DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS TO THE PANCREATIC ENDOCRINE LINEAGE

[0001] The present invention claims priority to application serial number 61/110,278, filed October 31, 2008.

FIELD OF THE INVENTION

[0002] The present invention provides methods to promote the differentiation of pluripotent stem cells. In particular, the present invention provides a method to increase the expression of markers associated with the pancreatic endocrine lineage.

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, HNF-3beta, GATA4, Mixl1, CXCR4 and Sox-17.

[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] 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.

[0007] 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.

[0008] 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.

[0009] 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^{+}/HNF-3beta^{+} 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] In one example, Benvenistry *et al.* states: "We conclude that over-expression of Pdx1 enhanced expression of pancreatic enriched genes, induction of insulin expression may require additional signals that are only present *in vivo*" (Benvenistry *et al*, Stem Cells 2006; 24:1923–1930).

[0021] Therefore, there still remains a significant need to develop conditions for establishing pluripotent stem cell lines that can be expanded to address the current clinical needs, while retaining the potential to differentiate into pancreatic endocrine cells, pancreatic hormone expressing cells, or pancreatic hormone secreting cells. We have taken an alternative approach to improve the efficiency of differentiating human embryonic stem cells toward pancreatic endocrine cells.

SUMMARY

[0022] In one embodiment, the present invention provides a method for differentiating pluripotent stem cells, comprising the steps of:

a. Culturing the pluripotent stem cells,

b. Differentiating the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage,

c. Differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage, and

d. Differentiating the cells expressing markers characteristic of the pancreatic endoderm lineage into cells expressing markers characteristic of the pancreatic endocrine lineage.

[0023] In one embodiment, the present invention provides a method for increasing the expression of markers associated with the pancreatic endocrine lineage comprising treating cells expressing markers characteristic of the pancreatic endocrine lineage with medium comprising a sufficient amount of a TGF-β receptor agonist to cause an increase in expression of markers associated with the pancreatic endocrine lineage.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figure 1 shows an outline of the differentiation protocol employed in the present invention.

[0025] Figure 2 shows the expression of markers associated with the various stages of the differentiation protocol employed in the present invention. Panel a) shows the expression of CXCR4 as determined by FACS in cells of the human embryonic stem cell line H1 that had been differentiated to stage 1. Panel b) shows the expression of markers associated with stage 1, as determined by real-time PCR, in cells of the human embryonic stem cell line H1 that had been differentiated to stage 1.

[0026] Figure 3 shows dithizone staining of cells of the human embryonic stem cell line H1 that were differentiated to stage 6 of the differentiation protocol employed in the present invention and subsequently treated with activin A (stage 7). Panel a) shows a phase contrast of cells stained with dithizone prior to passage through a 40μm cell strainer. Panel b) shows a phase contrast of cells stained with dithizone that were able to pass through a 40μm cell strainer. Panel c shows a phase contrast of cells stained with dithizone that were not able to pass through a 40μm cell strainer.

[0027] Figure 4 shows the expression of pdx-1 (panel a), nkx6-1 (panel b), Pax4 (panel c), nkx2.2 (panel d), insulin (panel e) and glucagon (panel f) as determined by real-time PCR, in cells of the human embryonic stem cell line H1 that were differentiated to stage 6 of the differentiation protocol employed in the present invention and treated with activin A following differentiation to stage 6, according to the methods described in Example 2.

DETAILED DESCRIPTION

[0028] 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

[0029] 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.

[0030] 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).

[0031] 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.

[0032] "β-cell lineage" refers to cells with positive gene expression for the transcription factor PDX-1 and at least one of the following transcription factors: NGN-3, Nkx2.2, Nkx6.1, NeuroD, Isl-1, HNF-3 beta, MAFA, Pax4, and Pax6. Cells expressing markers characteristic of the β cell lineage include β cells.

[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: SOX-17, GATA-4, HNF-3 beta, GSC, Cer1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA-6, 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: PDX-1, HNF-1beta, PTF-1 alpha, HNF-6, or HB9. Cells expressing markers characteristic of the pancreatic endoderm lineage include pancreatic endoderm cells, primitive gut tube cells, and posterior foregut cells.

