METHODS FOR IDENTIFYING FACTORS FOR DIFFERENTIATING DEFINITIVE ENDODERM

Related Applications

[0001] This application is a continuation-in-part of U.S. Patent Application Number 11/115,868, entitled PDX1 EXPRESSING ENDODERM, filed April 26, 2005, which claims priority under 35 U.S.C. § 119(e) as a nonprovisional application of U.S. Provisional Patent Application Number 60/566,293, entitled PDX1 EXPRESSING ENDODERM, filed April 27, 2004; U.S. Provisional Patent Application Number 60/587,942, entitled CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM, filed July 14, 2004; and U.S. Provisional Patent Application Number 60/586,566, entitled CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM, filed July 9, 2004; this application is also a continuation-in-part of U.S. Patent Application Number 11/021,618, entitled DEFINITIVE ENDODERM, filed December 23, 2004, which claims priority under 35 U.S.C. § 119(e) as a nonprovisional application to U.S. Provisional Patent Application Number 60/587,942, entitled CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM, filed July 14, 2004; and U.S. Provisional Patent Application Number 60/586,566, entitled CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM, filed July 9, 2004. and U.S. Provisional Patent Application Number 60/532,004, entitled DEFINITIVE ENDODERM, filed December 23, 2003.

Field of the Invention

[0002] The present invention relates to the fields of medicine and cell biology. In particular, the present invention relates the identification of factors that are useful for differentiating definitive endoderm cells into other cell types.

Background

[0003] Human pluripotent stem cells, such as embryonic stem (ES) cells and embryonic germ (EG) cells, were first isolated in culture without fibroblast feeders in 1994 (Bongso et al., 1994) and with fibroblast feeders (Hogan, 1997). Later, Thomson, Reubinoff and Shamblott established continuous cultures of human ES and EG cells using mitotically inactivated mouse feeder layers (Reubinoff et al., 2000; Shamblott et al., 1998; Thomson et al., 1998).

[0004] Human ES and EG cells (hESCs) offer unique opportunities for investigating early stages of human development as well as for therapeutic intervention in several disease

states, such as diabetes mellitus and Parkinson's disease. For example, the use of insulin-producing β-cells derived from hESCs would offer a vast improvement over current cell therapy procedures that utilize cells from donor pancreases for the treatment of diabetes. However, presently it is not known how to generate an insulin-producing β-cell from hESCs. As such, current cell therapy treatments for diabetes mellitus, which utilize islet cells from donor pancreases, are limited by the scarcity of high quality islet cells needed for transplant. Cell therapy for a single Type I diabetic patient requires a transplant of approximately 8×10^8 pancreatic islet cells. (Shapiro et al., 2000; Shapiro et al., 2001a; Shapiro et al., 2001b). As such, at least two healthy donor organs are required to obtain sufficient islet cells for a successful transplant. Human embryonic stem cells offer a source of starting material from which to develop substantial quantities of high quality differentiated cells for human cell therapies.

[0005] Two properties that make hESCs uniquely suited to cell therapy applications are pluripotence and the ability to maintain these cells in culture for prolonged periods. Pluripotency is defined by the ability of hESCs to differentiate to derivatives of all 3 primary germ layers (endoderm, mesoderm, ectoderm) which, in turn, form all somatic cell types of the mature organism in addition to extraembryonic tissues (e.g. placenta) and germ cells. Although pluripotency imparts extraordinary utility upon hESCs, this property also poses unique challenges for the study and manipulation of these cells and their derivatives. Owing to the large variety of cell types that may arise in differentiating hESC cultures, the vast majority of cell types are produced at very low efficiencies. Additionally, success in evaluating production of any given cell type depends critically on defining appropriate markers. Achieving efficient, directed differentiation is of great importance for therapeutic application of hESCs.

[0006] In order to use hESCs as a starting material to generate cells that are useful in cell therapy applications, it would be advantageous to overcome the foregoing problems. Additionally, it would be beneficial to identify factors which promote the differentiation of precursor cells derived from hESCs to cell types useful for cell therapies.

Summary of the Invention

[0007] Embodiments of the present invention relate to methods of identifying one or more differentiation factors that are useful for differentiating cells in a cell population comprising PDX1-positive (PDX1-expressing) endoderm cells and/or PDX1-negative endoderm cells (endoderm cells which do not significantly express PDX1), such as definitive endoderm cells, into cells that are useful for cell therapy. For example, some embodiments of the methods described herein relate to methods of identifying factors capable of promoting the differentiation of definitive endoderm cells into cells which are precursors for tissues and/or organs which include, but are not limited to, pancreas, liver, lungs, stomach, intestine, thyroid, thymus, pharynx,

gallbladder and urinary bladder. In some embodiments, such precursor cells are PDX1-positive endoderm cells. In other embodiments, such precursor cells are endoderm cells that do not significantly express PDX1.

[0008] In some embodiments of the methods described herein, cell cultures or cell populations of definitive endoderm cells are contacted or otherwise provided with a candidate (test) differentiation factor. In preferred embodiments, the definitive endoderm cells are human definitive endoderm cells. In more preferred embodiments, the human definitive endoderm cells are multipotent cells that can differentiate into cells of the gut tube or organs derived therefrom.

[0009] In other embodiments of the methods described herein, cell cultures or cell populations of PDX1-positive endoderm cells are contacted or otherwise provided with a candidate differentiation factor. In preferred embodiments, the PDX1-positive endoderm cells are human PDX1-positive endoderm cells. In certain embodiments, the human PDX1-positive endoderm cells are PDX1-positive foregut/midgut endoderm cells. In more preferred embodiments, the human PDX1-positive endoderm cells are PDX1-positive foregut endoderm cells. In other preferred embodiments, the human PDX1-positive endoderm cells are PDX1-positive endoderm cells of the posterior portion of the foregut. In especially preferred embodiments, the human PDX1-positive foregut endoderm cells are multipotent cells that can differentiate into cells, tissues or organs derived from the anterior portion of the gut tube.

[0010] As related to the methods described herein, the candidate differentiation factor may be one that is known to cause cell differentiation or one that is not known to cause cell differentiation. In certain embodiments, the candidate differentiation factor can be a polypeptide, such as a growth factor. In some embodiments, the growth factor includes, but is not limited to, FGF10, FGF4, FGF2, Wnt3A or Wnt3B. In other embodiments, the candidate differentiation factor can be a small molecule. In particular embodiments, the small molecule is a retinoid compound, such as retinoic acid. Alternatively, in some embodiments, the candidate differentiation factor is not a retinoid, is not a foregut differentiation factor or is not a member of the TGFβ superfamily. In other embodiments, the candidate differentiation factor is any molecule other than a retinoid compound, a foregut differentiation factor, or a member of the TGFβ superfamily of growth factors, such as activins A and B. In still other embodiments, the candidate differentiation factor is a factor that was not previously known to cause the differentiation of definitive endoderm cells.

[0011] Additional embodiments of the methods described herein relate to testing candidate differentiation factors at a plurality of concentrations. For example, a candidate differentiation factor may cause the differentiation of definitive endoderm cells and/or PDX1-positive endoderm cells only at concentrations above a certain threshold. Additionally, a candidate differentiation factor can cause the same cell to differentiate into a first cell type when

provided at a low concentration and a second cell type when provided at a higher concentration. In some embodiments, the candidate differentiation factor is provided at one or more concentrations ranging from about 0.1 ng/ml to about 10 mg/ml.

[0012] Prior to or at approximately the same time as contacting or otherwise providing the cell culture or cell population comprising definitive endoderm cells and/or PDX1-positive endoderm cells with the candidate differentiation factor, at least one marker is selected and evaluated so as to determine its expression. This step may be referred to as the first marker evaluation step. Alternatively, this step may be referred to as determining expression of a marker at a first time point. The marker can be any marker that is useful for monitoring cell differentiation, however, preferred markers include, but are not limited to, sex determining region Y-box 17 (SOX17), pancreatic-duodenal homeobox factor-1 (PDX1), albumin, hepatocyte specific antigen (HAS), prospero-related homeobox 1 (PROX1), thyroid transcription factor 1 (TITF1), villin, alpha fetoprotein (AFP), cytochrome P450 7A (CYP7A), tyrosine aminotransferase (TAT), hepatocyte nuclear factor 4a (HNF4a), CXC-type chemokine receptor 4 (CXCR4), von Willebrand factor (VWF), vascular cell adhesion molecule-1 (VACM1), apolipoprotein A1 (APOA1), glucose transporter-2 (GLUT2), alpha-1-antitrypsin (AAT), glukokinase (GLUKO), and human hematopoietically expressed homeobox (hHEX) and CDX2.

