones".

[0019] In another example, Fisk *et al.* reports a system for producing pancreatic islet cells from human embryonic stem cells (US2006/0040387A1). 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. The cells were then cultured with TGF-β antagonists such as Noggin in combination with EGF or betacellulin to generate PDX1 positive cells. The terminal differentiation was induced by nicotinamide.

[0020] There still remains a significant need to develop *in vitro* methods to generate a functional insulin expressing cell, that more closely resemble a β cell. The present invention takes an alternative approach to improve the efficiency of differentiating pluripotent stem cells toward insulin expressing cells, by generating a population of pancreatic precursor cells utilizing an agent that degrades retinoic acid.

SUMMARY

[0021] In one embodiment, the present invention provides a method utilizing an agent that degrades retinoic acid to produce a population of pancreatic endocrine precursor cells.

[0022] In one embodiment, formation of the population of pancreatic endocrine precursor cells is achieved utilizing a step-wise differentiation protocol, wherein a population of pluripotent stem cells is first differentiated into a population of cells expressing markers characteristic of the definitive endoderm lineage. Next, the population of

cells expressing markers characteristic of the definitive endoderm lineage is then differentiated into a population of primitive gut tube cells. Next, the population of primitive gut tube cells is then differentiated into a population of posterior foregut cells. Next, the population of posterior foregut cells is then differentiated into a population of endocrine precursor cells by treating the of posterior foregut cells with a medium supplemented with an agent that degrades retinoic acid.

[0023] In one embodiment, the population of endocrine precursor cells is further differentiated into a population of cells expressing markers characteristic of the pancreatic endocrine lineage.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figure 1 shows real-time PCR data obtained from samples obtained from cells at stages III-IV, of the protocol outlined in Example 1, for a) PAX4, b) NGN3, c) PDX1, d) NEUROD, e) NKX6.1, f) CDX2, and g) Albumin. The y-axis is fold expression over undifferentiated H1 cells. Panel h shows NGN3 immunostaining for control and CYP26A treated cultures at stage IV.

[0025] Figure 2 shows real-time PCR data obtained from samples obtained from cells at stages III-IV, of the protocol outlined in Example 2, for a) NGN3, b) NEUROD, c) CDX2, d) NKX6.1 and, e) PDX1. The y-axis is fold expression over undifferentiated H1 cells.

[0026] Figure 3 shows phase images of cells at stages I-VI of protocol outlined in Example 3.

[0027] Figure 4 shows FACS plots for the expression of NKX6.1 in cells at stages IV-VII of the protocol outlined in Example 3.

[0028] Figure 5 shoes immunostaining images for PDX1, NKX6.1 and CDX2 in cells at stages V and VII of the protocol outlined in Example 3.

DETAILED DESCRIPTION

[0029] For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections that describe or illustrate certain features, embodiments or applications of the present invention.

Definitions

[0030] Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to 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), as well as to give rise to tissues of multiple germ layers following transplantation and to contribute substantially to most, if not all, tissues following injection into blastocysts.

[0031] Stem cells are classified by their developmental potential as: (1) totipotent, meaning able to give rise to all embryonic and extraembryonic cell types; (2) pluripotent, meaning able to give rise to all embryonic cell types; (3) multipotent, meaning able to give rise to a subset of cell lineages but all within a particular tissue, organ, or physiological system (for example, hematopoietic stem cells (HSC) can produce progeny that include HSC (self- renewal), blood cell restricted oligopotent progenitors, and all cell types and elements (e.g., platelets) that are normal components of the blood); (4) oligopotent, meaning able to give rise to a more restricted subset of cell lineages than multipotent stem cells; and (5) unipotent, meaning able to give rise to a single cell lineage (e.g., spermatogenic stem cells).

[0032] 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 or differentiation-induced 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 what cells it can give rise to. 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.

[0033] "Cells expressing markers characteristic of the definitive endoderm lineage", or "Stage 1 cells", or "Stage 1", as used herein, refers to cells expressing at least one of the following markers: SOX17, GATA4, HNF3 beta, GSC, CER1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA6, CXCR4, C-Kit, CD99, or OTX2. Cells expressing markers characteristic of the definitive endoderm lineage include primitive streak precursor cells, primitive streak cells, mesendoderm cells and definitive endoderm cells.

[0034] "Cells expressing markers characteristic of the pancreatic endoderm lineage", as used herein, refers to cells expressing at least one of the following markers: PDX1, NKX6.1, HNF1 beta, PTF1 alpha, HNF6, HNF4 alpha, SOX9, HB9 or PROX1. Cells expressing markers characteristic of the pancreatic endoderm lineage include pancreatic endoderm cells, primitive gut tube cells, and posterior foregut cells.

[0035] "Definitive endoderm", 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 the following markers: HNF3 beta, GATA4, SOX17, Cerberus, OTX2, goosecoid, C-Kit, CD99, and MIXL1.

[0036] "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. 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.

[0037] "Pancreatic endocrine precursor cell", as used herein, refers to a cell expressing at least one of the following markers: NGN3, NEUROD, or NKX2.2.

[0038] "Posterior foregut cell", as used herein, refers to a cell expressing at least one of the following markers: PDX1 or HNF6.

[0039] "Immature pancreatic hormone expressing cells" as used herein, refers to a cell expressing at least one of the following markers: Insulin, glucagon, somatostatin, MAFB, PDX1, ARX, NKX6.1, NKX2.2, or NEUROD.