[0035] "Cells expressing markers characteristic of the pancreatic endocrine lineage", or "Stage 5 cells", or "Stage 5", as used herein, refers to cells expressing at least one of the following markers: NGN-3, NeuroD, Islet-1, PDX-1, NKX6.1, Pax-4, Ngn-3, or PTF-1 alpha. Cells expressing markers characteristic of the pancreatic endocrine lineage include pancreatic endocrine cells, pancreatic hormone expressing cells, and pancreatic hormone secreting cells, and cells of the β-cell lineage.

[0036] "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: HNF-3 beta, GATA-4, SOX-17, Cerberus, OTX2, goosecoid, C-Kit, CD99, and Mixl1.

[0037] "Extraembryonic endoderm", as used herein, refers to a population of cells expressing at least one of the following markers: SOX-7, AFP, and SPARC.

[0038] "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.

[0039] "Mesendoderm cell", as used herein, refers to a cell expressing at least one of the following markers: CD48, eomesodermin (EOMES), SOX-17, DKK4, HNF-3 beta, GSC, FGF17, GATA-6.

[0040] "Pancreatic endocrine cell", or "pancreatic hormone expressing cell", as used herein, refers to a cell capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.

[0041] "Pancreatic endoderm cell", or "Stage 4 cells", or "Stage 4", as used herein, refers to a cell capable of expressing at least one of the following markers: NGN-3, NeuroD, Islet-1, PDX-1, PAX-4, NKX2.2.

[0042] "Pancreatic hormone producing cell", as used herein, refers to a cell capable of producing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.

[0043] "Pancreatic hormone secreting cell" or "Stage 6 cells", or "Stage 6", as used herein, refers to a cell capable of secreting at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide.

[0044] "Posterior foregut cell" or "Stage 3 cells", or "Stage 3", as used herein, refers to a cell capable of secreting at least one of the following markers: PDX-1, HNF-1, PTF-1A, HNF-6, HB-9, PROX-1.

[0045] "Pre-primitive streak cell", as used herein, refers to a cell expressing at least one of the following markers: Nodal, or FGF8.

[0046] "Primitive gut tube cell" or "Stage 2 cells", or "Stage2", as used herein, refers to a cell capable of secreting at least one of the following markers: HNF-1, or HNF-4A.

[0047] "Primitive streak cell", as used herein, refers to a cell expressing at least one of the following markers: Brachyury, Mix-like homeobox protein, or FGF4.

Isolation, Expansion and Culture of Pluripotent Stem Cells

Characterization of Pluripotent Stem Cells

[0048] 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 Oct-4 and TERT, as detected by RT-PCR.

[0049] 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.

[0050] 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

[0051] 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 10-12 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).

[0052] 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

[0053] In one embodiment, 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 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.

[0054] For example, Reubinoff et al (Nature Biotechnology 18: 399 - 404 (2000)) and Thompson *et al* (Science 6 November 1998: Vol. 282. no. 5391, pp. 1145 – 1147) disclose the culture of pluripotent stem cell lines from human blastocysts using a mouse embryonic fibroblast feeder cell layer.

[0055] Richards *et al*, (Stem Cells 21: 546-556, 2003) evaluated a panel of 11 different human adult, fetal and neonatal feeder cell layers for their ability to support human pluripotent stem cell culture. Richards *et al*, states: "human embryonic stem cell lines cultured on adult skin fibroblast feeders retain human embryonic stem cell morphology and remain pluripotent".

[0056] US20020072117 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. US20020072117 also discloses the use of the cell lines as a primary feeder cell layer.

[0057] In another example, Wang *et al* (Stem Cells 23: 1221-1227, 2005) discloses methods for the long-term growth of human pluripotent stem cells on feeder cell layers derived from human embryonic stem cells.

[0058] In another example, 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.

[0059] In a further example, Miyamoto *et al* (Stem Cells 22: 433-440, 2004) disclose a source of feeder cells obtained from human placenta.

[0060] Amit *et al* (Biol. Reprod 68: 2150-2156, 2003) discloses a feeder cell layer derived from human foreskin.

[0061] In another example, Inzunza *et al* (Stem Cells 23: 544-549, 2005) disclose a feeder cell layer from human postnatal foreskin fibroblasts.

[0062] US6642048 discloses media that support the growth of primate pluripotent stem (pPS) cells in feeder-free culture, and cell lines useful for production of such media. US6642048 states: "This invention includes mesenchymal and fibroblast-like cell lines obtained from embryonic tissue or differentiated from embryonic stem cells. Methods for deriving such cell lines, processing media, and growing stem cells using the conditioned media are described and illustrated in this disclosure."