[0013] After sufficient time has passed since contacting or otherwise providing cell culture or cell population comprising definitive endoderm cells and/or PDX1-positive endoderm cells with the candidate differentiation factor, the expression of the at least one marker in the cell culture or cell population is again evaluated. This step may be referred to as the second marker evaluation step. Alternatively, this step may be referred to as determining expression of a marker at a second time point. In preferred embodiments, the marker evaluated at the first and second time points is the same marker.

[0014] In some embodiments of the methods described herein, it is further determined whether the expression of the at least one marker at the second time point has increased or decreased as compared to the expression of this marker at the first time point. An increase or decrease in the expression of the at least one marker indicates that the candidate differentiation factor is capable of promoting the differentiation of the definitive endoderm cells and/or the PDX1-positive endoderm cells. Sufficient time between contacting or otherwise providing a cell culture or cell population comprising definitive endoderm cells and/or PDX1-positive endoderm cells with the candidate differentiation factor and determining expression of the at least one marker at the second time point can be as little as from about 1 hour to as much as about 10 days. In some embodiments, the expression of the at least one marker is evaluated multiple times subsequent to contacting or otherwise providing the cell culture or cell population comprising definitive endoderm cells and/or PDX1-positive endoderm cells with the candidate

differentiation factor. In certain embodiments, marker expression is evaluated by Q-PCR. In other embodiments, marker expression is evaluated by immunocytochemistry.

[0015] Additional embodiments of the present invention relate to a method of identifying a differentiation factor capable of promoting the differentiation of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells. In such methods, PDX1-negative definitive endoderm cells are contacted with a candidate differentiation factor and it is determined whether PDX1 expression in the cell population after contact with the candidate differentiation factor has increased as compared to PDX1 expression in the cell population before contact with the candidate differentiation factor. An increase in the PDX1 expression in the cell population indicates that the candidate differentiation factor is capable of promoting the differentiation of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells. In some embodiments, PDX1 expression is determined by quantitative polymerase chain reaction (Q-PCR). Some embodiments of the foregoing method further comprise the step of determining expression of the HOXA13 and/or the HOXC6 gene in the cell population before and after contact with the candidate differentiation factor. In some embodiments, the candidate differentiation factor is a small molecule, for example, a retinoid, such as RA. In others, the candidate differentiation factor is a polypeptide, for example, a growth factor, such as FGF-10.

[0016] Still other embodiments of the present invention relate to a method of identifying a differentiation factor capable of promoting the differentiation of PDX1-positive foregut endoderm cells. In such methods, PDX1-positive foregut endoderm cells are contacted with a candidate differentiation factor and it is determined whether expression of a marker in the population is increased or decreased after contact with the candidate differentiation factor as compared to the expression of the same marker in the population before contact with the candidate differentiation factor. An increase or decrease in the expression of the marker indicates that the candidate differentiation factor is capable of promoting the differentiation of PDX1-positive foregut endoderm cells. In some embodiments, marker expression is determined by Q-PCR. In some embodiments, the candidate differentiation factor is a small molecule, for example, a retinoid, such as RA. In others, the candidate differentiation factor is a polypeptide, for example, a growth factor, such as FGF-10.

[0017] Yet other embodiments of the present invention relate to cells differentiated by the methods described herein. Such cells include but are not limited to precursors of the pancreas, liver, lungs, stomach, intestine, thyroid, thymus, pharynx, gallbladder and urinary bladder. In some embodiments, the cells may be terminally differentiated. Other embodiments described herein relate to cell cultures and/or cell populations comprising the above-described cells.

[0018] In certain jurisdictions, there may not be any generally accepted definition of the term "comprising." As used herein, the term "comprising" is intended to represent "open"

language which permits the inclusion of any additional elements. With this in mind, additional embodiments of the present inventions are described with reference to the numbered paragraphs below:

[0019] 1. A method of identifying a differentiation factor capable of promoting the differentiation of human definitive endoderm cells in a cell population comprising human cells, said method comprising the steps of: (a) obtaining a cell population comprising human definitive endoderm cells; (b) providing a candidate differentiation factor to said cell population; (c) determining expression of a marker in said cell population at a first time point; (d) determining expression of the same marker in said cell population at a second time point, wherein said second time point is subsequent to said first time point and wherein said second time point is subsequent to providing said cell population with said candidate differentiation factor; and (e) determining if expression of the marker in said cell population at said second time point is increased or decreased as compared to the expression of the marker in said cell population at said first time point, wherein an increase or decrease in expression of said marker in said cell population indicates that said candidate differentiation factor is capable of promoting the differentiation of said human definitive endoderm cells.

[0020] 2. The method of paragraph 1, wherein said human definitive endoderm cells comprise at least about 10% of the human cells in said cell population.

[0021] 3. The method of paragraph 1, wherein human feeder cells are present in said cell population and wherein at least about 10% of the human cells other than said feeder cells are definitive endoderm cells.

[0022] 4. The method of paragraph 1, wherein said human definitive endoderm cells comprise at least about 90% of the human cells in said cell population.

[0023] 5. The method of paragraph 1, wherein said human feeder cells are present in said cell population and wherein at least about 90% of the human cells other than said feeder cells are definitive endoderm cells.

[0024] 6. The method of paragraph 1, wherein said human definitive endoderm cells differentiate into cells, tissues or organs derived from the gut tube in response to said candidate differentiation factor.

[0025] 7. The method of paragraph 1, wherein said human definitive endoderm cells differentiate into pancreatic precursor cells in response to said candidate differentiation factor.

[0026] 8. The method of paragraph 7, wherein said marker is selected from the group consisting of pancreatic-duodenal homeobox factor-1 (PDX1), homeobox A13 (HOXA13) and homeobox C6 (HOXC6).

[0027] 9. The method of paragraph 1, wherein said human definitive endoderm cells differentiate into liver precursor cells in response to said candidate differentiation factor.

[0028] 10. The method of paragraph 9, wherein said marker is selected from the group consisting of albumin, prospero-related homeobox 1 (PROX1) and hepatocyte specific antigen (HSA).

[0029] 11. The method of paragraph 1, wherein said human definitive endoderm cells differentiate into lung precursor cells in response to said candidate differentiation factor.

[0030] 12. The method of paragraph 11, wherein said marker is thyroid transcription factor 1 (TITF1).

[0031] 13. The method of paragraph 1, wherein said human definitive endoderm cells differentiate into intestinal precursor cells in response to said candidate differentiation factor.

[0032] 14. The method of paragraph 13, wherein said marker is selected from the group consisting of villin and caudal type homeobox transcription factor 2 (CDX2).

[0033] 15. The method of paragraph 1, wherein said first time point is prior to providing said candidate differentiation factor to said cell population.

[0034] 16. The method of paragraph 1, wherein said first time point is at approximately the same time as providing said candidate differentiation factor to said cell population.

[0035] 17. The method of paragraph 1, wherein said first time point is subsequent to providing said candidate differentiation factor to said cell population.

[0036] 18. The method of paragraph 1, wherein expression of said marker is increased.

[0037] 19. The method of paragraph 1, wherein expression of said marker is decreased.

[0038] 20. The method of paragraph 1, wherein expression of said marker is determined by quantitative polymerase chain reaction (Q-PCR).

[0039] 21. The method of paragraph 1, wherein expression of said marker is determined by immunocytochemistry.

[0040] 22. The method of paragraph 1, wherein said marker is selected from the group consisting of pancreatic-duodenal homeobox factor-1 (PDX1), homeobox A13 (HOXA13) and homeobox C6 (HOXC6).

[0041] 23. The method of paragraph 1, wherein said marker is selected from the group consisting of albumin, prospero-related homeobox 1 (PROX1) and hepatocyte specific antigen (HSA).

[0042] 24. The method of paragraph 1, wherein said marker is selected from the group consisting of villin and caudal type homeobox transcription factor 2 (CDX2).

[0043] 25. The method of paragraph 1, wherein said marker is thyroid transcription factor 1 (TITF1).

[0044] 26. The method of paragraph 1, wherein said differentiation factor comprises a foregut differentiation factor.

[0045] 27. The method of paragraph 1, wherein said differentiation factor comprises a small molecule.

[0046] 28. The method of paragraph 1, wherein said differentiation factor comprises a retinoid.

[0047] 29. The method of paragraph 1, wherein said differentiation factor comprises retinoic acid.

[0048] 30. The method of paragraph 1, wherein said differentiation factor comprises a polypeptide.

[0049] 31. The method of paragraph 1, wherein said differentiation factor comprises a growth factor.

[0050] 32. The method of paragraph 1, wherein said differentiation factor comprises FGF-10.

[0051] 33. The method of paragraph 1, wherein said differentiation factor comprises FGF-2.

[0052] 34. The method of paragraph 1, wherein said differentiation factor comprises Wnt3B.