[0040] "Primitive gut tube cell", as used herein, refers to a cell expressing at least one of the following markers: HNF1 beta, or HNF4 alpha.

[0041] "Pancreatic endocrine cell", or "Pancreatic hormone expressing cell", or "Cells expressing markers characteristic of the pancreatic endocrine lineage" as used herein, refers to a cell capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.

Isolation, Expansion and Culture of Pluripotent Stem Cells

Characterization of Pluripotent Stem Cells

[0042] Pluripotent stem cells may express one or more of the stage-specific embryonic antigens (SSEA) 3 and 4, and markers detectable using antibodies designated Tra-1-60 and Tra-1-81 (Thomson *et al.*, Science 282:1145, 1998). Differentiation of pluripotent stem cells *in vitro* results in the loss of SSEA-4, Tra 1-60, and Tra 1-81 expression (if present) and increased expression of SSEA-1. Undifferentiated pluripotent stem cells typically have alkaline phosphatase activity, which can be detected by fixing the cells with 4% paraformaldehyde, and then developing with Vector Red as a substrate, as described by the manufacturer (Vector Laboratories, Burlingame Calif.). Undifferentiated pluripotent stem cells also typically express OCT4 and TERT, as detected by RT-PCR.

[0043] Another desirable phenotype of propagated pluripotent stem cells is a potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm tissues. Pluripotency of pluripotent stem cells can be confirmed, for example, by injecting cells into severe combined immunodeficient (SCID) mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining them histologically for evidence of cell types from the 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.

[0044] 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.

Sources of Pluripotent Stem Cells

[0045] The types of pluripotent stem cells that may be used include established lines of pluripotent cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, 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, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell). Also contemplated is use of the compositions of this disclosure during the initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, GA).

[0046] In one embodiment, human embryonic stem cells are prepared as described by Thomson *et al.* (U.S. Pat. No. 5,843,780; Science 282:1145, 1998; Curr. Top. Dev. Biol. 38:133 ff., 1998; Proc. Natl. Acad. Sci. U.S.A. 92:7844, 1995).

Culture of Pluripotent Stem Cells

[0047] In one embodiment, pluripotent stem cells are cultured on a layer of feeder cells that support the pluripotent stem cells in various ways. Alternatively, pluripotent stem cells are 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 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 is supported using a chemically defined medium.

[0048] In one embodiment, pluripotent stem cells may be cultured on a mouse embryonic fibroblast feeder cell layer according to the methods disclosed in Reubinoff *et al* (Nature Biotechnology 18: 399 - 404 (2000)). Alternatively, pluripotent stem cells may be cultured on a mouse embryonic fibroblast feeder cell layer according to the methods disclosed in Thompson *et al* (Science 6 November 1998: Vol. 282. no. 5391, pp. 1145 – 1147). Alternatively, pluripotent stem cells may be cultured on any one of the feeder cell layers disclosed in Richards *et al*, (Stem Cells 21: 546-556, 2003).

[0049] In one embodiment, pluripotent stem cells may be cultured on a human feeder cell layer according to the methods disclosed in Wang *et al* (Stem Cells 23: 1221-1227, 2005). In an alternate embodiment, pluripotent stem cells may be cultured on the human feeder cell layer disclosed in Stojkovic *et al* (Stem Cells 2005 23: 306-314, 2005). Alternatively, pluripotent stem cells may be cultured on the human feeder cell layer disclosed in Miyamoto *et al* (Stem Cells 22: 433-440, 2004). Alternatively, pluripotent stem cells may be cultured on the human feeder cell layer disclosed in Amit *et al* (Biol. Reprod 68: 2150-2156, 2003). Alternatively, pluripotent stem cells may be cultured on the human feeder cell layer disclosed in Inzunza *et al* (Stem Cells 23: 544-549, 2005).

[0050] In one embodiment, pluripotent stem cells may be cultured in culture media derived according to the methods disclosed in US20020072117. Alternatively, pluripotent stem cells may be cultured in culture media derived according to the methods disclosed in US6642048. Alternatively, pluripotent stem cells may be cultured in

culture media derived according to the methods disclosed in WO2005014799. Alternatively, pluripotent stem cells may be cultured in culture media derived according to the methods disclosed in Xu *et al* (Stem Cells 22: 972-980, 2004). Alternatively, pluripotent stem cells may be cultured in culture media derived according to the methods disclosed in US20070010011. Alternatively, pluripotent stem cells may be cultured in culture media derived according to the methods disclosed in US20050233446. Alternatively, pluripotent stem cells may be cultured in culture media derived according to the methods disclosed in US6800480. Alternatively, pluripotent stem cells may be cultured in culture media derived according to the methods disclosed in WO2005065354.

[0051] In one embodiment, pluripotent stem cells may be cultured according to the methods disclosed in Cheon *et al* (BioReprod DOI:10.1095/biolreprod.105.046870, October 19, 2005). Alternatively, pluripotent stem cells may be cultured according to the methods disclosed in Levenstein *et al* (Stem Cells 24: 568-574, 2006). Alternatively, pluripotent stem cells may be cultured according to the methods disclosed in US20050148070. Alternatively, pluripotent stem cells may be cultured according to the methods disclosed in US20050244962. Alternatively, pluripotent stem cells may be cultured according to the methods disclosed in WO2005086845.