[0063] In another example, WO2005014799 discloses conditioned medium for the maintenance, proliferation and differentiation of mammalian cells. WO2005014799 states: "The culture medium produced in accordance with the present invention is conditioned by the cell secretion activity of murine cells, in particular, those differentiated and immortalized transgenic hepatocytes, named MMH (Met Murine Hepatocyte)."

[0064] In another example, Xu *et al* (Stem Cells 22: 972-980, 2004) discloses conditioned medium obtained from human embryonic stem cell derivatives that have been genetically modified to over express human telomerase reverse transcriptase.

[0065] In another example, US20070010011 discloses a chemically defined culture medium for the maintenance of pluripotent stem cells.

[0066] An alternative culture system employs serum-free medium supplemented with growth factors capable of promoting the proliferation of embryonic stem cells. For example, 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.

[0067] In another example, 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.

[0068] In another example, US20050148070 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.

[0069] In another example, US20050233446 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.

[0070] In another example, US6800480 states "In one embodiment, 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 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. The medium further includes non-essential amino acids, an anti-oxidant, and a first growth factor selected from the group consisting of nucleosides and a pyruvate salt."

[0071] In another example, US20050244962 states: "In one aspect the invention provides a method of culturing primate embryonic stem cells. One cultures the stem cells in a culture 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. In a preferred form, the fibroblast feeder layer, previously required to sustain a stem cell culture, is rendered unnecessary by the addition of sufficient fibroblast growth factor."

[0072] In a further example, WO2005065354 discloses a defined, isotonic culture medium that is essentially feeder-free and serum-free, comprising: a. a basal medium; b. an amount of bFGF sufficient to support growth of substantially undifferentiated mammalian stem cells; c. an amount of insulin sufficient to support growth of substantially undifferentiated mammalian stem cells; and d. an amount of ascorbic acid sufficient to support growth of substantially undifferentiated mammalian stem cells.

[0073] In another example, WO2005086845 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-beta (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.

[0074] 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, a 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.

[0075] 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.

[0076] 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.

[0077] 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 Hormone Producing Cells from Pluripotent Stem Cells

[0078] In one embodiment, the present invention provides a method for producing pancreatic hormone producing 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 the definitive endoderm lineage,

c. Differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage, and

d. Differentiating the cells expressing markers characteristic of the pancreatic endoderm lineage into cells expressing markers characteristic of the pancreatic endocrine lineage.

[0079] In one aspect of the present invention, the pancreatic endocrine cell is a pancreatic hormone producing cell. In an alternate aspect, 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: NGN-3, Nkx2.2, Nkx6.1, NeuroD, Isl-1, HNF-3 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.

[0080] 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, UTF-1, ZFP42, SSEA-3, SSEA-4, Tra1-60, Tra1-81.

[0081] Markers characteristic of the definitive endoderm lineage are selected from the group consisting of SOX-17, GATA4, Hnf-3beta, 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.

[0082] Markers characteristic of the pancreatic endoderm lineage are selected from the group consisting of Pdx1, HNF-1beta, PTF1a, HNF-6, 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.

[0083] Markers characteristic of the pancreatic endocrine lineage are selected from the group consisting of NGN-3, NeuroD, Islet-1, Pdx-1, NKX6.1, Pax-4, Ngn-3, 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.

[0084] 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: NGN-3, Nkx2.2, Nkx6.1, NeuroD, Isl-1, HNF-3 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

[0085] Pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by any method in the art or by any method proposed in this invention.

[0086] For example, 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).

[0087] For example, 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).

[0088] For example, 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).

[0089] For example, 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).

[0090] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A in the absence of serum, then culturing the cells with activin A and serum, and then culturing the cells with activin A and serum of a different concentration. An example of this method is disclosed in Nature Biotechnology 23, 1534 - 1541 (2005).

[0091] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A in the absence of serum, then culturing the cells with activin A with serum of another concentration. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2005.

[0092] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in medium containing activin A and a Wnt ligand in the absence of serum, then removing the Wnt ligand and culturing the cells with activin A with serum. An example of this method is disclosed in Nature Biotechnology 24, 1392 - 1401 (2006).

[0093] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

[0094] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.