[0053] 35. The method of paragraph 1, wherein said differentiation factor is not a foregut differentiation factor.

[0054] 36. The method of paragraph 1, wherein said differentiation factor is not a retinoid.

[0055] 37. The method of paragraph 1, wherein said differentiation factor is not retinoic acid.

[0056] 38. The method of paragraph 1, wherein said differentiation factor is provided to said cell population at a concentration of between about 0.1 ng/ml to about 10 mg/ml.

[0057] 39. The method of paragraph 1, wherein said differentiation factor is provided to said cell population at a concentration of between about 1 ng/ml to about 1 mg/ml

[0058] 40. The method of paragraph 1, wherein said differentiation factor is provided to said cell population at a concentration of between about 10 ng/ml to about 100 μg/ml.

[0059] 41. The method of paragraph 1, wherein said differentiation factor is provided to said cell population at a concentration of between about 100 ng/ml to about 10 μg/ml.

[0060] 42. The method of paragraph 1, wherein said differentiation factor is provided to said cell population at a concentration of about 1 μg/ml.

[0061] 43. The method of paragraph 1, wherein said differentiation factor is provided to said cell population at a concentration of about 100 ng/ml.

[0062] It will be appreciated that the methods and compositions described above relate to cells cultured *in vitro*. However, the above-described *in vitro* differentiated cell compositions may be used for *in vivo* applications.

[0063] Additional embodiments of the present invention may also be found in United States Provisional Patent Application No. 60/532,004, entitled DEFINITIVE ENDODERM, filed December 23, 2003; U.S. Provisional Patent Application Number 60/566,293, entitled PDX1 EXPRESSING ENDODERM, filed April 27, 2004; U.S. Provisional Patent Application Number 60/586,566, entitled CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM, filed July 9, 2004; U.S. Provisional Patent Application Number 60/587,942, entitled CHEMOKINE CELL SURFACE RECEPTOR FOR THE ISOLATION OF DEFINITIVE ENDODERM, filed July 14, 2004; U.S. Patent Application Number 11/021,618, entitled DEFINITIVE ENDODERM, filed December 23, 2004, and U.S. Patent Application Number 11/115,868, entitled PDX1 EXPRESSING ENDODERM, filed April 26, 2005.

Brief Description of the Drawings

[0064] Figure 1 is a schematic of a proposed differentiation pathway for the production of beta-cells from hESCs. The first step in the pathway commits the ES cell to the definitive endoderm lineage and also represents the first step prior to further differentiation events to pancreatic endoderm, endocrine endoderm, or islet/beta-cells. The second step in the pathway shows the conversion of SOX17-positive/PDX1-negative definitive endoderm to PDX1-positive foregut endoderm. Some factors useful for mediating these transitions are italicized. Relevant markers for defining the target cells are underlined.

[0065] Figure 2 is a diagram of the human SOX17 cDNA which displays the positions of conserved motifs and highlights the region used for the immunization procedure by GENOVAC.

[0066] Figure 3 is a relational dendrogram illustrating that SOX17 is most closely related to SOX7 and somewhat less to SOX18. The SOX17 proteins are more closely related among species homologs than to other members of the SOX group F subfamily within the same species.

[0067] Figure 4 is a Western blot probed with the rat anti-SOX17 antibody. This blot demonstrates the specificity of this antibody for human SOX17 protein over-expressed in

fibroblasts (lane 1) and a lack of immunoreactivity with EGFP (lane 2) or the most closely related SOX family member, SOX7 (lane 3).

[0068] Figures 5A-B are micrographs showing a cluster of $SOX17^+$ cells that display a significant number of AFP^+ co-labeled cells (A). This is in striking contrast to other $SOX17^+$ clusters (B) where little or no AFP^+ cells are observed.

[0069] Figures 6A-C are micrographs showing parietal endoderm and SOX17. Panel A shows immunocytochemistry for human Thrombomodulin (TM) protein located on the cell surface of parietal endoderm cells in randomly differentiated cultures of hES cells. Panel B is the identical field shown in A double-labeled for TM and SOX17. Panel C is the phase contrast image of the same field with DAPI labeled nuclei. Note the complete correlation of DAPI labeled nuclei and SOX17 labeling.

[0070] Figures 7A-B are bar charts showing SOX17 gene expression by quantitative PCR (Q-PCR) and anti-SOX17 positive cells by SOX17-specific antibody. Panel A shows that activin A increases SOX17 gene expression while retinoic acid (RA) strongly suppresses SOX17 expression relative to the undifferentiated control media (SR20). Panel B shows the identical pattern as well as a similar magnitude of these changes is reflected in $SOX17^+$ cell number, indicating that Q-PCR measurement of SOX17 gene expression is very reflective of changes at the single cell level.

[0071] Figure 8A is a bar chart which shows that a culture of differentiating hESCs in the presence of activin A maintains a low level of AFP gene expression while cells allowed to randomly differentiate in 10% fetal bovine serum (FBS) exhibit a strong upregulation of AFP. The difference in expression levels is approximately 7-fold.

[0072] Figures 8B-C are images of two micrographs showing that the suppression of AFP expression by activin A is also evident at the single cell level as indicated by the very rare and small clusters of AFP^+ cells observed in activin A treatment conditions (bottom) relative to 10% FBS alone (top).

[0073] Figures 9A-B are comparative images showing the quantitation of the AFP^+ cell number using flow cytometry. This figure demonstrates that the magnitude of change in AFP gene expression (Figure 8A) in the presence (right panel) and absence (left panel) of activin A exactly corresponds to the number of AFP^+ cells, further supporting the utility of Q-PCR analyses to indicate changes occurring at the individual cell level.

[0074] Figures 10A-F are micrographs which show that exposure of hESCs to nodal, activin A and activin B (NAA) yields a striking increase in the number of $SOX17^+$ cells over the period of 5 days (A-C). By comparing to the relative abundance of $SOX17^+$ cells to the total number of cells present in each field, as indicated by DAPI stained nuclei (D-F), it can be seen that approximately 30-50% of all cells are immunoreactive for SOX17 after five days treatment with NAA.

[0075] Figure 11 is a bar chart which demonstrates that activin A (0, 10, 30 or 100 ng/ml) dose-dependently increases SOX17 gene expression in differentiating hESCs. Increased expression is already robust after 3 days of treatment on adherent cultures and continues through subsequent 1, 3 and 5 days of suspension culture as well.

[0076] Figures 12A-C are bar charts which demonstrate the effect of activin A on the expression of MIXL1 (panel A), GATA4 (panel B) and HNF3b (panel C). Activin A dose-dependent increases are also observed for three other markers of definitive endoderm; MIXL1, GATA4 and HNF3b. The magnitudes of increased expression in response to activin dose are strikingly similar to those observed for SOX17, strongly indicating that activin A is specifying a population of cells that co-express all four genes ($SOX17^+$, $MIXL1^+$, $GATA4^+$ and $HNF3b^+$).

[0077] Figures 13A-C are bar charts which demonstrate the effect of activin A on the expression of AFP (panel A), SOX7 (panel B) and SPARC (panel C). There is an activin A dose-dependent decrease in expression of the visceral endoderm marker AFP. Markers of primitive endoderm (SOX7) and parietal endoderm (SPARC) remain either unchanged or exhibit suppression at some time points indicating that activin A does not act to specify these extra-embryonic endoderm cell types. This further supports the fact that the increased expression of SOX17, MIXL1, GATA4, and HNF3b are due to an increase in the number of definitive endoderm cells in response to activin A.

[0078] Figures 14A-B are bar charts showing the effect of activin A on ZIC1 (panel A) and Brachyury expression (panel B) Consistent expression of the neural marker ZIC1 demonstrates that there is not a dose-dependent effect of activin A on neural differentiation. There is a notable suppression of mesoderm differentiation mediated by 100 ng/ml of activin A treatment as indicated by the decreased expression of brachyury. This is likely the result of the increased specification of definitive endoderm from the mesendoderm precursors. Lower levels of activin A treatment (10 and 30 ng/ml) maintain the expression of brachyury at later time points of differentiation relative to untreated control cultures.

[0079] Figures 15A-B are micrographs showing decreased parietal endoderm differentiation in response to treatment with activins. Regions of TM^{hi} parietal endoderm are found through the culture (A) when differentiated in serum alone, while differentiation to TM^+ cells is scarce when activins are included (B) and overall intensity of TM immunoreactivity is lower.

[0080] Figures 16A-D are micrographs which show marker expression in response to treatment with activin A and activin B. hESCs were treated for four consecutive days with activin A and activin B and triple labeled with SOX17, AFP and TM antibodies. Panel A - SOX17; Panel B - AFP; Panel C - TM; and Panel D - Phase/DAPI. Notice the numerous SOX17 positive cells (A) associated with the complete absence of AFP (B) and TM (C) immunoreactivity.

