DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

CROSS REFERENCE TO RELATED APPLICATION

[0001] The present invention claims priority to application serial number 61/226,936, filed July 20, 2009.

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 to produce cells capable of producing insulin following transplantation into an animal.

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] 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).

[00010] 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).

[0010] 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).

[0011] 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.).

[0012] 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).

[0013] 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".

[0014] However, the mouse model of embryonic stem cell development may not exactly mimic the developmental program in higher mammals, such as, for example, humans.

[0015] 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).

[0016] 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).

[0017] 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".

[0018] 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.

[0019] 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).

[0020] In another example, Grapin-Botton *et al.* states: "Early activation of Ngn3 almost exclusively induced glucagon+ cells while depleting the pool of pancreas progenitors. As from E11.5, PDX-1 progenitors became competent to differentiate into insulin [positive] and PP [positive] cells" (Johansson KA *et al*, Developmental Cell 12, 457–465, March 2007).

[0021] The expression of NGN3 in cells expressing markers characteristic of the pancreatic endoderm lineage may reduce the ability of the cells to further differentiate into insulin expressing cells. Previous studies have showed that cells expressing markers characteristic of the pancreatic endoderm lineage that express NGN3 are more likely to produce glucagon expressing cells than insulin expressing cells, when subjected to further differentiation. However, NGN3 expression is required to form pancreatic endocrine cells, or pancreatic endocrine precursor cells (cells that can form, for example glucagon, or insulin expressing cells). Therefore, the temporal regulation of NGN3 is important in guiding the ultimate fate of pancreatic endocrine precursor cells toward insulin expressing cells.

[0022] 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 insulin expressing cells. The present invention takes an alternative approach to improve the efficiency of differentiating human embryonic stem cells toward insulin expressing cells, by generating a population of cells

expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3. The methods of the present invention maintain NGN3 expression at a minimal level until the differentiation of pancreatic endoderm toward pancreatic endocrine precursor cells is commenced.

SUMMARY

[0023] In one embodiment, the present invention provides a population of cells expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3. In one embodiment, the population of cells is capable of producing C-peptide following implantation into an animal.

[0024] In one embodiment, the present invention provides a method to differentiate a population of pluripotent stem cells into a population of cells expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3, 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, and

c. Differentiating the cells expressing markers characteristic of the definitive endoderm lineage into cells expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3 by treating cells expressing markers characteristic of the definitive endoderm lineage with a first medium supplemented with FGF7, followed by culturing the cells in a second medium supplemented with FGF7, a factor capable of inhibiting BMP, a TGF-β receptor agonist, retinoic acid, and a hedgehog signaling pathway inhibitor.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Figure 1 shows the effect of activin A on the expression of NKX6.1, NGN3, PDX1, PTF1 alpha, and ARX at stage 3 day 4, in cells treated according to the methods described in Example 1. Duplicate samples were collected for real-time PCR analysis. The plots represent fold induction for each gene relative to the control group (light grey bars). The dark gray bars represent cells treated with FGF7, cyclopamine-KAAD, retinoic acid, 20

ng/ml activin A and noggin. Black bars represent cells treated with FGF7, cyclopamine-KAAD, retinoic acid, 50ng/ml activin A and noggin.

[0026] Figure 2 shows immunofluorescence images, showing the expression of NKX6.1 (panels a, c, and e) and NGN3 (panels b, d and f) in cells treated with FGF7 + Noggin + retinoic acid + KAAD-cyclopamine (panel a and b), or cells treated with FGF7 + Noggin + retinoic acid + KAAD-cyclopamine + 20 ng/ml activin A (panel c and d), and cells treated with FGF7 + Noggin + retinoic acid + KAAD-cyclopamine + Alk5 inhibitor II.

[0027] Figure 3 shows immunofluorescence images, showing the expression of PDX1 (panels a and c), CDX2 (panel b and d) in cells treated with DMEM-high glucose supplemented with 1%B27+ FGF7 + Noggin + retinoic acid + KAAD-cyclopamine + 20 ng/ml activin A (panels a and b), and cells treated with DMEM/F12 supplemented with 1%B27+ FGF7 + Noggin + retinoic acid + KAAD-cyclopamine + 20 ng/ml activin A (panels c and d).