[0095] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 60/990,529.

[0096] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 61/076,889.

[0097] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 61/076,900.

[0098] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 61/076,908.

[0099] For example, pluripotent stem cells may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage by treating the pluripotent stem cells according to the methods disclosed in US patent application Ser. No. 61/076,915.

Differentiation of Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage

[0100] Formation of cells expressing markers characteristic of the definitive endoderm lineage 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 is detected when cells begin to express them.

[0101] 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 definitive endoderm lineage.

[0102] 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)).

[0103] 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, UTF-1, ZFP42, SSEA-3, SSEA-4, Tra1-60, Tra1-81.

[0104] 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.

Formation of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage

[0105] Cells expressing markers characteristic of the definitive endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage by any method in the art or by any method proposed in this invention.

[0106] For example, cells expressing markers characteristic of the definitive endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 24, 1392 - 1401 (2006).

[0107] For example, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with a fibroblast growth factor and the hedgehog signaling pathway inhibitor KAAD-cyclopamine, then removing the medium containing the fibroblast growth factor and KAAD-cyclopamine and subsequently culturing the cells in medium containing retinoic acid, a fibroblast growth factor and KAAD-cyclopamine. An example of this method is disclosed in Nature Biotechnology 24, 1392 - 1401 (2006).

[0108] In one aspect of the present invention, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with retinoic acid and at

least one fibroblast growth factor for a period of time, according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

[0109] In one aspect of the present invention, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage with retinoic acid and at least one fibroblast growth factor for a period of time, according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.

[0110] In one aspect of the present invention, cells expressing markers characteristic of the definitive endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage, by treating the cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in US patent application Ser. No. 60/990,529.

Detection of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage

[0111] Markers characteristic of the pancreatic endoderm lineage are well known to those skilled in the art, and additional markers characteristic of the pancreatic endoderm lineage continue to be identified. These markers can be used to confirm that the cells treated in accordance with the present invention have differentiated to acquire the properties characteristic of the pancreatic endoderm lineage. Pancreatic endoderm lineage specific markers include the expression of one or more transcription factors such as, for example, Hlxb9, PTF-1a, PDX-1, HNF-6, HNF-1beta.

[0112] 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 pancreatic endoderm lineage.

[0113] 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)).

Formation of Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage

[0114] Cells expressing markers characteristic of the pancreatic endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage by any method in the art or by any method disclosed in this invention.

[0115] For example, cells expressing markers characteristic of the pancreatic endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage according to the methods disclosed in D'Amour *et al*, Nature Biotechnology 24, 1392 - 1401 (2006).

[0116] For example, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing DAPT and exendin 4, then removing the medium containing DAPT and exendin 4 and subsequently culturing the cells in medium containing exendin 1, IGF-1 and HGF. An example of this method is disclosed in Nature Biotechnology 24, 1392 - 1401 (2006).

[0117] For example, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing exendin 4, then removing the medium containing exendin 4 and subsequently culturing the cells in medium containing exendin 1, IGF-1 and HGF. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

[0118] For example, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic

of the pancreatic endoderm lineage in medium containing DAPT and exendin 4. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

[0119] For example, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by culturing the cells expressing markers characteristic of the pancreatic endoderm lineage in medium containing exendin 4. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

[0120] In one aspect of the present invention, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 11/736,908, assigned to LifeScan, Inc.

[0121] In one aspect of the present invention, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 11/779,311, assigned to LifeScan, Inc.

[0122] In one aspect of the present invention, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage with a factor that inhibits the Notch signaling pathway, according to the methods disclosed in US patent application Ser. No. 60/953,178, assigned to LifeScan, Inc.

[0123] In one aspect of the present invention, cells expressing markers characteristic of the pancreatic endoderm lineage are further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage, by treating the cells expressing markers characteristic of the pancreatic endoderm lineage according to the methods disclosed in US patent application Ser. No. 60/990,529.

[0124] In one aspect of the present invention, the present invention provides a method for increasing the expression of markers associated with the pancreatic endocrine lineage comprising treating cells expressing markers characteristic of the pancreatic endocrine lineage with medium comprising a sufficient amount of a TGF-β receptor agonist to cause an increase in expression of markers associated with the pancreatic endocrine lineage.

[0125] The TGF-β receptor agonist may be any agent capable of binging to, and activating the TGF-β receptor. In one embodiment, the TGF-β receptor agonist is selected from the group consisting of activin A, activin B, and activin C.