[0081] Figure 17 is a micrograph showing the appearance of definitive endoderm and visceral endoderm in vitro from hESCs. The regions of visceral endoderm are identified by $AFP^{hi}/SOX17^{lo/-}$ while definitive endoderm displays the complete opposite profile, $SOX17^{hi}/AFP^{lo/-}$. This field was selectively chosen due to the proximity of these two regions to each other. However, there are numerous times when $SOX17^{hi}/AFP^{lo/-}$ regions are observed in absolute isolation from any regions of AFP^{hi} cells, suggesting the separate origination of the definitive endoderm cells from visceral endoderm cells.

[0082] Figure 18 is a diagram depicting the TGFβ family of ligands and receptors. Factors activating AR Smads and BR Smads are useful in the production of definitive endoderm from human embryonic stem cells (see, *J Cell Physiol.*187:265-76).

[0083] Figure 19 is a bar chart showing the induction of SOX17 expression over time as a result of treatment with individual and combinations of TGFβ factors.

[0084] Figure 20 is a bar chart showing the increase in $SOX17^+$ cell number with time as a result of treatment with combinations of TGFβ factors.

[0085] Figure 21 is a bar chart showing induction of SOX17 expression over time as a result of treatment with combinations of TGFβ factors.

[0086] Figure 22 is a bar chart showing that activin A induces a dose-dependent increase in $SOX17^+$ cell number.

[0087] Figure 23 is a bar chart showing that addition of Wnt3a to activin A and activin B treated cultures increases SOX17 expression above the levels induced by activin A and activin B alone.

[0088] Figures 24A-C are bar charts showing differentiation to definitive endoderm is enhanced in low FBS conditions. Treatment of hESCs with activins A and B in media containing 2% FBS (2AA) yields a 2-3 times greater level of SOX17 expression as compared to the same treatment in 10% FBS media (10AA) (panel A). Induction of the definitive endoderm marker MIXL1 (panel B) is also affected in the same way and the suppression of AFP (visceral endoderm) (panel C) is greater in 2% FBS than in 10% FBS conditions.

[0089] Figures 25A-D are micrographs which show $SOX17^+$ cells are dividing in culture. SOX17 immunoreactive cells are present at the differentiating edge of an hESC colony (C, D) and are labeled with proliferating cell nuclear antigen (PCNA) (panel B) yet are not co-labeled with OCT4 (panel C). In addition, clear mitotic figures can be seen by DAPI labeling of nuclei in both $SOX17^+$ cells (arrows) as well as $OCT4^+$, undifferentiated hESCs (arrowheads) (D).

[0090] Figure 26 is a bar chart showing the relative expression level of CXCR4 in differentiating hESCs under various media conditions.

[0091] Figures 27A-D are bar charts that show how a panel of definitive endoderm markers share a very similar pattern of expression to CXCR4 across the same differentiation treatments displayed in Figure 26.

[0092] Figures 28A-E are bar charts showing how markers for mesoderm (BRACHYURY, MOX1), ectoderm (SOX1, ZIC1) and visceral endoderm (SOX7) exhibit an inverse relationship to CXCR4 expression across the same treatments displayed in Figure 26.

[0093] Figures 29A-F are micrographs that show the relative difference in SOX17 immunoreactive cells across three of the media conditions displayed in Figures 26-28.

[0094] Figures 30A-C are flow cytometry dot plots that demonstrate the increase in $CXCR4^+$ cell number with increasing concentration of activin A added to the differentiation media.

[0095] Figures 31A-D are bar charts that show the $CXCR4^+$ cells isolated from the high dose activin A treatment (A100-CX+) are even further enriched for definitive endoderm markers than the parent population (A100).

[0096] Figure 32 is a bar chart showing gene expression from $CXCR4^+$ and $CXCR4^-$ cells isolated using fluorescence-activated cell sorting (FACS) as well as gene expression in the parent populations. This demonstrates that the $CXCR4^+$ cells contain essentially all the CXCR4 gene expression present in each parent population and the $CXCR4^-$ populations contain very little or no CXCR4 gene expression.

[0097] Figures 33A-D are bar charts that demonstrate the depletion of mesoderm (BRACHYURY, MOX1), ectoderm (ZIC1) and visceral endoderm (SOX7) gene expression in the CXCR4+ cells isolated from the high dose activin A treatment which is already suppressed in expression of these non-definitive endoderm markers.

[0098] Figures 34A-M are bar charts showing the expression patterns of marker genes that can be used to identify definitive endoderm cells. The expression analysis of definitive endoderm markers, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 is shown in panels G-L, respectively. The expression analysis of previously described lineage marking genes, SOX17, SOX7, SOX17/SOX7, TM, ZIC1, and MOX1 is shown in panels A-F, respectively. Panel M shows the expression analysis of CXCR4. With respect to each of panels A-M, the column labeled hESC indicates gene expression from purified human embryonic stem cells; 2NF indicates cells treated with 2% FBS, no activin addition; 0.1A100 indicates cells treated with 0.1% FBS, 100 ng/ml activin A; 1A100 indicates cells treated with 1% FBS, 100 ng/ml activin A; and 2A100 indicates cells treated with 2% FBS, 100 ng/ml activin A.

[0099] Figure 35 is a chart which shows the relative expression of the PDX1 gene in a culture of hESCs after 4 days and 6 days with and without activin in the presence of retinoic acid (RA) and fibroblast growth factor-10 (FGF-10) added on day 4.

[0100] Figures 36A-F are charts which show the relative expression of marker genes in a culture of hESCs after 4 days and 6 days with and without activin in the presence of retinoic acid (RA) and fibroblast growth factor-10 (FGF-10) added on day 4. The panels show the relative levels of expression of the following marker genes: (A) SOX17; (B) SOX7; (C) AFP; (D) SOX1; (E) ZIC1; and (F) NFM.

[0101] Figures 37A-C are charts which show the relative expression of marker genes in a culture of hESCs after 4 days and 8 days with and without activin in the presence or absence of combinations of retinoic acid (RA), fibroblast growth factor-10 (FGF-10) and fibroblast growth factor-4 (FGF-4) added on day 4. The panels show the relative levels of expression of the following marker genes: (A) PDX1; (B) SOX7; and (C) NFM.

[0102] Figures 38A-G are charts which show the relative expression of marker genes in a culture of definitive endoderm cells contacted with 50 ng/ml FGF-10 in combination with either 1 μM, 0.2 μM or 0.04 μM retinoic acid (RA) added on day 4. The panels show the relative levels of expression of the following marker genes: (A) PDX1; (B) HOXA3; (C) HOXC6; (D) HOXA13; (E) CDX1; (F) SOX1; and (G) NFM.

[0103] Figures 39A-E are charts which show the relative expression of marker genes in a culture of hESCs after 4 days and 8 days with and without activin in the presence of combinations of retinoic acid (RA), fibroblast growth factor-10 (FGF-10) and one of the following: serum replacement (SR), fetal bovine serum (FBS) or B27. The panels show the relative levels of expression of the following marker genes: (A) PDX1; (B) SOX7; (C) AFP; (D) ZIC1; and (E) NFM.

[0104] Figures 40A-B are charts which show the relative expression of marker genes for pancreas (PDX1, HNF6) and liver (HNF6) in a culture of hESCs after 6 days (just prior to addition of RA) and at 9 days (three days after exposure to RA). Various conditions were included to compare the addition of activin B at doses of 10 ng/ml (a10), 25 ng/ml (a25) or 50 ng/ml (a50) in the presence of either 25 ng/ml (A25) or 50 ng/ml (A50) activin A. The condition without any activin A or activin B (NF) serves as the negative control for definitive endoderm and PDX1-positive endoderm production. The panels show the relative levels of expression of the following marker genes: (A) PDX1and (B) HNF6.

[0105] Figures 41A-C are charts which show the relative expression of marker genes in a culture of hESCs with 100 ng/ml (A100), 50 ng/ml (A50) or without (NF) activin A at 5 days (just prior to retinoic acid addition) and at 2, 4, and 6 days after RA exposure (day 7, 9, and 11, respectively). The percentage label directly under each bar indicates the FBS dose during days 3-5 of differentiation. Starting at day 7, cells treated with RA (R) were grown in RPMI medium comprising 0.5% FBS. The RA concentration was 2 μM on day 7, 1 μM on day 9 and 0.2 μM on

day 11. The panels show the relative levels of expression of the following marker genes: (A) PDX1; (B) ZIC1; (C) SOX7.