[0052] 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, for example, those derived from basement membrane or that may form part of adhesion molecule receptor-ligand couplings. In one embodiment, the suitable culture substrate is MATRIGEL® (Becton Dickenson). MATRIGEL® is a soluble preparation from Engelbreth-Holm Swarm tumor cells that gels at room temperature to form a reconstituted basement membrane.

[0053] Other extracellular matrix components and component mixtures 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.

[0054] The pluripotent stem cells may be plated onto the substrate in a suitable distribution and in the presence of a medium that 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.

[0055] Suitable culture media may be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM), Gibco # 11965-092; Knockout Dulbecco's modified Eagle's medium (KO DMEM), Gibco #10829-018; Ham's F12/50% DMEM basal medium; 200 mM L-glutamine, Gibco # 15039-027; non-essential amino acid solution, Gibco 11140-050; β-mercaptoethanol, Sigma # M7522; human recombinant basic fibroblast growth factor (bFGF), Gibco # 13256-029.

Formation of Pancreatic Endocrine Precursor Cells from Pluripotent Stem Cells

[0056] The present invention provides methods for the formation of a population of pancreatic precursor cells from a population of pluripotent stem cells. In one embodiment, the present invention provides methods to further differentiate the pancreatic endocrine precursor cells into cells expressing markers of the pancreatic endocrine lineage.

[0057] In one embodiment, the present invention provides a method for producing of pancreatic precursor cells, comprising the steps of:

a. Culturing a population of pluripotent stem cells,

b. Differentiating the population of pluripotent stem cells into a population of cells expressing markers characteristic of the definitive endoderm lineage;

c. Differentiating the population of cells expressing markers characteristic of the definitive endoderm lineage into a population of primitive gut tube cells;

d. Differentiating the population of primitive gut tube cells into a population of posterior foregut cells; and

e. Differentiating the population of posterior foregut cells into a population of pancreatic endocrine precursor cells by treating the population of posterior

foregut cells with a medium supplemented with an agent that degrades retinoic acid.

[0058] The population of pancreatic endocrine precursor cells may be further treated to form a population of cells expressing markers characteristic of the pancreatic endocrine lineage.

[0059] 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 cells expressing markers characteristic of the desired cell type.

[0060] Methods for assessing expression of protein and nucleic acid markers in cultured or isolated cells are standard in the art. These include quantitative reverse transcriptase polymerase chain reaction (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)).

[0061] 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.

[0062] 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, such as CXCR4, expressed by cells expressing markers characteristic of the definitive endoderm lineage.

[0063] Pluripotent stem cells suitable for use in the present invention include, for example, 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 for use in the present invention 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.

[0064] Markers characteristic of the definitive endoderm lineage are selected from the group consisting of SOX17, GATA4, HNF3 beta, GSC, CER1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4, CD48, eomesodermin (EOMES), DKK4, FGF17, GATA6, CXCR4, C-Kit, CD99, and OTX2. 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 aspect of the present invention, a cell expressing markers characteristic of the definitive endoderm lineage is a primitive streak precursor cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a mesendoderm cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a definitive endoderm cell.

[0065] Markers characteristic of the pancreatic endoderm lineage (which includes primitive gut tube cells and posterior foregut cells) are selected from the group consisting of PDX1, NKX6.1, HNF1 beta, PTF1 alpha, HNF6, HNF4 alpha, SOX9, HB9 and PROX1. 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 aspect of the present invention, a cell expressing markers characteristic of the pancreatic endoderm lineage is a pancreatic endoderm cell.

[0066] Markers characteristic of the pancreatic endocrine lineage are selected from the group consisting of NGN3, NEUROD, ISL1, PDX1, NKX6.1, PAX4, NGN3, and PTF-1 alpha. In one embodiment, a pancreatic endocrine cell is capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide. 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 aspect of the present invention, a cell expressing markers characteristic of the pancreatic endocrine lineage is a pancreatic endocrine cell. The pancreatic endocrine cell may be a pancreatic hormone-expressing cell. Alternatively, the pancreatic endocrine cell may be a pancreatic hormone-secreting cell.

[0067] In one aspect of the present invention, the pancreatic endocrine cell is a cell expressing markers characteristic of the β cell lineage. A cell expressing markers characteristic of the β cell lineage expresses PDX1 and at least one of the following transcription factors: NGN3, NKX2.2, NKX6.1, NEUROD, ISL1, HNF3 beta, MAFA, PAX4, and PAX6. In one aspect of the present invention, a cell expressing markers characteristic of the β cell lineage is a β cell.

Formation of Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage from Pluripotent Stem Cells

[0068] Populations of cells expressing markers characteristic of the definitive endoderm lineage may be formed from populations of pluripotent stem cells by any method in the art.

[0069] For example, populations of pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 23, 1534 - 1541 (2005).

[0070] For example, populations of pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in Shinozaki *et al*, Development 131, 1651 - 1662 (2004).

[0071] For example, populations of pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in McLean *et al*, Stem Cells 25, 29 - 38 (2007).

[0072] For example, populations of pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 24, 1392 - 1401 (2006).

[0073] For example, populations of pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in US patent application Ser. No. 11/736,908.

[0074] For example, populations of pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in US patent application Ser. No. 11/779,311.

[0075] For example, populations of pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in US patent application Ser. No. 12/493,741.

[0076] For example, populations of pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in US patent application Ser. No. 12/494,789.