[0126] In an alternate embodiment, the the TGF-β receptor agonist may be a peptide variant of activin A. Examples of such peptide variants are disclosed in US patent application Ser. No. 61/076,889, assigned to Centocor R&D, Inc.

[0127] In one embodiment, the cells expressing markers characteristic of the pancreatic endocrine lineage are treated with an amount of activin A sufficient to cause an increase in expression of markers associated with the pancreatic endocrine lineage for about one to about five days. Alternatively, the cells expressing markers characteristic of the pancreatic endocrine lineage are treated with an amount of activin A sufficient to cause an increase in expression of markers associated with the pancreatic endocrine lineage for about three to about five days. Alternatively, the cells expressing markers characteristic of the pancreatic endocrine lineage are treated with an amount of activin A sufficient to cause an increase in expression of markers associated with the pancreatic endocrine lineage for about five days.

[0128] In an alternate embodiment, the TGF-β receptor agonist is selected from the group consisting of GDF-8, GDF 11, and GDF-15. In one embodiment, the cells expressing markers characteristic of the pancreatic endocrine lineage are treated with the TGF-β receptor agonist selected from the group consisting of GDF-8, GDF 11, and GDF-15 and at least one other factor. In one embodiment, the at least one other factor is a cyclic aniline-pyridinotriazine compound. Examples of such cyclic aniline-pyridinotriazine compounds are disclosed in US patent application Ser. No. 61/076,900, assigned to Centocor R&D, Inc. In an alternate embodiment, the at least one other factor is an aniline-pyridinotriazine compound. Examples of such aniline-

pyridinotriazine compounds are disclosed in US patent application Ser. No. 61/076,908, assigned to Centocor R&D, Inc. In an alternate embodiment, the at least one other factor is a compound disclosed in US patent application Ser. No. 61/076,915, assigned to Centocor R&D, Inc.

[0129] In one aspect of the present invention, the cells expressing markers characteristic of the pancreatic endocrine lineage are subsequently enriched. The enrichment step may be performed prior to treatment of the cells expressing markers characteristic of the pancreatic endocrine lineage with the TGF-β receptor agonist. Alternatively, the enrichment step may be performed after the cells expressing markers characteristic of the pancreatic endocrine lineage have been treated with the TGF-β receptor agonist.

[0130] In one embodiment, the enrichment is achieved by obtaining a suspension of cells expressing markers characteristic of the pancreatic endocrine lineage and passing the suspension of cells through a 40μm cell strainer.

Detection of Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage

[0131] Markers characteristic of cells of the pancreatic endocrine lineage are well known to those skilled in the art, and additional markers characteristic of the pancreatic endocrine lineage continue to be identified. These markers can be used to confirm that the cells treated in accordance with the present invention have differentiated to acquire the properties characteristic of the pancreatic endocrine lineage. Pancreatic endocrine lineage specific markers include the expression of one or more transcription factors such as, for example, NGN-3, NeuroD, Islet-1.

[0132] Markers characteristic of cells of the β cell lineage are well known to those skilled in the art, and additional markers characteristic of the β cell lineage continue to be identified. These markers can be used to confirm that the cells treated in accordance with the present invention have differentiated to acquire the properties characteristic of the β-cell lineage. β cell lineage specific characteristics include the expression of one or more transcription factors such as, for example, Pdx1 (pancreatic and duodenal homeobox gene-1), Nkx2.2, Nkx6.1, Isl1, Pax6, Pax4, NeuroD, Hnf1b, Hnf-6, Hnf-3beta, and MafA, among others. These transcription factors are well established in

the art for identification of endocrine cells. See, e.g., Edlund (Nature Reviews Genetics 3: 524-632 (2002)).

[0133] 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 pancreatic endocrine lineage. Alternatively, 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 β cell lineage.

[0134] 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)).

[0135] In one aspect of the present invention, the efficiency of differentiation is determined by measuring the percentage of insulin positive cells in a given cell culture following treatment. In one embodiment, the methods of the present invention produce about 100% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 90% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 80% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 70% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 60% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 50% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 40% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 30% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce

about 20% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 10% insulin positive cells in a given culture. In an alternate embodiment, the methods of the present invention produce about 5% insulin positive cells in a given culture.