[0106] Figures 42A-B are charts which show the relative expression of marker genes in a culture of hESCs treated first with activin A in low FBS to induce definitive endoderm (day 5) and then with fresh (A25R) medium comprising 25 ng/ml activin A and RA or various conditioned media (MEFCM, CM#2, CM#3 and CM#4) and RA to induce PDX1-expressing endoderm. Marker expression was determined on days 5, 6, 7, 8 and 9. The panels show the relative levels of expression of the following marker genes: (A) PDX1; (B) CDX1.

[0107] Figure 43 is a chart which shows the relative expression of PDX1 in a culture of hESCs treated first with activin A in low FBS to induce definitive endoderm and followed by fresh media comprising activin A and retinoic acid (A25R) or varying amounts of RA in conditioned media diluted into fresh media. Total volume of media is 5 ml in all cases.

[0108] Figure 44 is a Western blot showing PDX1 immunoprecipitated from RA-treated definitive endoderm cells 3 days (d8) and 4 days (d9) after the addition of RA and 50 ng/ml activin A.

[0109] Figure 45 is a summary chart displaying the results of a fluorescence-activated cell sort (FACs) of PDX1-positive foregut endoderm cells genetically tagged with a EGFP reporter under control of the PDX1 promoter.

[0110] Figure 46 is a chart showing relative PDX1 expression levels normalized to housekeeping genes for sorted populations of live cells (Live), EGFP-negative cells (Neg) and EGFP-positive cells (GFP+).

[0111] Figure 47 is a chart showing relative PDX1 expression levels normalized to housekeeping genes for sorted populations of live cells (Live), EGFP-negative cells (Neg), the half of the EGFP-positive cell population that has the lowest EGFP signal intensity (Lo) and the half of the EGFP-positive cell population that has the highest EGFP signal intensity (Hi).

[0112] Figures 48A-E are a charts showing the relative expression levels normalized to housekeeping genes of five pancreatic endoderm markers in sorted populations of live cells (Live), EGFP-negative cells (Neg) and EGFP-positive cells (GFP+). Panels: A – NKX2.2; B – GLUT2; C – HNF3β; D – KRT19 and E – HNF4α.

[0113] Figure 49 are a charts showing the relative expression levels normalized to housekeeping genes of two non-pancreatic endoderm markers in sorted populations of live cells (Live), EGFP-negative cells (Neg) and EGFP-positive cells (GFP+). Panels: A – ZIC1 and B – GFAP.

[0114] Figures 50A-D show the *in vivo* differentiation of definitive endoderm cells that are transplanted under the kidney capsule of immunocompromised mice. Panels: A – hetatoxylin-eosin staining showing gut-tube-like structures; B – antibody immunoreactivity

against hepatocyte specific antigen (liver); C – antibody immunoreactivity against villin (intestine); and D – antibody immunoreactivity against CDX2 (intestine).

[0115] Figures 51A-C are charts showing the normalized relative expression levels of markers for liver (albumin and PROX1) and lung (TITF1) tissues in cells contacted with Wnt3B at 20 ng/ml, FGF2 at 5 ng/ml or FGF2 at 100 ng/ml on days 5-10. DE refers to definitive endoderm. Panels: A – albumin, B – PROX1, and C – TITF1.

[0116] Figures 52A-L are charts showing the normalized relative expression levels of markers for liver (AFP, AAT, hHEX, GLUT2, APOA1 and VCAM1) and lung (VWF and CXR4) tissues in cells contacted with Wnt3A at 20-50 ng/ml, FGF2 at 5 ng/ml or FGF2 at 100 ng/ml on days 5-10 and BMP4 on days 9 and 10. DE refers to definitive endoderm. Panels: A – AFP, B – AAT, C – GLUKO, D – hHEX, E – TAT, F – hNF4a, G – CYP7A, H – GLUT2, I – APOA1, J – VCAM1, K – VWF, and L – CXCR4.

Detailed Description

[0117] A crucial stage in early human development termed gastrulation occurs 2-3 weeks after fertilization. Gastrulation is extremely significant because it is at this time that the three primary germ layers are first specified and organized (Lu et al., 2001; Schoenwolf and Smith, 2000). The ectoderm is responsible for the eventual formation of the outer coverings of the body and the entire nervous system whereas the heart, blood, bone, skeletal muscle and other connective tissues are derived from the mesoderm. Definitive endoderm is defined as the germ layer that is responsible for formation of the entire gut tube which includes the esophagus, stomach and small and large intestines, and the organs which derive from the gut tube such as the lungs, liver, thymus, parathyroid and thyroid glands, gall bladder and pancreas (Grapin-Botton and Melton, 2000; Kimelman and Griffin, 2000; Tremblay et al., 2000; Wells and Melton, 1999; Wells and Melton, 2000). A very important distinction should be made between the definitive endoderm and the completely separate lineage of cells termed primitive endoderm. The primitive endoderm is primarily responsible for formation of extra-embryonic tissues, mainly the parietal and visceral endoderm portions of the placental yolk sac and the extracellular matrix material of Reichert's membrane.

[0118] During gastrulation, the process of definitive endoderm formation begins with a cellular migration event in which mesendoderm cells (cells competent to form mesoderm or endoderm) migrate through a structure called the primitive streak. Definitive endoderm is derived from cells, which migrate through the anterior portion of the streak and through the node (a specialized structure at the anterior-most region of the streak). As migration occurs, definitive endoderm populates first the most anterior gut tube and culminates with the formation of the posterior end of the gut tube.

[0119] Definitive endoderm and endoderm cells derived therefrom represent important multipotent starting points for the derivation of cells which make up terminally differentiated tissues and/or organs derived from the definitive endoderm lineage. Such cells, tissues and/or organs are extremely useful in cell therapies. As such, the methods described herein for identifying differentiation factors capable of causing the differentiation of definitive endoderm cells and/or PDX1 expressing endoderm cells to other cells types derived from the definitive endoderm cell lineage are beneficial for the advancement of cell therapy.

[0120] In particular, some embodiments of the present invention relate to methods of identifying one or more differentiation factors that are useful for differentiating cells in a cell population comprising PDX1-positive endoderm cells and/or definitive endoderm cells into cells that are capable of promoting the differentiation of definitive endoderm cells into cells which are precursors for tissues and/or organs which include, but are not limited to, pancreas, liver, lungs, stomach, intestine, thyroid, thymus, pharynx, gallbladder and urinary bladder.

[0121] Additional aspects which relate to compositions of definitive endoderm cells, PDX1-positive endoderm as well as methods and compositions useful for producing such cells are also described herein.

Definitions

[0122] Certain terms and phrases as used throughout this application have the meanings provided as follows:

[0123] As used herein, "embryonic" refers to a range of developmental stages of an organism beginning with a single zygote and ending with a multicellular structure that no longer comprises pluripotent or totipotent cells other than developed gametic cells. In addition to embryos derived by gamete fusion, the term "embryonic" refers to embryos derived by somatic cell nuclear transfer.

[0124] As used herein, "multipotent" or "multipotent cell" refers to a cell type that can give rise to a limited number of other particular cell types.

[0125] As used herein, "expression" refers to the production of a material or substance as well as the level or amount of production of a material or substance. Thus, determining the expression of a specific marker refers to detecting either the relative or absolute amount of the marker that is expressed or simply detecting the presence or absence of the marker.

[0126] As used herein, "marker" refers to any molecule that can be observed or detected. For example, a marker can include, but is not limited to, a nucleic acid, such as a transcript of a specific gene, a polypeptide product of a gene, a non-gene product polypeptide, a glycoprotein, a carbohydrate, a glycolipd, a lipid, a lipoprotein or a small molecule (for example, molecules having a molecular weight of less than 10,000 amu)

[0127] When used in connection with cell cultures and/or cell populations, the term "portion" means any non-zero amount of the cell culture or cell population, which ranges from a single cell to the entirety of the cell culture or cells population.

[0128] With respect to cells in cell cultures or in cell populations, the phrase "substantially free of" means that the specified cell type of which the cell culture or cell population is free, is present in an amount of less than about 5% of the total number of cells present in the cell culture or cell population.

[0129] As used herein, "retinoid" refers to retinol, retinal or retinoic acid as well as derivatives of any of these compounds.

[0130] By "conditioned medium" is meant, a medium that is altered as compared to a base medium.

[0131] As used herein, "foregut/midgut" refers to cells of the anterior portion of the gut tube as well as cells of the middle portion of the gut tube, including cells of the foregut/midgut junction.