[0028] Figure 4 shows the effect of activin A, activin B, TGFβ2, GDF11 and GDF8 on the expression of NKX6.1, NGN3, and PDX1 at stage 3 day 4 in cells treated according to the methods described in Example 1. Duplicate samples were collected for real-time PCR analysis. The plots represent fold induction for each gene relative to the group treated with FGF7+cyclopamine-KAAD+retinoic acid + Noggin.

[0029] Figure 5 shows the effect of Noggin and Alk5 inhibitor II treatment on the expression of NGN3, NEUROD, NKX2.2 and PAX6 (panel a), and NKX6.1, PDX1and PTF1 alpha (panel b) at stage 4 day 3 in cells treated according to the methods described in Example 2. Duplicate samples were collected for real-time PCR analysis. The plots represent fold induction for each gene relative to the basal medium only (DMEM-high glucose+1% B27) group (Light grey bars). The dark gray bars represent cells treated with Noggin and Alk5 inhibitor II.

[0030] Figure 6 shows immunofluorescence images in cells treated with FGF7 + Noggin + retinoic acid + KAAD-cyclopamine + activin A for four days, followed by Noggin and Alk5 inhibitor II for three days, as described in Example 2. Panel a shows the expression of NKX6.1, NGN3 and an overlay of NKX6.1 and NGN3. Panel b shows the expression of PDX1, NGN3 and an overlay of PDX1 and NGN3.

[0031] Figure 7 shows the expression of NGN3, PAX4, PDX1, NKX6.1, NEUROD, insulin and glucagon in cells at stage 4, day 3 (light grey bars), or stage 5, day 3 (dark grey bars), or stage 5, day 7 (black bars), of the treatment protocol described in Example 3. Duplicate samples were collected for real-time PCR analysis. The plots represent fold induction for each gene relative to the expression detected at stage 4 day one.

[0032] Figure 8 shows immunofluorescence images, showing expression of insulin, glucagon and NKX6.1, in cells at stage 5 day 7 of the treatment protocol described in Example 3. Overlays of insulin and glucagon expression, and insulin and NKX6.1 expression are also shown.

[0033] Figure 9 shows circulating human C-peptide (panel a) and non-fasting blood glucose level (panel b) in STZ induced diabetic SCID-beige mice that received the cells of the present invention under the kidney capsule. C-peptide levels and blood glucose levels were detected at the times indicated.

DETAILED DESCRIPTION

[0034] 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

[0035] 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.

[0036] 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).

[0037] 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.

[0038] "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.

[0039] "Cells expressing markers characteristic of the pancreatic endoderm lineage", as used herein, refers to cells expressing at least one of the following markers: PDX1, HNF1 beta, PTF1 alpha, HNF6, NKX6.1, or HB9. Cells expressing markers characteristic of

the pancreatic endoderm lineage include pancreatic endoderm cells, primitive gut tube cells, and posterior foregut cells.

[0040] "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.

[0041] "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.

[0042] "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.

Isolation, Expansion and Culture of Pluripotent Stem Cells

Characterization of Pluripotent Stem Cells

[0043] 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.

[0044] 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.

[0045] 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

[0046] 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).

[0047] 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

[0048] 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.

[0049] 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.

[0050] 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".

[0051] 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.

[0052] 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.

[0053] 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.

[0054] In a further example, Miyamoto *et al* (Stem Cells 22: 433-440, 2004) disclose a source of feeder cells obtained from human placenta.

[0055] Amit *et al* (Biol. Reprod 68: 2150-2156, 2003) discloses a feeder cell layer derived from human foreskin.

[0056] In another example, Inzunza *et al* (Stem Cells 23: 544-549, 2005) disclose a feeder cell layer from human postnatal foreskin fibroblasts.

[0057] 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."

[0058] 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)."

[0059] 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.

[0060] In another example, US20070010011 discloses a chemically defined culture medium for the maintenance of pluripotent stem cells.

[0061] 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.

[0062] 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.

[0063] 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.

[0064] 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.

[0065] 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."

[0066] 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."

[0067] 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.

[0068] 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.

[0069] 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.

[0070] 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.

[0071] 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.

[0072] 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 Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage from Pluripotent Stem Cells

[0073] In one embodiment, the present invention provides a method for producing cells expressing markers characteristic of the pancreatic endoderm lineage 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, and

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

[0074] In one aspect of the present invention, the cells expressing markers characteristic of the pancreatic endoderm lineage co-express PDX1, NKX6.1, but do not express CDX-2 and NGN3.