[0136] In one aspect of the present invention, the efficiency of differentiation is determined by measuring glucose-stimulated insulin secretion, as detected by measuring the amount of C-peptide released by the cells. In one embodiment, cells produced by the methods of the present invention produce about 1000ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 900ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 800ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 700ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 600ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 500ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 400ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 500ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 400ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 300ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 200ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 100ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 90ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 80ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 70ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 60ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 50ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce

about 40ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 30ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 20ng C-peptide/pg DNA. In an alternate embodiment, cells produced by the methods of the present invention produce about 10ng C-peptide/pg DNA.

Therapies

[0137] In one aspect, the present invention provides a method for treating a patient suffering from, or at risk of developing, Type1 diabetes. This method involves culturing pluripotent stem cells, differentiating the pluripotent stem cells in vitro into a β-cell lineage, and implanting the cells of a β-cell lineage into a patient.

[0138] In yet another aspect, this invention provides a method for treating a patient suffering from, or at risk of developing, Type 2 diabetes. This method involves culturing pluripotent stem cells, differentiating the cultured cells *in vitro* into a β-cell lineage, and implanting the cells of a β-cell lineage into the patient.

[0139] If appropriate, the patient can be further treated with pharmaceutical agents or bioactives that facilitate the survival and function of the transplanted cells. These agents may include, for example, insulin, members of the TGF-β family, including TGF-β1, 2, and 3, bone morphogenic proteins (BMP-2, -3, -4, -5, -6, -7, -11, -12, and -13), fibroblast growth factors-1 and -2, platelet-tderived growth factor-AA, and –BB, platelet rich plasma, insulin growth factor (IGF-I, II) growth differentiation factor (GDF-5, -6, -7, -8, -10, -15), vascular endothelial cell-derived growth factor (VEGF), pleiotrophin, endothelin, among others. Other pharmaceutical compounds can include, for example, nicotinamide, glucagon like peptide-I (GLP-1) and II, GLP-1 and 2 mimetibody, Exendin-4, retinoic acid, parathyroid hormone, MAPK inhibitors, such as, for example, compounds disclosed in U.S. Published Application 2004/0209901 and U.S. Published Application 2004/0132729.

[0140] The pluripotent stem cells may be differentiated into an insulin-producing cell prior to transplantation into a recipient. In a specific embodiment, the pluripotent stem cells are fully differentiated into β-cells, prior to transplantation into a recipient. Alternatively, the pluripotent stem cells may be transplanted into a recipient in an

undifferentiated or partially differentiated state. Further differentiation may take place in the recipient.

[0141] Definitive endoderm cells or, alternatively, pancreatic endoderm cells, or, alternatively, β 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.

[0142] To enhance further differentiation, survival or activity of the implanted cells, additional factors, such as growth factors, antioxidants or anti-inflammatory agents, can be administered before, simultaneously with, or after the administration of the cells. In certain embodiments, growth factors are utilized to differentiate the administered cells *in vivo*. 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.

[0143] 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.

[0144] In one aspect, this invention provides a method for treating a patient suffering from, or at risk of developing diabetes. This method involves culturing pluripotent stem cells, differentiating the cultured cells *in vitro* into a β-cell lineage, and incorporating the cells into a three-dimensional support. 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.

[0145] Support materials suitable for use for purposes of the present invention include tissue templates, conduits, barriers, and reservoirs useful for tissue repair. In particular,

synthetic and natural materials in the form of foams, sponges, gels, hydrogels, textiles, and nonwoven structures, which have been used *in vitro* and *in vivo* to reconstruct or regenerate biological tissue, as well as to deliver chemotactic agents for inducing tissue growth, are suitable for use in practicing the methods of the present invention. See, for example, the materials disclosed in U.S. Patent 5,770,417, U.S. Patent 6,022,743, U.S. Patent 5,567,612, U.S. Patent 5,759,830, U.S. Patent 6,626,950, U.S. Patent 6,534,084, U.S. Patent 6,306,424, U.S. Patent 6,365,149, U.S. Patent 6,599,323, U.S. Patent 6,656,488, U.S. Published Application 2004/0062753 A1, U.S. Patent 4,557,264and U.S. Patent 6,333,029.