Definitive Endoderm Cells and Processes Related Thereto

[0132] Embodiments described herein relate to novel, defined processes for the production of definitive endoderm cells in culture by differentiating pluripotent cells, such as stem cells into multipotent definitive endoderm cells. As described above, definitive endoderm cells do not differentiate into tissues produced from ectoderm or mesoderm, but rather, differentiate into the gut tube as well as organs that are derived from the gut tube. In certain preferred embodiments, the definitive endoderm cells are derived from hESCs. Such processes can provide the basis for efficient production of human endodermal derived tissues such as pancreas, liver, lung, stomach, intestine, thyroid and thymus. For example, production of definitive endoderm may be the first step in differentiation of a stem cell to a functional insulin-producing β-cell. To obtain useful quantities of insulin-producing β-cells, high efficiency of differentiation is desirable for each of the differentiation steps that occur prior to reaching the pancreatic islet/β-cell fate. Since differentiation of stem cells to definitive endoderm cells represents perhaps the earliest step towards the production of functional pancreatic islet/β-cells (as shown in Figure 1), high efficiency of differentiation at this step is particularly desirable.

[0133] In view of the desirability of efficient differentiation of pluripotent cells to definitive endoderm cells, some aspects of the differentiation processes described herein relate to *in vitro* methodology that results in approximately 50-80% conversion of pluripotent cells to definitive endoderm cells. Typically, such methods encompass the application of culture and growth factor conditions in a defined and temporally specified fashion. Further enrichment of the cell population for definitive endoderm cells can be achieved by isolation and/or purification of

the definitive endoderm cells from other cells in the population by using a reagent that specifically binds to definitive endoderm cells. As such, some embodiments described herein relate to definitive endoderm cells as well as methods for producing and isolating and/or purifying such cells.

[0134] In order to determine the amount of definitive endoderm cells in a cell culture or cell population, a method of distinguishing this cell type from the other cells in the culture or in the population is desirable. Accordingly, certain embodiments described herein relate to cell markers whose presence, absence and/or relative expression levels are specific for definitive endoderm and methods for detecting and determining the expression of such markers.

[0135] In some embodiments described herein, the presence, absence and/or level of expression of a marker is determined by quantitative PCR (Q-PCR). For example, the amount of transcript produced by certain genetic markers, such as SOX17, CXCR4, OCT4, AFP, TM, SPARC, SOX7, MIXL1, GATA4, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1, CRIP1 and other markers described herein is determined by quantitative Q-PCR. In other embodiments, immunohistochemistry is used to detect the proteins expressed by the above-mentioned genes. In still other embodiments, Q-PCR and immunohistochemical techniques are both used to identify and determine the amount or relative proportions of such markers.

[0136] By using methods, such as those described above, to determine the expression of one or more appropriate markers, it is possible to identify definitive endoderm cells, as well as determine the proportion of definitive endoderm cells in a cell culture or cell population. For example, in some embodiments of the present invention, the definitive endoderm cells or cell populations that are produced express the SOX17 and/or the CXCR4 gene at a level of about 2 orders of magnitude greater than non-definitive endoderm cell types or cell populations. In other embodiments, the definitive endoderm cells or cell populations that are produced express the SOX17 and/or the CXCR4 gene at a level of more than 2 orders of magnitude greater than non-definitive endoderm cell types or cell populations. In still other embodiments, the definitive endoderm cells or cell populations that are produced express one or more of the markers selected from the group consisting of SOX17, CXCR4, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 at a level of about 2 or more than 2 orders of magnitude greater than non-definitive endoderm cell types or cell populations. In some embodiments described herein, definitive endoderm cells do not substantially express PDX1.

[0137] Embodiments described herein also relate to definitive endoderm compositions. For example, some embodiments relate to cell cultures comprising definitive endoderm, whereas others relate to cell populations enriched in definitive endoderm cells. Some preferred embodiments relate to cell cultures which comprise definitive endoderm cells, wherein at least about 50-80% of the cells in culture are definitive endoderm cells. An especially preferred embodiment relates to cells cultures comprising human cells, wherein at least about 50-80% of

the human cells in culture are definitive endoderm cells. Because the efficiency of the differentiation procedure can be adjusted by modifying certain parameters, which include but are not limited to, cell growth conditions, growth factor concentrations and the timing of culture steps, the differentiation procedures described herein can result in about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or greater than about 95% conversion of pluripotent cells to definitive endoderm. In other preferred embodiments, conversion of a pluripotent cell population, such as a stem cell population, to substantially pure definitive endoderm cell population is contemplated.

[0138] The compositions and methods described herein have several useful features. For example, the cell cultures and cell populations comprising definitive endoderm as well as the methods for producing such cell cultures and cell populations are useful for modeling the early stages of human development. Furthermore, the compositions and methods described herein can also serve for therapeutic intervention in disease states, such as diabetes mellitus. For example, since definitive endoderm serves as the source for only a limited number of tissues, it can be used in the development of pure tissue or cell types.

Production of Definitive Endoderm from Pluripotent Cells

[0139] Processes for differentiating pluripotent cells to produce cell cultures and enriched cell populations comprising definitive endoderm is described below and in US Patent No. 11/021,618, entitled DEFINITIVE ENDODERM, filed December 23, 2004. In some of these processes, the pluripotent cells used as starting material are stem cells. In certain processes, definitive endoderm cell cultures and enriched cell populations comprising definitive endoderm cells are produced from embryonic stem cells. A preferred method for deriving definitive endoderm cells utilizes human embryonic stem cells as the starting material for definitive endoderm production. Such pluripotent cells can be cells that originate from the morula, embryonic inner cell mass or those obtained from embryonic gonadal ridges. Human embryonic stem cells can be maintained in culture in a pluripotent state without substantial differentiation using methods that are known in the art. Such methods are described, for example, in US Patent Nos. 5,453,357, 5,670,372, 5,690,926 5,843,780, 6,200,806 and 6,251,671.

[0140] In some processes for producing definitive endoderm cells, hESCs are maintained on a feeder layer. In such processes, any feeder layer which allows hESCs to be maintained in a pluripotent state can be used. One commonly used feeder layer for the cultivation of human embryonic stem cells is a layer of mouse fibroblasts. More recently, human fibroblast feeder layers have been developed for use in the cultivation of hESCs (see US Patent Application No. 2002/0072117). Alternative processes for producing definitive endoderm permit the maintenance of pluripotent hESC without the use of a feeder layer. Methods of maintaining

pluripotent hESCs under feeder-free conditions have been described in US Patent Application No. 2003/0175956.

[0141] The human embryonic stem cells used herein can be maintained in culture either with or without serum. In some embryonic stem cell maintenance procedures, serum replacement is used. In others, serum free culture techniques, such as those described in US Patent Application No. 2003/0190748, are used.

[0142] Stem cells are maintained in culture in a pluripotent state by routine passage until it is desired that they be differentiated into definitive endoderm. In some processes, differentiation to definitive endoderm is achieved by providing to the stem cell culture a growth factor of the TGFβ superfamily in an amount sufficient to promote differentiation to definitive endoderm. Growth factors of the TGFβ superfamily which are useful for the production of definitive endoderm are selected from the Nodal/Activin or BMP subgroups. In some preferred differentiation processes, the growth factor is selected from the group consisting of Nodal, activin A, activin B and BMP4. Additionally, the growth factor Wnt3a and other Wnt family members are useful for the production of definitive endoderm cells. In certain differentiation processes, combinations of any of the above-mentioned growth factors can be used.

[0143] With respect to some of the processes for the differentiation of pluripotent stem cells to definitive endoderm cells, the above-mentioned growth factors are provided to the cells so that the growth factors are present in the cultures at concentrations sufficient to promote differentiation of at least a portion of the stem cells to definitive endoderm cells. In some processes, the above-mentioned growth factors are present in the cell culture at a concentration of at least about 5 ng/ml, at least about 10 ng/ml, at least about 25 ng/ml, at least about 50 ng/ml, at least about 75 ng/ml, at least about 100 ng/ml, at least about 200 ng/ml, at least about 300 ng/ml, at least about 400 ng/ml, at least about 500 ng/ml, at least about 1000 ng/ml, at least about 2000 ng/ml, at least about 3000 ng/ml, at least about 4000 ng/ml, at least about 5000 ng/ml or more than about 5000 ng/ml.

[0144] In certain processes for the differentiation of pluripotent stem cells to definitive endoderm cells, the above-mentioned growth factors are removed from the cell culture subsequent to their addition. For example, the growth factors can be removed within about one day, about two days, about three days, about four days, about five days, about six days, about seven days, about eight days, about nine days or about ten days after their addition. In a preferred processes, the growth factors are removed about four days after their addition.