Formation of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage

[0077] Cells expressing markers characteristic of the pancreatic endoderm lineage include pancreatic endoderm cells, primitive gut tube cells, and posterior foregut cells. In one embodiment, populations of cells expressing markers characteristic of the definitive endoderm lineage formed by the methods of the present invention are further differentiated into populations of cells expressing markers characteristic of the pancreatic endoderm lineage by any method in the art.

[0078] For example, populations of cells expressing markers characteristic of the definitive endoderm lineage obtained according to the methods of the present invention may be further differentiated into populations of cells expressing markers characteristic of the pancreatic endoderm lineage by treating the population of cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 24, 1392 - 1401 (2006).

[0079] For example, populations of cells expressing markers characteristic of the definitive endoderm lineage obtained according to the methods of the present invention may be further differentiated into populations of cells expressing markers characteristic of the pancreatic endoderm lineage by treating the population of cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in US patent application Ser. No. 11/736,908.

Formation of a Population of Pancreatic Endocrine Precursor Cells

[0080] In one embodiment, the present invention provides a method for producing of pancreatic precursor cells, comprising the steps of:

a. Culturing a population of pluripotent stem cells,

b. Differentiating the population of pluripotent stem cells into a population of cells expressing markers characteristic of the definitive endoderm lineage;

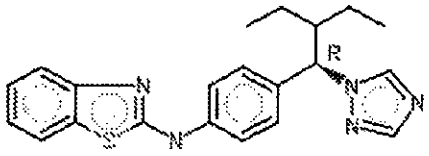
c. Differentiating the population of cells expressing markers characteristic of the definitive endoderm lineage into a population of primitive gut tube cells;

d. Differentiating the population of primitive gut tube cells into a population of posterior foregut cells; and

e. Differentiating the population of posterior foregut cells into a population of pancreatic endocrine precursor cells by treating the population of posterior foregut cells with a medium supplemented with an agent that degrades retinoic acid.

[0081] In one embodiment, the agent that degrades retinoic acid is a CYP26A inhibitor. The CYP26A inhibitor may be used at a concentration from about 1 nM to about 1000 nM. Alternatively, the CYP26A inhibitor may be used at a concentration from about 10 nM to about 100 nM.

[0082] Any CYP26A inhibitor is suitable for use in the present invention. For example, the CYP26A inhibitor may be selected from the compounds disclosed in US Patent No. 7,468,391. Alternatively, the CYP26A inhibitor may be selected from the compounds disclosed in US Patent Application No. 2005/0187298A1. Alternatively, the CYP26A inhibitor may be selected from the compounds disclosed in US Patent Application No. 2004/0106216A1. Alternatively, the CYP26A inhibitor may be selected from the compounds disclosed in WO2005058301A1. Alternatively, the CYP26A inhibitor may be selected from the compounds disclosed in PNAS May 12, 2009 vol. 106 no. 19 7864-7869. In one embodiment, the CYP26A inhibitor is N-{4-[2-Ethyl-1-(1H-1, 2, 4-triazol-1-yl)butyl]phenyl}-1, 3-benzothiazol-2-amine. See Formula 1.



Formula 1.

[0083] In one embodiment, the medium supplemented with an agent that degrades retinoic acid is further supplemented with at least one factor selected from the group consisting of a factor capable of inhibiting BMP, a TGFβ receptor signaling inhibitor, vitamin A and a PKC activator.

[0084] In one embodiment, the factor capable of inhibiting BMP is noggin. Noggin may be used at a concentration from about 50ng/ml to about 500μg/ml. In one embodiment, noggin is used at a concentration of 100ng/ml.

[0085] In one embodiment, the TGFβ receptor signaling inhibitor is an inhibitor of ALK5. In one embodiment, the inhibitor of ALK5 is ALK5 inhibitor II. The ALK5 inhibitor II may be used at a concentration from about 0.1 μM to about 10 μM. In one embodiment, ALK5 inhibitor II is used at a concentration of 1 μM.

[0086] In one embodiment, the PKC activator is selected from the group consisting of (2S, 5S)-(E, E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadiemoylamino) benzolactam, Indolactam V (ILV), phorbol-12-myristate-13-acetate (PMA), and phorbol-12,13-dibutyrate (PDBu). In one embodiment, the protein kinase C activator is (2S, 5S)-(E, E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadiemoylamino) benzolactam. (2S, 5S)-(E, E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadiemoylamino) benzolactam may be used at a concentration from about 20nM to about 500nM. (2S, 5S)-(E, E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadiemoylamino) benzolactam, is referred to herein as "TPB".

Formation of Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage

[0087] In one embodiment, the populations of pancreatic endocrine precursor cells produced by the methods of the present invention are further differentiated into populations of cells expressing markers characteristic of the pancreatic endocrine lineage by any method in the art.

[0088] For example, populations of cells expressing markers characteristic of the pancreatic endoderm lineage may be further differentiated into populations of cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the population of cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

[0089] For example, populations of cells expressing markers characteristic of the pancreatic endoderm lineage may be further differentiated into populations of cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the population of cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

[0090] For example, populations of cells expressing markers characteristic of the pancreatic endoderm lineage may be further differentiated into populations of cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the population of cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in US patent application Ser. No. 11/736,908.

[0091] For example, populations of cells expressing markers characteristic of the pancreatic endoderm lineage may be further differentiated into populations of cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the population of cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in US patent application Ser. No. 11/779,311.

[0092] For example, populations of cells expressing markers characteristic of the pancreatic endoderm lineage may be further differentiated into populations of cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the population of cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in US patent application Ser. No. 60/953,178.