Differentiation of Pluripotent Stem Cells into Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage

[0075] 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.

[0076] 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.

[0077] 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).

[0078] 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).

[0079] 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).

[0080] 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).

[0081] 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).

[0082] 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.

[0083] 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).

[0084] 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.

[0085] 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.

[0086] 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.

[0087] 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.

[0088] 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.

[0089] 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.

[0090] 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 into Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage

[0091] In one embodiment, cells expressing markers characteristic of the definitive endoderm lineage are differentiated into cells expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3, by culturing the cells expressing markers characteristic of the definitive endoderm lineage in a first medium supplemented with FGF7, followed by culturing the cells in a second medium supplemented with FGF7, a factor capable of inhibiting BMP, a TGFβ receptor agonist, retinoic acid, and a hedgehog signaling pathway inhibitor.

[0092] In one embodiment, FGF7 may be used at a concentration from about 50pg/ml to about 50μg/ml. In one embodiment, FGF7 is used at a concentration of 50ng/ml.

[0093] In one embodiment, the factor capable of inhibiting BMP is noggin. Noggin may be used at a concentration from about 500ng/ml to about 500μg/ml. In one embodiment, noggin is used at a concentration of 100ng/ml.

[0094] In one embodiment, the TGFβ receptor agonist is selected from the group consisting of activin A, activin B, TGFβ-I, TGFβ-II, GDF-8, and GDF-11.

[0095] Activin A may be used at a concentration from about 2ng/ml to 100ng/ml. In one embodiment, activin A is used at a concentration of 20ng/ml. In an alternate embodiment, activin A is used at a concentration of 50ng/ml.

[0096] Activin B may be used at a concentration from about 2ng/ml to 100ng/ml. In one embodiment, activin B is used at a concentration of 20ng/ml. In an alternate embodiment, activin B is used at a concentration of 50ng/ml.

[0097] TGFβ-I may be used at a concentration from about 2ng/ml to 100ng/ml. In one embodiment, TGFβ-I is used at a concentration of 20ng/ml. In an alternate embodiment, TGFβ-I is used at a concentration of 50ng/ml.

[0098] TGFβ-II may be used at a concentration from about 2ng/ml to 100ng/ml. In one embodiment, TGFβ-II is used at a concentration of 20ng/ml. In an alternate embodiment, TGFβ-II is used at a concentration of 50ng/ml.

[0099] GDF-8 may be used at a concentration from about 2ng/ml to 100ng/ml. In one embodiment, GDF-8 is used at a concentration of 20ng/ml. In an alternate embodiment, GDF-8 is used at a concentration of 50ng/ml.

[0100] GDF-11 may be used at a concentration from about 2ng/ml to 100ng/ml. In one embodiment, GDF-11 is used at a concentration of 20ng/ml. In an alternate embodiment, GDF-11 is used at a concentration of 50ng/ml.

[0101] Retinoic acid may be used at a concentration from about 1nM to about 1mM. In one embodiment, retinoic acid is used at a concentration of 1μM.

[0102] In one embodiment, the hedgehog signaling pathway inhibitor is cyclopamine-KAAD. Cyclopamine-KAAD may be used at a concentration from about 0.025μM to about 2.5μM. In one embodiment, cyclopamine-KAAD is used at a concentration of 0.25μM.

[0103] 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.

[0104] 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)).

[0105] 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.

[0106] 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.

[0107] 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.

[0108] 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.

[0109] Markers characteristic of the pancreatic endoderm lineage are selected from the group consisting of PDX1, HNF1 beta, PTF1 alpha, HNF6, 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.

Formation of Cells Expressing Markers Characteristic of the Pancreatic Endocrine Lineage

[0110] In one embodiment, the cells expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3, produced by the methods of the present invention may be further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage.

[0111] 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 proposed in this invention.

[0112] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention 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.

[0113] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention 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 (Sigma-Aldrich, MO) and exendin 4. An example of this method is disclosed in D' Amour *et al*, Nature Biotechnology, 2006.

[0114] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention 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.

[0115] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention 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.

[0116] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention 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.

[0117] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention 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.

[0118] For example, cells expressing markers characteristic of the pancreatic endoderm lineage obtained according to the methods of the present invention 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. 60/990,529, assigned to LifeScan, Inc.

[0119] 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.

[0120] 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.