[0146] To form a support incorporated with a pharmaceutical agent, the pharmaceutical agent can be mixed with the polymer solution prior to forming the support. Alternatively, a pharmaceutical agent could be coated onto a fabricated support, preferably in the presence of a pharmaceutical carrier. The pharmaceutical agent may be present as a liquid, a finely divided solid, or any other appropriate physical form. Alternatively, excipients may be added to the support to alter the release rate of the pharmaceutical agent. In an alternate embodiment, the support is incorporated with at least one pharmaceutical compound that is an anti-inflammatory compound, such as, for example compounds disclosed in U.S. Patent 6,509,369.

[0147] The support may be incorporated with at least one pharmaceutical compound that is an anti-apoptotic compound, such as, for example, compounds disclosed in U.S. Patent 6,793,945.

[0148] The support may also be incorporated with at least one pharmaceutical compound that is an inhibitor of fibrosis, such as, for example, compounds disclosed in U.S. Patent 6,331,298.

[0149] The support may also be incorporated with at least one pharmaceutical compound that is capable of enhancing angiogenesis, such as, for example, compounds disclosed in U.S. Published Application 2004/0220393 and U.S. Published Application 2004/0209901.

[0150] The support may also be incorporated with at least one pharmaceutical compound that is an immunosuppressive compound, such as, for example, compounds disclosed in U.S. Published Application 2004/0171623.

[0151] The support may also be incorporated with at least one pharmaceutical compound that is a growth factor, such as, for example, members of the TGF-β family, including TGF-β1, 2, and 3, bone morphogenic proteins (BMP-2, -3,-4, -5, -6, -7, -11, -12, and -13), fibroblast growth factors-1 and -2, platelet-derived growth factor-AA, and –BB, platelet rich plasma, insulin growth factor (IGF-I, II) growth differentiation factor (GDF-5, -6, -8, -10, -15), vascular endothelial cell-derived growth factor (VEGF), pleiotrophin, endothelin, among others. Other pharmaceutical compounds can include, for example, nicotinamide, hypoxia inducible factor 1-alpha, glucagon like peptide-I (GLP-1), GLP-1 and GLP-2 mimetibody, and II, Exendin-4, nodal, noggin, NGF, retinoic acid, parathyroid hormone, tenascin-C, tropoelastin, thrombin-derived peptides, cathelicidins, defensins, laminin, biological peptides containing cell- and heparin-binding domains of adhesive extracellular matrix proteins such as fibronectin and vitronectin, MAPK inhibitors, such as, for example, compounds disclosed in U.S. Published Application 2004/0209901 and U.S. Published Application 2004/0132729.

[0152] The incorporation of the cells of the present invention into a scaffold can be achieved by the simple depositing of cells onto the scaffold. Cells can enter into the scaffold by simple diffusion (J. Pediatr. Surg. 23 (1 Pt 2): 3-9 (1988)). Several other approaches have been developed to enhance the efficiency of cell seeding. For example, spinner flasks have been used in seeding of chondrocytes onto polyglycolic acid scaffolds (Biotechnol. Prog. 14(2): 193-202 (1998)). Another approach for seeding cells is the use of centrifugation, which yields minimum stress to the seeded cells and enhances seeding efficiency. For example, Yang *et al.* developed a cell seeding method (J.Biomed. Mater. Res. 55(3): 379-86 (2001)), referred to as Centrifugational Cell Immobilization (CCI).

[0153] The present invention is further illustrated, but not limited by, the following examples.

EXAMPLES

Example 1

Differentiation of Human Embryonic Stem Cells of the Cell Line H1 to Pancreatic Endocrine Cells in the Absence of Fetal Bovine Serum

[0154] Cells of the human embryonic stem cells line H1 at passage 52 were cultured on MATRIGEL™ (1:30 dilution) coated dishes and exposed to RPMI medium supplemented with 2% BSA (Catalog# 152401, MP Biomedical, Ohio), 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 media supplemented with 2% BSA and 100 ng/ml activin A plus 8 ng/ml of bFGF for an additional two days. Next the cultures were treated with DMEM/F12 + 2% BSA + 50 ng/ml FGF7 + 0.25 μM Cyclopamine- KAAD (#239804, Calbiochem, CA) for two days, followed by four days incubation in DMEM/F12 + 1% B27 (Invitrogen, CA) + 50 ng/ml FGF7 + 0.25 μM Cyclopamine- KAAD + 2 μM Retinoic acid (RA) (Sigma, MO) + 100 ng/ml of Noggin (R & D Systems, MN).