[0093] For example, populations of cells expressing markers characteristic of the pancreatic endoderm lineage may be further differentiated into populations of cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the population of cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in US patent application Ser. No. 60/990,529.

[0094] The present invention is further illustrated, but not limited by, the following examples.

EXAMPLES

Example 1

Differentiation of Cells of the Human Embryonic Stem Cell Line H1 into Pancreatic Endocrine Precursor Cells in Cell Culture Medium Lacking FBS and Containing an Inhibitor of CYP26A

[0095] Cells of the human embryonic stem cells line H1 (p40-p50) were cultured on MATRIGEL®-coated dishes (1:30 dilution) (BD Biosciences; Cat # 356231) in MEF-CM (mouse embryonic fibroblast conditioned medium) as colonies and differentiated into pancreatic endocrine precursor cells as follows:

a. Stage I (Definitive Endoderm): Human embryonic stem cells were cultured in RPMI medium supplemented with 2% fatty acid-free BSA (Catalog# 68700, Proliant, IA), and 100 ng/ml activin A (R&D Systems, MN) plus 20 ng/ml WNT-3a (Catalog# 1324-WN-002, R&D Systems, MN) plus 8 ng/ml of bFGF (Catalog# 100-18B, PeproTech, NJ), for one day, followed by treatment with RPMI medium supplemented with 2% BSA and 100 ng/ml activin A plus 8 ng/ml of bFGF for an additional two days, then

b. Stage II (Primitive gut tube): Cells were treated with RPMI + 2% fatty acid-free BSA and 50 ng/ml FGF7, for two days, then

c. Stage III (Posterior foregut): Cells were treated with DMEM/High-Glucose supplemented with 1:200 dilution of ITS-X (Invitrogen, CA) and 0.1% BSA (Lipid Rich) (Invitrogen, Ca No. 11021-045), 50 ng/ml FGF7,

0.25 μM SANT-1, 2 μM Retinoic acid (RA) (Sigma, MO), 100 ng/ml of Noggin (R & D Systems, MN), 2.5 μM 4-[4-(4-Fluorophenyl)-1-(3-phenylpropyl)-5-pyridin-4-yl-1H-imidazol-2-yl]but-3-yn-1-ol (a P38 inhibitor disclosed in US Patent 6,521,655), and activin A at 20 ng/ml for five days, then

d. Stage IV (Pancreatic endocrine precursor): Cells were treated with DMEM/High-Glucose supplemented with 1:200 dilution of ITS-X (Invitrogen, CA) and 0.1% BSA (Invitrogen, Ca), 100 ng/ml Noggin, 1 μM ALK5 inhibitor (SD-208, disclosed in Molecular Pharmacology 2007 72:152-161), 500 nM TPB (α-Amyloid Precursor Protein Modulator) (Catalogue #565740, EMD, CA), and 10-100 nM of the CYP26A inhibitor N-{4-[2-Ethyl-1-(1H-1, 2, 4-triazol-1-yl)butyl]phenyl}-1, 3-benzothiazol-2-amine, and 10-100 nM Vitamin A (Catalogue# R7632, Sigma, MO) for four days, or

[0096] In some of the cultures, the Stage IV was extended to six days. mRNA was isolated at stages III and IV for real-time PCR analysis of pancreatic related genes. As shown in Figure 1, addition of the CYP26A inhibitor at stage IV significantly boosted expression of endocrine precursor markers (NGN3, Pax4, NeuroD) along with the pancreatic endoderm marker NKX6.1 in a dose dependent manner. Addition of vitamin A along with the CYP26A inhibitor did not significantly modify expression of pancreatic endoderm or endocrine precursor markers. Furthermore, addition of the CYP26A inhibitor at stage IV decreased expression of CDX2 (an intestinal marker) and albumin (a liver marker). Immunostaining for NGN3 (Catalogue# AF3444, R & D systems, MN) at stage IV clearly showed a significant boost in expression of NGN3 for cultures treated with 100 nM of the CYP26A inhibitor.

Example 2

An Alternative Method for the Differentiation of Cells of the Human Embryonic Stem Cell Line H1 into Pancreatic Endocrine Precursor Cells in Cell Culture Medium Lacking FBS and Containing an Inhibitor of CYP26A

[0097] Cells of the human embryonic stem cells line H1 (p40-p52) were seeded as single cells at a density of 100000 cells/cm^2 on MATRIGEL®-coated dishes (1:30 dilution)

(BD Biosciences; Cat # 356231) in MEF-CM (mouse embryonic fibroblast conditioned media supplemented) with 16 ng/ml of FGF2 (Catalog# 100-18B, PeproTech, NJ) and 10 μM of Y27632 (Rock inhibitor, Catalogue# Y0503, Sigma, MO). 72 hrs post seeding, cultures were differentiated into definitive endoderm (DE) as follows:

a. Stage I (Definitive Endoderm): Human embryonic stem cells were treated with MCDB-131 (Catalogue# 10372-019, Invitrogen, CA) medium supplemented with 2% fatty acid-free BSA (Catalog# 68700, Proliant, IA), 0.0025 g/ml sodium bicarbonate (Catalogue # S3187, Sigma, MO), 1X GlutaMax™ (Catalogue # 35050-079, Invitrogen, Ca) and 100 ng/ml activin A (R&D Systems, MN) plus 20 ng/ml WNT-3a (Catalog# 1324-WN-002, R&D Systems, MN) for one day, followed by treatment with MCDB-131 medium supplemented with 2% BSA, sodium bicarbonate, Glutamax, and 100 ng/ml activin A for an additional three days, then