[0145] Cultures of definitive endoderm cells can be grown in medium containing reduced serum or no serum. Under certain culture conditions, serum concentrations can range from about 0.05% v/v to about 20% v/v. For example, in some differentiation processes, the serum concentration of the medium can be less than about 0.05% (v/v), less than about 0.1%

(v/v), less than about 0.2% (v/v), less than about 0.3% (v/v), less than about 0.4% (v/v), less than about 0.5% (v/v), less than about 0.6% (v/v), less than about 0.7% (v/v), less than about 0.8% (v/v), less than about 0.9% (v/v), less than about 1% (v/v), less than about 2% (v/v), less than about 3% (v/v), less than about 4% (v/v), less than about 5% (v/v), less than about 6% (v/v), less than about 7% (v/v), less than about 8% (v/v), less than about 9% (v/v), less than about 10% (v/v), less than about 15% (v/v) or less than about 20% (v/v). In some processes, definitive endoderm cells are grown without serum or with serum replacement. In still other processes, definitive endoderm cells are grown in the presence of B27. In such processes, the concentration of B27 supplement can range from about 0.1% v/v to about 20% v/v.

Monitoring the Differentiation of Pluripotent Cells to Definitive Endoderm

[0146] The progression of the hESC culture to definitive endoderm can be monitored by determining the expression of markers characteristic of definitive endoderm. In some processes, the expression of certain markers is determined by detecting the presence or absence of the marker. Alternatively, the expression of certain markers can be determined by measuring the level at which the marker is present in the cells of the cell culture or cell population. In such processes, the measurement of marker expression can be qualitative or quantitative. One method of quantitating the expression of markers that are produced by marker genes is through the use of quantitative PCR (Q-PCR). Methods of performing Q-PCR are well known in the art. Other methods which are known in the art can also be used to quantitate marker gene expression. For example, the expression of a marker gene product can be detected by using antibodies specific for the marker gene product of interest. In certain processes, the expression of marker genes characteristic of definitive endoderm as well as the lack of significant expression of marker genes characteristic of hESCs and other cell types is determined.

[0147] As described further in the Examples below, a reliable marker of definitive endoderm is the SOX17 gene. As such, the definitive endoderm cells produced by the processes described herein express the SOX17 marker gene, thereby producing the SOX17 gene product. Other markers of definitive endoderm are MIXL1, GATA4, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1. Since definitive endoderm cells express the SOX17 marker gene at a level higher than that of the SOX7 marker gene, which is characteristic of primitive and visceral endoderm (see Table 1), in some processes, the expression of both SOX17 and SOX7 is monitored. In other processes, expression of the both the SOX17 marker gene and the OCT4 marker gene, which is characteristic of hESCs, is monitored. Additionally, because definitive endoderm cells express the SOX17 marker gene at a level higher than that of the AFP, SPARC or Thrombomodulin (TM) marker genes, the expression of these genes can also be monitored.

[0148] Another marker of definitive endoderm is the CXCR4 gene. The CXCR4 gene encodes a cell surface chemokine receptor whose ligand is the chemoattractant SDF-1. The principal roles of the CXCR4 receptor-bearing cells in the adult are believed to be the migration of hematopoetic cells to the bone marrow, lymphocyte trafficking and the differentiation of various B cell and macrophage blood cell lineages [Kim, C., and Broxmeyer, H. J. Leukocyte Biol. 65, 6-15 (1999)]. The CXCR4 receptor also functions as a coreceptor for the entry of HIV-1 into T-cells [Feng, Y., et al. Science, 272, 872-877 (1996)]. In an extensive series of studies carried out by [McGrath, K.E. et al. Dev. Biology 213, 442-456 (1999)], the expression of the chemokine receptor CXCR4 and its unique ligand, SDF-1 [Kim, C., and Broxmyer, H., J. Leukocyte Biol. 65, 6-15 (1999)], were delineated during early development and adult life in the mouse. The CXCR4/SDF1 interaction in development became apparent when it was demonstrated that if either gene was disrupted in transgenic mice [Nagasawa et al. Nature, 382, 635-638 (1996)], Ma, Q., et al Immunity, 10, 463-471 (1999)] it resulted in late embryonic lethality. McGrath et al. demonstrated that CXCR4 is the most abundant chemokine receptor messenger RNA detected during early gastrulating embryos (E7.5) using a combination of RNase protection and *in situ* hybridization methodologies. In the gastrulating embryo, CXCR4/SDF-1 signaling appears to be mainly involved in inducing migration of primitive-streak germlayer cells and is expressed on definitive endoderm, mesoderm and extraembryonic mesoderm present at this time. In E7.2-7.8 mouse embryos, CXCR4 and alpha-fetoprotein are mutually exclusive indicating a lack of expression in visceral endoderm [McGrath, K.E. et al. Dev. Biology 213, 442-456 (1999)].

[0149] Since definitive endoderm cells produced by differentiating pluripotent cells express the CXCR4 marker gene, expression of CXCR4 can be monitored in order to track the production of definitive endoderm cells. Additionally, definitive endoderm cells produced by the methods described herein express other markers of definitive endoderm including, but not limited to, SOX17, MIXL1, GATA4, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1. Since definitive endoderm cells express the CXCR4 marker gene at a level higher than that of the SOX7 marker gene, the expression of both CXCR4 and SOX7 can be monitored. In other processes, expression of both the CXCR4 marker gene and the OCT4 marker gene, is monitored. Additionally, because definitive endoderm cells express the CXCR4 marker gene at a level higher than that of the AFP, SPARC or Thrombomodulin (TM) marker genes, the expression of these genes can also be monitored.

[0150] It will be appreciated that expression of CXCR4 in endodermal cells does not preclude the expression of SOX17. As such, definitive endoderm cells produced by the processes described herein will substantially express SOX17 and CXCR4 but will not substantially express AFP, TM, SPARC or PDX1.

[0151] It will be appreciated that SOX17 and/or CXCR4 marker expression is induced over a range of different levels in definitive endoderm cells depending on the differentiation conditions. As such, in some embodiments described herein, the expression of the SOX17 marker and/or the CXCR4 marker in definitive endoderm cells or cell populations is at least about 2-fold higher to at least about 10,000-fold higher than the expression of the SOX17 marker and/or the CXCR4 marker in non-definitive endoderm cells or cell populations, for example pluripotent stem cells. In other embodiments, the expression of the SOX17 marker and/or the CXCR4 marker in definitive endoderm cells or cell populations is at least about 4-fold higher, at least about 6-fold higher, at least about 8-fold higher, at least about 10-fold higher, at least about 15-fold higher, at least about 20-fold higher, at least about 40-fold higher, at least about 80-fold higher, at least about 100-fold higher, at least about 150-fold higher, at least about 200-fold higher, at least about 500-fold higher, at least about 750-fold higher, at least about 1000-fold higher, at least about 2500-fold higher, at least about 5000-fold higher, at least about 7500-fold higher or at least about 10,000-fold higher than the expression of the SOX17 marker and/or the CXCR4 marker in non-definitive endoderm cells or cell populations, for example pluripotent stem cells. In some embodiments, the expression of the SOX17 marker and/or CXCR4 marker in definitive endoderm cells or cell populations is infinitely higher than the expression of the SOX17 marker and/or the CXCR4 marker in non-definitive endoderm cells or cell populations, for example pluripotent stem cells.

[0152] It will also be appreciated that in some embodiments described herein, the expression of markers selected from the group consisting of GATA4, MIXL1, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 in definitive endoderm cells or cell populations is increased as compared to the expression of GATA4, MIXL1, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 in non-definitive endoderm cells or cell populations.

[0153] Additionally, it will be appreciated that there is a range of differences between the expression level of the SOX17 marker and the expression levels of the OCT4, SPARC, AFP, TM and/or SOX7 markers in definitive endoderm cells. Similarly, there exists a range of differences between the expression level of the CXCR4 marker and the expression levels of the OCT4, SPARC, AFP, TM and/or SOX7 markers in definitive endoderm cells. As such, in some embodiments described herein, the expression of the SOX17 marker or the CXCR4 marker is at least about 2-fold higher to at least about 10,000-fold higher than the expression of OCT4, SPARC, AFP, TM and/or SOX7 markers. In other embodiments, the expression of the SOX17 marker or the CXCR4 marker is at least about 4-fold higher, at least about 6-fold higher, at least about 8-fold higher, at least about 10-fold higher, at least about 15-fold higher, at least about 20-fold higher, at least about 40-fold higher, at least about 80-fold higher, at least about 100-fold higher, at least about 150-fold higher, at least about 200-fold higher, at least about 500-fold

higher, at least about 750-fold higher, at least about 1000-fold higher, at least about 2500-fold higher, at least about 5000-fold higher, at least about 7500-fold higher or at least about 10,000-fold higher than the expression of OCT4, SPARC, AFP, TM and/or SOX7 markers. In some embodiments, OCT4, SPARC, AFP, TM and/or SOX7 markers are not significantly expressed in definitive endoderm cells.

[0154] It will also be appreciated that in some embodiments described herein, the expression of markers selected from the group consisting of GATA4, MIXL1, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 in definitive endoderm cells is increased as compared to the expression of OCT4, SPARC, AFP, TM and/or SOX7 in definitive endoderm cells.