Therapies

[0121] In one aspect, the present invention provides a method for treating a patient suffering from, or at risk of developing, Type1 diabetes. In one embodiment, the 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. In an alternate embodiment, the method involves culturing pluripotent stem cells, differentiating the pluripotent stem cells *in vitro* into cells expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1, NKX6.1, but do not express CDX2

and NGN3, and implanting the cells of the pancreatic endoderm lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3 into a patient.

[0122] In yet another aspect, this invention provides a method for treating a patient suffering from, or at risk of developing, Type 2 diabetes. In one embodiment, the 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. In an alternate embodiment, the method involves culturing pluripotent stem cells, differentiating the pluripotent stem cells *in vitro* into cells expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3, and implanting the cells of the pancreatic endoderm lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3 into a patient.

[0123] 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-derived 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.

[0124] 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.

[0125] 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.

[0126] 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.

[0127] 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.

[0128] 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.

[0129] 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.

[0130] 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.

[0131] 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.

[0132] 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.

[0133] 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.

[0134] 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.

[0135] 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.

[0136] 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).

[0137] The present invention is further illustrated, but not limited by, the following examples.

EXAMPLES

Example 1

Differentiation of Human Pluripotent Stem Cells into Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3

[0138] This example demonstrates that activin A can be used in combination with Noggin and retinoic Acid to facilitate the up-regulation of NKX6.1 expression. Briefly, cells of the human embryonic stem cell line H1 were cultured on MATRIGEL™ (1:30 dilution) coated dishes and RPMI medium supplemented with 2% BSA, 100 ng/ml activin A, 20 ng/ml WNT-3a, 8 ng/ml of bFGF for one day, followed by treatment with RPMI media supplemented with 2% BSA, 100 ng/ml activin A, 8 ng/ml of bFGF for an additional two days (Stage 1), then

a. DMEM/F12 + 2% BSA + 50 ng/ml FGF7 for three days (Stage 2), then

b. DMEM-High glucose + 1% B27 + 50 ng/ml FGF7 + 0.25 μM Cyclopamine-KAAD + 2 μM Retinoic acid (RA) + 100 ng/ml of Noggin + 20ng/ml activin A, or 50 ng/ml activin A for four days (Stage 3).

[0139] As a control, separate populations of cells were treated with DMEM High glucose, supplemented with 1% B27, 50 ng/ml FGF7, 0.25 μM Cyclopamine- KAAD, 2 μM Retinoic acid (RA), and 100 ng/ml of Noggin.

[0140] Cultures were sampled in duplicate on stage 3 day 4, and analyzed for the expression of pancreatic markers using real-time PCR.

[0141] As shown in Figure 1, there was a dramatic increase of NKX6.1 expression at stage 3 day 4, compared to samples obtained from cells receiving no activin A. The increase in expression of NKX6.1, mediated by activin A, increased in proportion to the activin A dose. A down-regulation of NGN3 expression was also observed in the cells at stage 3 day 4. To determine whether the TGF-beta pathway was involved in facilitating the formation of pancreatic endoderm cells that co-expressed PDX1 and NKX-6.1, cells were treated as follows:

[0142] Cells of the human embryonic stem cell line H1 were cultured on MATRIGEL-coated plates (1:30 dilution), and differentiated into pancreatic endocrine precursor cells using the following protocol:

a. RPMI medium (Catalogue#22400, Invitrogen, Ca) 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 (Stage 1), then

b. DMEM/F12 (Catalogue#11330, Invitrogen, Ca) + 2% BSA + 50 ng/ml FGF7 for three days (Stage 2), then

c. Treatment 1: DMEM (high glucose) + 1% B27 (Invitrogen, CA) + 50 ng/ml FGF7, 0.25 μM Cyclopamine-KAAD, 2 μM retinoic acid (RA) and 100 ng/ml of Noggin for four days (Stage 3), or

d. Treatment 2: DMEM (high glucose) + 1% B27 (Invitrogen, CA) + 50 ng/ml FGF7, 0.25 μM Cyclopamine-KAAD, 2 μM retinoic acid (RA), 100 ng/ml of Noggin, 20ng/ml activin A for four days (Stage 3), or

e. Treatment 3: DMEM (high glucose) + 1% B27 (Invitrogen, CA) + 50 ng/ml FGF7, 0.25 μM Cyclopamine-KAAD, 2 μM retinoic acid (RA), 100 ng/ml of Noggin, 1 μM ALK5 inhibitor II (Alexis Biochemical) for four days (Stage 3).