b. Stage II (Primitive gut tube): Cells were treated with MCDB-131 + 2% fatty acid-free BSA and 50 ng/ml FGF7 and for three days, then

c. Stage III (Posterior foregut): Cells were treated with MCDB-131/High-Glucose (25 mM glucose) supplemented with 1:200 dilution of ITS-X (Invitrogen, CA), 1X GlutaMax™ (Catalogue # 35050-079, Invitrogen, Ca), 0.0025 g/ml sodium bicarbonate (Catalogue # S3187, Sigma, MO), 0.1% BSA (Lipid Rich) (Invitrogen, Ca No. 11021-045), 50 ng/ml FGF7, 0.25 μM SANT-1, 2 μM Retinoic acid (RA) (Sigma, MO), 2.5 μM 4-[4-(4-Fluorophenyl)-1-(3-phenylpropyl)-5-pyridin-4-yl-1H-imidazol-2-yl]but-3-yn-1-ol (a p38 inhibitor, disclosed in US Patent 6,521,655), 100 nM LDN-193189 (BMP receptor inhibitor, Catalogue # 04-0019, Stemgent, CA), 500 nM of the CYP26A inhibitor N-{4-[2-Ethyl-1-(1H-1, 2, 4-triazol-1-yl)butyl]phenyl}-1, 3-benzothiazol-2-amine, and activin A at 20 ng/ml for four days, then

d. Stage IV (Pancreatic endocrine precursor): Cells were treated MCDB-131/High-Glucose (25 mM glucose) supplemented with 1:200 dilution of ITS-X (Invitrogen, CA) and 0.1% BSA (Invitrogen, Ca), 1X GlutaMax™

(Catalogue # 35050-079, Invitrogen, Ca), 0.0025 g/ml sodium bicarbonate (Catalogue # S3187, Sigma, MO), 1 μM ALK5 inhibitor (SD-208, disclosed in Molecular Pharmacology 2007 72:152-161), 500 nM PDBu (PKC activator) (Catalogue #P1269, Sigma, MO), 100 nM LDN-193189 (BMP receptor inhibitor, Catalogue # 04-0019, Stemgent, CA), 0.25 μM SANT-1 (#S4572, Sigma, MO), and 500 nM of the CYP26A inhibitor N-{4-[2-Ethyl-1-(1H-1, 2, 4-triazol-1-yl)butyl]phenyl}-1, 3-benzothiazol-2-amine for seven days, or

e. Stage IV (Pancreatic endocrine precursor): Cells were treated MCDB-131/High-Glucose (25 mM glucose) supplemented with 1:200 dilution of ITS-X (Invitrogen, CA) and 0.1% BSA (Invitrogen, Ca), 1X GlutaMax™ (Catalogue # 35050-079, Invitrogen, Ca), 0.0025 g/ml sodium bicarbonate (Catalogue # S3187, Sigma, MO), 1 μM ALK5 inhibitor (SD-208, disclosed in Molecular Pharmacology 2007 72:152-161), 500 nM PDBu (PKC activator) (Catalogue #P1269, Sigma, MO), 100 nM LDN-193189 (BMP receptor inhibitor, Catalogue # 04-0019, Stemgent, CA), 0.25 μM SANT-1 (#S4572, Sigma, MO) for seven days.

[0098] mRNA was isolated at stages III and IV for real-time PCR analysis of pancreatic related genes. Similar to the results observed in Example 1 above, addition of CYP26A inhibitor to stage IV enhanced expression of pancreatic endocrine precursor markers, such as NGN3 and NeuroD. (See Figure 2). Addition of the inhibitor to both stages III and IV further enhanced expression of NGN3 and NeuroD. Surprisingly, addition of the CYP26A inhibitor to stage III (in the presence of retinoic acid) significantly down regulated PDX-1 and NKX6.1, while enhancing expression of CDX2. These results suggest that the optimal stage for addition of CYP26A inhibitor is stage IV.

Example 3

An Alternative Method for the Differentiation of Cells of the Human Embryonic Stem Cell Line H1 into Pancreatic Endocrine Cells in Cell Culture Medium Lacking FBS and Containing an Inhibitor of CYP26A

[0099] Cells of the human embryonic stem cells line H1 (p40-p52) were seeded as single cells at a density of 100000 cells/cm^2 on MATRIGEL®-coated dishes (1:30 dilution) (BD Biosciences; Cat # 356231) in MEF-CM (mouse embryonic fibroblast conditioned media supplemented) with 16 ng/ml of FGF2 (Catalog# 100-18B, PeproTech, NJ) and 10 μM of Y27632 (Rock inhibitor, Catalogue# Y0503, Sigma, MO). 72 hrs post seeding, cultures were differentiated into definitive endoderm (DE) as follows:

a. Stage I (Definitive Endoderm): Human embryonic stem cells cultured as single cells on Matrigel-coated dishes were treated with MCDB-131 (Catalogue# 10372-019, Invitrogen, CA) medium supplemented with 2% fatty acid-free BSA (Catalog# 68700, Proliant, IA), 0.0025 g/ml sodium bicarbonate (Catalogue # S3187, Sigma, MO), 1X GlutaMax™ (Catalogue # 35050-079, Invitrogen, Ca) and 100 ng/ml GDF-8 (R&D Systems, MN) plus 2.5 μM of the GSK3B inhibitor 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 for one day, followed by treatment with MCDB-131 medium supplemented with 2% BSA, sodium bicarbonate, Glutamax, and 100 ng/ml GDF-8 for an additional three days, then