[0155] Cells were differentiated into pancreatic endocrine cells by incubating the cells in DMEM/F12 + 1% B27 (Invitrogen, CA) + 100 ng/ml Noggin + 1 μM DAPT (a gamma-secretase inhibitor) (Catalog# 565784, Calbiochem, CA) + 1 μM ALK5 inhibitor II (Catalog# 616452, Calbiochem, Ca) + 100 ng/ml of Netrin-4 (R&D Systems, MN) for three days followed by additional seven days incubation in DMEM/F12 + 1% B27 (Invitrogen, CA) + 1 μM ALK5 inhibitor II (Calbiochem, Ca). A last stage was added to further mature the endocrine cultures, which consisted of a seven day treatment with DMEM/F12 + 1% B27 (Invitrogen, CA). Except for the last stage, all other stages included daily media changes. An outline of the procedure is depicted in Figure 1. At each stage cell number was calculated using a hemocytometer and RNA was collected for PCR analysis. All samples were colleted in triplicate.

[0156] Figure 2 panels a and b displays CXCR4 expression in cells as measured by FACS and real-time PCR data for at the three day time point, corresponding to stage 1. Fold change in expression is shown relative to undifferentiated H1 ES cells.

Example 2

Addition of Activin A to Stage 6 Cultures Significantly Enhanced the Expression of Pancreatic Endocrine Markers

[0157] Cells of the human embryonic stem cells line H1 at passage 43 were cultured on MATRIGEL™ (1:30 dilution) coated dishes and differentiated to pancreatic endocrine cells according to the methods described in Example 1. At stage 6, some of the cultures were treated with 10 ng/ml of activin A (R&D Systems, MN) for seven days. At day 7, cells were treated for 5 mins at room temperature with 1X Accutase (Sigma, MO) followed by removal of Accutase and addition of DMEM/12 + 1% B27. The attached cells were removed using a cell scarper and gently resuspended and passed through a 40 μm cell strainer. The flow through and cells retained on the strainer were removed and samples collected for PCR and staining for Dithizone. Dithizone (DTZ) is a dye that selectively binds to zinc, which has been shown to be present within islets. Figure 3, panels a-c depicts DTZ stained cultures before and after separation using a 40 μm cell strainer. A significant (approximately 80%) portion of clusters greater than 40 μm were stained positive for DTZ. This provides for a rapid and simple method of enrichment of endocrine rich clusters. Figure 4, panels a-f shows the gene expression profile of cells treated at stage 6 +/- 10 ng/ml of activin A and subsequently enriched using a 40 μm filter. Addition of activin A to stage 6, significantly boosted expression of all key pancreatic endocrine markers.

[0158] 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.

What is claimed is:

1. A method of increasing the expression of markers associated with the pancreatic endocrine lineage comprising treating cells expressing markers associated with the pancreatic endocrine lineage with medium comprising a sufficient amount of a TGF-β receptor agonist to cause an increase in expression of markers associated with the pancreatic endocrine lineage.

2. The method of claim 1, wherein the TGF-β receptor agonist is selected from the group consisting of activin A, activin B, activin C, peptide variants of activin A, GDF-8, GDF-11 and GDF-15.

3. The method of claim 1, wherein the cells expressing markers associated with the pancreatic endocrine lineage are enriched.

4. A method for differentiating pluripotent stem cells, comprising the steps of:

    a. Culturing the pluripotent stem cells,

    b. Differentiating the pluripotent stem cells into cells expressing markers characteristic of the definitive endoderm lineage,

    c. Differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage, and

    d. Differentiating the cells expressing markers characteristic of the pancreatic endoderm lineage into cells expressing markers characteristic of the pancreatic endocrine lineage.

5. The method of claim 4, wherein the expression of markers characteristic of the pancreatic endocrine lineage is increased by treating the cells expressing markers characteristic of the pancreatic endocrine lineage with medium comprising a sufficient amount of a TGF-β receptor agonist to cause an increase in expression of markers associated with the pancreatic endocrine lineage.

6. The method of claim 4, wherein the TGF-β receptor agonist is selected from the group consisting of activin A, activin B, activin C, peptide variants of activin A, GDF-8, GDF-11 and GDF-15.

7. The method of claim 4, wherein the cells expressing markers associated with the pancreatic endocrine lineage are enriched.