[0143] Cultures were sampled in duplicate on stage 3 day 4, and analyzed for expression of pancreatic markers using real-time PCR. Cultures also were fixed in parallel for immunofluorescence analysis.

[0144] Table 1 shows the relative expression levels of NKX6.1, NGN3 and PDX1 at stage 3 day 4 when normalized to the most minimal condition in this experiment (treatment 1).

Table 1

	NGN3	NKX6.1	PDX1
Treatment 1	1	1	1
Treatment 2	0.02	5.97	1.13
Treatment 3	5.64	0.02	0.65

[0145] Treatment 1 (FGF7, retinoic acid and Noggin) induced the expression of NKX6.1 and NGN3. See Figure 2, panels a and b. However, the addition of activin A (treatment 2) blocked the expression of NGN3, and significantly increased the number of NKX6.1 expressing cells. See Figure 2, panels c and d. These data suggest that activation of the TGFβ receptor pathway during the formation of a population of cells expressing markers characteristic of the pancreatic endoderm lineage results in a population of cells

expressing markers characteristic of the pancreatic endoderm lineage that do not express NGN3.

[0146] Incubation of cells with the TGFβ receptor inhibitor Alk5 inhibitor II confirmed this hypothesis (see Treatment 3). Treatment of cells in DMEM (high glucose) supplemented with 1% B27 (Invitrogen, CA), 50 ng/ml FGF7, 0.25 μM Cyclopamine-KAAD, 2 μM retinoic acid (RA), 100 ng/ml of Noggin, 1 μM ALK5 inhibitor II resulted in a decrease in the level of expression of NKX6.1. The level of expression observed was lower than that observed in cells that received treatment 1. See Table 1, and Figure 2, panel e. On the other hand, the number of NGN3 expressing cells was significantly increased. See Table 1, and Figure 2, panel f. No significant impact on the PDX1 expression was observed. These results suggest that the combination of Noggin, retinoid acid and activin A acts synergistically to specify a pancreatic precursor cell population that is positive for the expression of NKN6.1 and PDX1, but negative for the expression of NGN3.

[0147] As shown in Figure 3, panels a and b, most PDX1 expressing cells generated by using DMEM (Treatment 2 -DMEM (high glucose) + 1% B27 (Invitrogen, CA) + 50 ng/ml FGF7, 0.25 μM Cyclopamine-KAAD, 2 μM retinoic acid (RA), 100 ng/ml of Noggin, 20ng/ml activin A) did not express CDX2 at stage 3 day 4. This is in contrast with PDX1 expressing cells generated by using DMEM/F12 supplemented with 1% B27 (Invitrogen, CA) + 50 ng/ml FGF7, 0.25 μM Cyclopamine-KAAD, 2 μM retinoic acid (RA), 100 ng/ml of Noggin, 20ng/ml activin A , wherein most PDX1 expressing cells also expressed CDX2. See Figure 3, panels c and d.

[0148] A number of TGFβ receptor agonists were tested. Replacement of activin A in treatment 2 with either GDF-8, GDF-11, activin B, or TGFβ2 all produced similar results: Treatment of either GDF-8, GDF-11, activin B, or TGFβ2 for four days resulted in an increase in the expression of NKX6.1 and a down-regulation of NGN3. See Figure 4, panels a and c. No significant impact on PDX1 expression was observed. See Figure 4, panel b.

Example 2

Differentiation of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3 into Pancreatic Endocrine Precursor Cells

[0149] Previous studies showed that cells expressing markers characteristic of the pancreatic endoderm lineage are more likely to produce glucagon expressing cells than insulin expressing cells, when subjected to further differentiation. This may be due, in part, to the expression of NGN3 in the pancreatic endoderm cells. The methods of the present invention produce a population of pancreatic endoderm cells that do not express NGN3, and therefore would be more likely to differentiate into insulin expressing cells. However, NGN3 expression is required to form pancreatic endocrine cells, or pancreatic endocrine precursor cells (cells that can form, for example glucagon, or insulin expressing cells). Therefore, the temporal regulation of NGN3 is important in guiding the ultimate fate of pancreatic endocrine precursor cells.