b. Stage II (Primitive gut tube): Cells were treated with MCDB-131 + 2% fatty acid-free BSA and 50 ng/ml FGF7 and for two days, then

c. Stage III (Posterior foregut): Cells were treated with MCDB131/High-Glucose (25 mM glucose) supplemented with 1:200 dilution of ITS-X (Invitrogen, CA), 1X GlutaMax™ (Catalogue # 35050-079, Invitrogen, Ca), 0.0025 g/ml sodium bicarbonate (Catalogue # S3187, Sigma, MO), 0.1% BSA (Lipid Rich) (Invitrogen, Ca No. 11021-045), 50 ng/ml FGF7, 0.25 μM SANT-1, 2 μM Retinoic acid (RA) (Sigma, MO), 2.5 μM 4-[4-(4-Fluorophenyl)-1-(3-phenylpropyl)-5-pyridin-4-yl-1H-imidazol-2-yl]but-3-yn-1-ol, 100 nM LDN-193189 (BMP receptor inhibitor, Catalogue # 04-0019, Stemgent, CA), and activin A at 20 ng/ml for four days, then

d. Stage IV (Pancreatic precursor): Cells were treated MCDB131/High-Glucose (25 mM glucose) supplemented with 1:200 dilution of ITS-X (Invitrogen, CA) and 0.1% BSA (Invitrogen, Ca), 1X GlutaMax™ (Catalogue # 35050-079, Invitrogen, Ca), 0.0025 g/ml sodium bicarbonate (Catalogue # S3187, Sigma, MO), 100 nM LDN-193189 (BMP receptor inhibitor, Catalogue # 04-0019, Stemgent, CA), 50 nM PDBu (PKC activator) (Catalogue #P1269, Sigma, MO), 0.25 μM SANT-1 (#S4572, Sigma, MO), and 100 nM of the CYP26A inhibitor N-{4-[2-Ethyl-1-(1H-1, 2, 4-triazol-1-yl)butyl]phenyl}-1, 3-benzothiazol-2-amine for three days, then

e. Stage V (Pancreatic endocrine precursor): Cells were treated MCDB131/High-Glucose (25 mM glucose) supplemented with 1:200 dilution of ITS-X (Invitrogen, CA) and 0.1% BSA (Invitrogen, Ca), 1X GlutaMax™ (Catalogue # 35050-079, Invitrogen, Ca), 0.0025 g/ml sodium bicarbonate (Catalogue # S3187, Sigma, MO), 100 nM LDN-193189 (BMP receptor inhibitor, Catalogue # 04-0019, Stemgent, CA), 0.25 μM SANT-1 (#S4572, Sigma, MO), 2 μM ALK5 inhibitor (SD-208, disclosed in Molecular Pharmacology 2007 72:152-161) and 100 nM of the CYP26A inhibitor N-{4-[2-Ethyl-1-(1H-1, 2, 4-triazol-1-yl)butyl]phenyl}-1, 3-benzothiazol-2-amine for three days, then

f. Stage VI (Immature pancreatic hormone expressing cells): Cells were treated MCDB131/High-Glucose (25 mM glucose) supplemented with 1:200 dilution of ITS-X (Invitrogen, CA) and 0.1% BSA (Invitrogen, Ca), 1X GlutaMax™ (Catalogue # 35050-079, Invitrogen, Ca), 0.0025 g/ml sodium bicarbonate (Catalogue # S3187, Sigma, MO), 100 nM LDN-193189 (BMP receptor inhibitor, Catalogue # 04-0019, Stemgent, CA), and 2 μM ALK5 inhibitor (SD-208, disclosed in Molecular Pharmacology 2007 72:152-161) for three days, then

g. Stage VII (Pancreatic hormone expressing cells): Cells were treated MCDB131/High-Glucose (25 mM glucose) supplemented with 1:200 dilution of ITS-X (Invitrogen, CA) and 0.1% BSA (Invitrogen, Ca), 1X GlutaMax™ (Catalogue # 35050-079, Invitrogen, Ca), 0.0025 g/ml

sodium bicarbonate (Catalogue # S3187, Sigma, MO), 100 nM LDN-193189 (BMP receptor inhibitor, Catalogue # 04-0019, Stemgent, CA), 2 μM ALK5 inhibitor (SD-208, disclosed in Molecular Pharmacology 2007 72:152-161) and 100 nM Vitamin A (Catalogue# R7632, Sigma, MO) for three days.

[0100] In some of the cultures, stage VII was extended to 18 days. Samples were collected at stages V, VI, and for real-time PCR analysis, immune fluorescence (IF) staining and FACS analysis. For both FACS and immune fluorescence (IF) staining, the NKX6.1 antibody was obtained from University of Iowa hybridoma bank (Catalogue# F55A12), the CDX2 antibody was obtained from Abcam (Catalogue # ab76541, Cambridge, MA), and the PDX-1 antibody was purchased from Abcam (Catalogue# ab47267). Figure 3 highlights the morphology of cultures at various stages of differentiation. Following stage II, the cultures showed homogenous morphology throughout stages III-VI. Figure 4 depicts the expression of NKX6.1 as measured by FACS for various stages of differentiation. This figure highlights that the protocol disclosed in Example 3, can retain high expression of NKX6.1 through late stages of differentiation. Figure 5 shows IF staining for PDX1, NKX6.1, and CDX2 expression for stages V and stage VII of the protocol. Greater than 90% of the NKX6.1 positive cells were also PDX1 positive, while less than 10% of the cells stained positive for CDX2.