[0150] The present invention hypothesizes that NGN3 expression should be maintained at a minimal level until the differentiation of pancreatic endoderm toward pancreatic endocrine precursor cells is commenced.

[0151] Briefly, cells of the human embryonic stem cells line H1 were cultured on MATRIGEL™ coated dishes (1:30 dilution) with RPMI medium + 2% BSA + 100 ng/ml activin A + 20 ng/ml WNT-3a + 8 ng/ml of bFGF for one day followed by treatment with RPMI media + 2% BSA + 100 ng/ml activin A + 8 ng/ml of bFGF for an additional two days (Stage 1), then

a. DMEM/F12 + 2% BSA + 50 ng/ml FGF7 for three days (Stage 2), then

b. DMEM-High glucose + 1% B27 + 50 ng/ml FGF7 + 0.25 μM Cyclopamine-KAAD + 2 μM Retinoic acid (RA) + 100 ng/ml of Noggin + 20ng/ml Activin A for four days (Stage 3), then

c. DMEM-High glucose + 1% B27 + 100 ng/ml Noggin + 1 μM ALK5 inhibitor II for three days (Stage 4) , or

d. DMEM-High glucose + 1% B27 only for three days (Stage 4).

[0152] The above differentiation protocol was designed to test the ability of cells expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1 and NKX6.1, but do not express CDX2 and NGN3 to further differentiate into pancreatic endocrine precursor cells. Pancreatic endocrine precursor cells express NGN3.

[0153] Simply culturing the cells expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1 and NKX6.1, but do not express CDX2 and NGN3 in basal medium (DMEM-High glucose + 1% B27) did not result in the induction of NGN3 expression. See Figure 5, panel a, light grey bars. Similarly, the expression of NEUROD, NKX2.2, and PAX6 was not observed.

[0154] In contrast, in cells incubated in the presence of Alk5 inhibitor II, a significant increase in NGN3 expression was observed. See Figure 5, panel a, dark grey bars. The up regulation in the expression of NEUROD, NKX2.2, PAX4 and PAX6 was also observed, as well as an increase in the expression of PTF1 alpha. See Figure 5, panels a and b. The presence of Alk5 inhibitor II did not appear to affect the expression of PDX1 or NKX6.1. See Figure 5, panel b.

[0155] The increase in NGN3 expression, as detected by PCR, in the presence of Alk5 inhibitor II was also reflected in an increase in the number of cells that were positive for the presence of NGN3 protein, as detected by immunocytochemistry. See Figure 6. Analysis of the images revealed that the majority of the NGN3 expressing cells also co-expressed PDX1, but not NKX6.1. Furthermore, the majority of the NKX6.1 cells co-expressed PDX1. At this stage, the expression level of endocrine cells, as evidenced by the expression of example, insulin and glucagon, was minimal. Our results suggested that activation of TGF-beta pathway will facilitate the formation of a population of cells that co-express PDX1 and NKX6.1, and subsequent inhibition of the TGF-beta pathway will further induce the differentiation of endoderm into endocrine precursor cells.

Example 3

Differentiation of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage that co-express PDX1, NKX6.1, but do not express CDX2 and NGN3 into Pancreatic Endocrine Cells

[0156] This Example was designed to test the ability of cells expressing markers characteristic of the pancreatic endoderm lineage that co-express PDX1 and NKX6.1, but do not express CDX2 and NGN3 to further differentiate into pancreatic endocrine cells, from pancreatic endocrine precursor cells.

[0157] Briefly, cells of the human embryonic stem cells line H1 were cultured on MATRIGEL™ coated dishes (1:30 dilution) with RPMI medium + 2% BSA + 100 ng/ml activin A + 20 ng/ml WNT-3a + 8 ng/ml of bFGF for one day followed by treatment with RPMI media + 2% BSA + 100 ng/ml activin A + 8 ng/ml of bFGF for an additional two days (Stage 1), then

a. DMEM/F12 + 2% BSA + 50 ng/ml FGF7 for three days (Stage 2), then

b. DMEM-High glucose + 1% B27 + 50 ng/ml FGF7 + 0.25 μM Cyclopamine-KAAD + 2 μM Retinoic acid (RA) + 100 ng/ml of Noggin + 20ng/ml Activin A for four days (Stage 3), or

c. DMEM-High glucose + 1% B27 + 50 ng/ml FGF7 + 0.25 μM Cyclopamine-KAAD + 2 μM Retinoic acid (RA) + 100 ng/ml of Noggin + 20ng/ml Activin A for four days (Stage 3), then

d. DMEM-High glucose + 1% B27 + 100 ng/ml Noggin + 1 μM ALK5 inhibitor II for three days (Stage 4)

e. DMEM-High glucose + 1% B27 + 100 ng/ml Noggin + 1 μM ALK5 inhibitor II + Betacellulin 20ng/ml for five to seven days (Stage 5).

[0158] The expression of NGN3 and PAX4 declined from their maximum levels at stage four day three, reaching a lower level of expression at stage 5, day 7. During this time, the expression of endocrine markers, for example insulin and glucagon increased. See Figure 7. These data suggest that the cells of the present invention were able to form pancreatic endocrine cells from endocrine precursor cells.

[0159] Cells expressing either insulin alone, or glucagon alone, or both insulin and glucagon were observed. See Figure 8 and Table 2. By FACS analysis of cultures at stage 5, day 7 (Table 2), approximately 60 % of pancreatic endoderm precursors that co-express PDX1 and NKX6.1 differentiated were expressing the pan-endocrine marker synaptophysin.

The percentage of single insulin-expressing cells was 10.4%, single glucagon-expressing cells was 5.1%. In addition, there were20% are insulin and glucagon co-expressing cells. Among the cells that expressed insulin and no other pancreatic hormone, 60% co-expressed NKX6.1 (a marker for mature beta cells). These data suggest that more mature insulin expressing cells were formed by the methods of the present invention.

Table 2

	Insulin
Insulin+	10.4%
Glucagon+	5.1%
Insulin+/Glucagon+	20%
Synaptophysin+	60%
NKX6.1+	52%
NKX6.1+/Insulin+	6%

Example 4

Implantation of the Cells of the Present Invention into STZ Induced Diabetic Severe Combined Immunodeficient (SCID) – beige (Bg) Mice

[0160] Cells of the human embryonic stem cells line H1 were cultured on MATRIGEL® - coated dishes (1:30 dilution) with RPMI medium + 0.2% FBS + 100 ng/ml activin A + 20 ng/ml WNT-3a for one day followed by treatment with RPMI media + 0.5% FBS + 100 ng/ml activin A for an additional two days (Stage 1), then,

a. DMEM/F12+ 2% FBS + 50 ng/ml FGF7 for three days (Stage 2), then

b. DMEM-High glucose + 1% B27+ 0.25 μM Cyclopamine- KAAD + 2 μM Retinoic acid (RA) + 100 ng/ml of Noggin +50ng/ml FGF7+20ng/ml Activin A for four days (Stage 3), then

c. DMEM-High glucose + 1% B27 + 100 ng/ml Noggin + 1 μM ALK5 inhibitor II for four days (Stage 4).

[0161] Five to six-week-old male scid-beige mice (C.B-*Igh*-1b/GbmsTac-*Prkdc*^{scid}-*Lyst*^{bg} N7) were purchased from Taconic Farms. Mice were housed in microisolator cages with free access to sterilized food and water. In preparation for surgery, mice were identified by ear tagging and their body weight measured and their blood glucose determine by a hand held glucometer (One Touch, LifeScan).

[0162] Two weeks prior to transplant, mice were weighed and dosed with 80 mg/kg streptozotocin (Sigma) dissolved in acetate buffer with a pH of 4.5 on each of five consecutive days to induce diabetes. The blood glucose was monitored and only mice with blood glucose > 300 mg/dL were used as transplant recipients.

[0163] Mice were anesthetized with a mixture of isolflurane and oxygen and the surgical site was shaved with small animal clippers. Mice were dosed with 0.1 mg.kg Buprenex subcutaneously pre-operatively. The surgical site was prepared with successive washes of 70% isopropyl alcohol and 10% povidone-iodide.

[0164] Cells at the end of stage four were briefly treated with 1mg/ml dispase for five minutes and mechanically scored using a 1-ml glass pipette and subsequently transferred to non-adherent plates for culture overnight. During the preoperative preparation of the mice, the cells were centrifuged in a 1.5 ml microfuge tube and most of the supernatant removed, leaving just enough to collect the pellet of cells. The cells were collected into a Rainin Pos-D positive displacement pipette and the pipette was inverted to allow for the cells to settle by gravity. The excess media was dispensed leaving a packed cell preparation for transplant.