[0101] Publications cited throughout this document are hereby incorporated by reference in their entirety. Although the various aspects of the invention have been illustrated above by reference to examples and preferred embodiments, it will be appreciated that the scope of the invention is defined not by the foregoing description but by the following claims properly construed under principles of patent law.

Claims

1. A method to derive a population of pancreatic endocrine precursor cells from pluripotent stem cells comprising the steps of:

a. Culturing a population of pluripotent stem cells;

b. Differentiating the population of pluripotent stem cells into a population of cells expressing markers characteristic of the definitive endoderm lineage;

c. Differentiating the population of cells expressing markers characteristic of the definitive endoderm lineage into a population of primitive gut tube cells;

d. Differentiating the population of primitive gut tube cells into a population of posterior foregut cells; and

e. Treating the population of posterior foregut cells with a medium supplemented with a CYP26A inhibitor and with no added retinoic acid such that the population of posterior foregut cells differentiates into a population of pancreatic endocrine precursor cells, wherein expression of endocrine precursor markers is increased and wherein the differentiating in steps b., c. and d. comprises treatment in a medium lacking a CPY26A inhibitor.

2. The method of claim 1, wherein the CYP26A inhibitor is used at a concentration from about 1 nM to about 1000 nM.

3. The method of claim 1, wherein the CYP26A inhibitor is used at a concentration from about 10 nM to about 100 nM.

4. The method of claim 1, wherein the CYP26A inhibitor is N-{4-[2-Ethyl-1-(1H-1, 2, 4-triazol-1-yl)butyl]phenyl}-1, 3-benzothiazol-2-amine.

5. The method of claim 1, wherein treatment increases expression of the endocrine precursor markers NGN3, Pax and NeuroD.

6. The method of claim 1, wherein the treatment decreases the expression of CDX2 and albumin.

7. The method of claim 1, wherein the medium supplemented with the CYP26A inhibitor is further supplemented with at least one factor selected from the group consisting of a factor capable of inhibiting BMP, a TGFβ receptor signaling inhibitor, and a PKC activator.

8. The method of claim 7, wherein the factor capable of inhibiting BMP comprises noggin.

9. The method of claim 7, wherein the TGFβ receptor signaling inhibitor comprises an inhibitor of ALK5.

10. The method of claim 9, wherein the inhibitor of ALK5 is ALK5 inhibitor II.

11. The method of claim 7, wherein the PKC activator is selected from the group consisting of (2S, 5S)-(E, E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadiemoylamino) benzolactam, Indolactam V (ILV), phorbol-12-myristate-13-acetate (PMA), and phorbol-12,13-dibutyrate (PDBu).

12. A method to derive a population of pancreatic endocrine precursor cells from pluripotent stem cells comprising the steps of:

a. Differentiating pluripotent stem cells into a population of cells expressing markers characteristic of the definitive endoderm lineage (Stage I) by the culturing the pluripotent stem cells in a medium supplemented with activin A;

b. Differentiating the population of cells expressing markers characteristic of the definitive endoderm lineage (Stage I) into a population of primitive gut tube cells (Stage II) by culturing the Stage I cells in a medium supplemented with FGF7;

c. Differentiating the population of primitive gut tube cells (Stage II) into a population of posterior foregut cells (Stage III) by culturing the Stage II cells in a medium supplemented with retinoic acid and a P38 inhibitor; and

d. Differentiating the population of posterior foregut cells (Stage III) into a population of Stage IV cells by treating the Stage III cells with a media supplemented with a CPY26A inhibitor and with no added retinoic acid, wherein the treatment increases the expression of endocrine precursor markers and wherein the differentiating in steps a., b. and c. comprises treatment in a medium lacking a CPY26A inhibitor.

13. The method of claim 12, wherein the Stage IV cells are pancreatic endocrine precursors.

14. The method of claim 12, wherein treatment increases expression of the endocrine precursor markers NGN3, Pax and NeuroD.

15. The method of claim 12, wherein treatment decreases the expression of CDX2 and albumin.

16. The method of claim 12, wherein the CYP26A inhibitor is used at a concentration from about 1 nM to about 1000 nM.

17. The method of claim 12, wherein the medium in step c. is further supplemented with at least one or more of FGF7, activin A or a BMP receptor inhibitor.

18. The method of claim 12, wherein the medium supplemented with a CPY26A inhibitor is further supplemented with at least one factor selected from the group consisting of a factor capable of inhibiting BMP, a TGFβ receptor signaling inhibitor, and a PKC activator.

19. The method of claim 18, wherein the factor capable of inhibiting BMP comprises noggin.

20. The method of claim 18, wherein the TGFβ receptor signaling inhibitor comprises an inhibitor of ALK5.

21. The method of claim 20, wherein the inhibitor of ALK5 is ALK5 inhibitor II.

22. The method of claim 18, wherein the PKC activator is selected from the group consisting of (2S, 5S)-(E, E)-8-(5-(4-(Trifluoromethyl)phenyl)-2,4-pentadiemoylamino) benzolactam, Indolactam V (ILV), phorbol-12-myristate-13-acetate (PMA), and phorbol-12,13-dibutyrate (PDBu).