[0165] For transplantation, a 24G x ¾" I.V. catheter was used to penetrate the kidney capsule and the needle was removed. The catheter was then advanced under the kidney capsule to

the distal pole of the kidney. The Pos-D pipette tip was placed firmly in the hub of the catheter and the 5 million cells dispensed from the pipette through the catheter under the kidney capsule and delivered to the distal pole of the kidney. The kidney capsule was sealed with a low temperature cautery and the kidney was returned its original anatomical position. In parallel, cell aggregates containing 5 million cells were loaded into the 50-µl device using Post-D pipette tip. The 50-µl devices were purchased from TheraCyte, Inc (Irvine, CA). The device was sealed by medical adhesive silicone type A (Dow Corning, Cat#129109) after the loading, and implanted subcutaneously into SICD/Bg mice (animal Nos.3 and 4). The muscle was closed with continuous sutures using 5-0 vicryl and the skin closed with wound clips. Mice were dosed with 1.0 mg.kg Metacam subcutaneously post-operatively. The mouse was removed from the anesthesia and allowed to fully recover.

[0166] Following transplantation, mice were weighed once per week and blood glucose measured twice a week. At various intervals following transplantation, blood was drawn via the retro-orbital sinus into microfuge tubes containing a small amount of heparin. The blood was centrifuged and the plasma placed into a second microfuge tube and frozen on dry ice and then stored at -80°C until human c-peptide assay was performed. Human c-peptide levels were determined using the Mercodia/ALPCO Diagnotics Ultrasensitive C-peptide ELISA (Cat No. 80-CPTHU-E01, Alpco Diagnostics, NH) according to the manufacturer's instructions.

[0167] Human C-peptide was detected in animal serum as early as 4 weeks in kidney capsule group after transplantation and increased over time (Figure 9, panel a). At the end of two months, we were able to detect significant amount of circulating human C-peptide, 1.1± 0.5 ng/ml (Figure 9, panel a). Up to two months after transplantation, their non-fasting blood glucose levels consistently above 400 ng/dl. In this study, no administration of insulin was required. The rising serum levels of graft-derived insulin, greater than 1 ng/ml results in gradual decreases in hyperglycemia. At three months, we were able to detect significant amount of circulating human C-peptide in 90% STZ induced diabetic animals. Average circulating human C-peptide was 2± 0.96 ng/ml (n=8) (Figure 9, panel a). The blood glucose levels of 90% diabetic mice engrafted with hES cell-derived endocrine precursors were below 200 mg/dl and the levels were maintained. After we surgically removed the graft, blood glucose levels were increased to hyperglycemic

levels, shortly after graft removal, which suggested that engrafted human cells were solely responsible for maintaining the normoglycemia in STZ-treated mice (Figure 9, panel b).

[0168] This example demonstrates that PDX1 and NKX6.1 co-expressing cell population and the endocrine progenitor cell population derived from PDX-1 and NKX6.1 co-expressing cell population have the competency to further differentiate into insulin-secreting cells *in vivo*.

[0169] 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 to differentiate a population of human pluripotent stem cells into a purified population of pancreatic endoderm cells that co-express PDX-1 and NKX-6.1, but do not express CDX-2 and NGN-3 comprising the steps of:

(a) culturing human pluripotent stem cells,

(b) differentiating the human pluripotent stem cells into definitive endoderm cells by treating the human pluripotent stem cells with a medium supplemented with a TGF-β receptor agonist,

(c) differentiating the definitive endoderm cells into a population of pancreatic endoderm cells that co-express PDX1 and NKX6.1, but do not express CDX2 and NGN3 by treating the definitive endoderm cells with a first medium supplemented with FGF7, followed by culturing the cells in a second medium supplemented with FGF7, a factor capable of inhibiting BMP, retinoic acid, and a hedgehog signaling pathway inhibitor, and a TGF-β receptor agonist selected from the group consisting of: activin B, TGFβ-I, TGFβ-II, GDF-8, and GDF-11, and

(d) selecting cells which co-express PDX-1 and NKX6.1 and do not express CDX2 and NGN3 to obtain a purified population of pancreatic endoderm cells that co-express PDX-1 and NKX-6.1, but do not express CDX-2 and NGN-3.

2. The method of claim 1, wherein the hedgehog signaling pathway inhibitor is cyclopamine-KAAD.