Enrichment, Isolation and/or Purification of Definitive Endoderm

[0155] Definitive endoderm cells produced by any of the above-described processes can be enriched, isolated and/or purified by using an affinity tag that is specific for such cells. Examples of affinity tags specific for definitive endoderm cells are antibodies, ligands or other binding agents that are specific to a marker molecule, such as a polypeptide, that is present on the cell surface of definitive endoderm cells but which is not substantially present on other cell types that would be found in a cell culture produced by the methods described herein. In some processes, an antibody which binds to CXCR4 is used as an affinity tag for the enrichment, isolation or purification of definitive endoderm cells. In other processes, the chemokine SDF-1 or other molecules based on SDF-1 can also be used as affinity tags. Such molecules include, but not limited to, SDF-1 fragments, SDF-1 fusions or SDF-1 mimetics.

[0156] Methods for making antibodies and using them for cell isolation are known in the art and such methods can be implemented for use with the antibodies and definitive endoderm cells described herein. In one process, an antibody which binds to CXCR4 is attached to a magnetic bead and then allowed to bind to definitive endoderm cells in a cell culture which has been enzymatically treated to reduce intercellular and substrate adhesion. The cell/antibody/bead complexes are then exposed to a movable magnetic field which is used to separate bead-bound definitive endoderm cells from unbound cells. Once the definitive endoderm cells are physically separated from other cells in culture, the antibody binding is disrupted and the cells are replated in appropriate tissue culture medium.

[0157] Additional methods for obtaining enriched, isolated or purified definitive endoderm cell cultures or populations can also be used. For example, in some embodiments, the CXCR4 antibody is incubated with a definitive endoderm-containing cell culture that has been treated to reduce intercellular and substrate adhesion. The cells are then washed, centrifuged and resuspended. The cell suspension is then incubated with a secondary antibody, such as an FITC-conjugated antibody that is capable of binding to the primary antibody. The cells are then

washed, centrifuged and resuspended in buffer. The cell suspension is then analyzed and sorted using a fluorescence activated cell sorter (FACS). CXCR4-positive cells are collected separately from CXCR4-negative cells, thereby resulting in the isolation of such cell types. If desired, the isolated cell compositions can be further purified by using an alternate affinity-based method or by additional rounds of sorting using the same or different markers that are specific for definitive endoderm.

[0158] In still other processes, definitive endoderm cells are enriched, isolated and/or purified using a ligand or other molecule that binds to CXCR4. In some processes, the molecule is SDF-1 or a fragment, fusion or mimetic thereof.

[0159] In preferred processes, definitive endoderm cells are enriched, isolated and/or purified from other non-definitive endoderm cells after the stem cell cultures are induced to differentiate towards the definitive endoderm lineage. It will be appreciated that the above-described enrichment, isolation and purification procedures can be used with such cultures at any stage of differentiation.

[0160] In addition to the procedures just described, definitive endoderm cells may also be isolated by other techniques for cell isolation. Additionally, definitive endoderm cells may also be enriched or isolated by methods of serial subculture in growth conditions which promote the selective survival or selective expansion of the definitive endoderm cells.

[0161] Using the methods described herein, enriched, isolated and/or purified populations of definitive endoderm cells and or tissues can be produced *in vitro* from pluripotent cell cultures or cell populations, such as stem cell cultures or populations, which have undergone at least some differentiation. In some methods, the cells undergo random differentiation. In a preferred method, however, the cells are directed to differentiate primarily into definitive endoderm. Some preferred enrichment, isolation and/or purification methods relate to the *in vitro* production of definitive endoderm from human embryonic stem cells. Using the methods described herein, cell populations or cell cultures can be enriched in definitive endoderm content by at least about 2- to about 1000-fold as compared to untreated cell populations or cell cultures. In some embodiments, definitive endoderm cells can be enriched by at least about 5- to about 500-fold as compared to untreated cell populations or cell cultures. In other embodiments, definitive endoderm cells can be enriched from at least about 10- to about 200-fold as compared to untreated cell populations or cell cultures. In still other embodiments, definitive endoderm cells can be enriched from at least about 20- to about 100-fold as compared to untreated cell populations or cell cultures. In yet other embodiments, definitive endoderm cells can be enriched from at least about 40- to about 80-fold as compared to untreated cell populations or cell cultures. In certain embodiments, definitive endoderm cells can be enriched from at least about 2- to about 20-fold as compared to untreated cell populations or cell cultures.

Compositions Comprising Definitive Endoderm

[0162] Cell compositions produced by the above-described methods include cell cultures comprising definitive endoderm and cell populations enriched in definitive endoderm. For example, cell cultures which comprise definitive endoderm cells, wherein at least about 50-80% of the cells in culture are definitive endoderm cells, can be produced. Because the efficiency of the differentiation process can be adjusted by modifying certain parameters, which include but are not limited to, cell growth conditions, growth factor concentrations and the timing of culture steps, the differentiation procedures described herein can result in about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or greater than about 95% conversion of pluripotent cells to definitive endoderm. In processes in which isolation of definitive endoderm cells is employed, for example, by using an affinity reagent that binds to the CXCR4 receptor, a substantially pure definitive endoderm cell population can be recovered.

[0163] Some embodiments described herein relate to compositions, such as cell populations and cell cultures, that comprise both pluripotent cells, such as stem cells, and definitive endoderm cells. For example, using the methods described herein, compositions comprising mixtures of hESCs and definitive endoderm cells can be produced. In some embodiments, compositions comprising at least about 5 definitive endoderm cells for about every 95 pluripotent cells are produced. In other embodiments, compositions comprising at least about 95 definitive endoderm cells for about every 5 pluripotent cells are produced. Additionally, compositions comprising other ratios of definitive endoderm cells to pluripotent cells are contemplated. For example, compositions comprising at least about 1 definitive endoderm cell for about every 1,000,000 pluripotent cells, at least about 1 definitive endoderm cell for about every 100,000 pluripotent cells, at least about 1 definitive endoderm cell for about every 10,000 pluripotent cells, at least about 1 definitive endoderm cell for about every 1000 pluripotent cells, at least about 1 definitive endoderm cell for about every 500 pluripotent cells, at least about 1 definitive endoderm cell for about every 100 pluripotent cells, at least about 1 definitive endoderm cell for about every 10 pluripotent cells, at least about 1 definitive endoderm cell for about every 5 pluripotent cells, at least about 1 definitive endoderm cell for about every 2 pluripotent cells, at least about 2 definitive endoderm cells for about every 1 pluripotent cell, at least about 5 definitive endoderm cells for about every 1 pluripotent cell, at least about 10 definitive endoderm cells for about every 1 pluripotent cell, at least about 20 definitive endoderm cells for about every 1 pluripotent cell, at least about 50 definitive endoderm cells for about every 1 pluripotent cell, at least about 100 definitive endoderm cells for about every 1 pluripotent cell, at least about 1000 definitive endoderm cells for about every 1 pluripotent cell, at least about 10,000 definitive endoderm cells for about every 1 pluripotent cell, at least about 100,000

definitive endoderm cells for about every 1 pluripotent cell and at least about 1,000,000 definitive endoderm cells for about every 1 pluripotent cell are contemplated. In some embodiments, the pluripotent cells are human pluripotent stem cells. In certain embodiments the stem cells are derived from a morula, the inner cell mass of an embryo or the gonadal ridges of an embryo. In certain other embodiments, the pluripotent cells are derived from the gondal or germ tissues of a multicellular structure that has developed past the embryonic stage.

[0164] Some embodiments described herein relate to cell cultures or cell populations comprising from at least about 5% definitive endoderm cells to at least about 95% definitive endoderm cells. In some embodiments the cell cultures or cell populations comprise mammalian cells. In preferred embodiments, the cell cultures or cell populations comprise human cells. For example, certain specific embodiments relate to cell cultures comprising human cells, wherein from at least about 5% to at least about 95% of the human cells are definitive endoderm cells. Other embodiments relate to cell cultures comprising human cells, wherein at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90% or greater than 90% of the human cells are definitive endoderm cells. In embodiments where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0165] Further embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising human cells, such as human definitive endoderm cells, wherein the expression of either the SOX17 or the CXCR4 marker is greater than the expression of the OCT4, SPARC, alpha-fetoprotein (AFP), Thrombomodulin (TM) and/or SOX7 marker in at least about 5% of the human cells. In other embodiments, the expression of either the SOX17 or the CXCR4 marker is greater than the expression of the OCT4, SPARC, AFP, TM and/or SOX7 marker in at least about 10% of the human cells, in at least about 15% of the human cells, in at least about 20% of the human cells, in at least about 25% of the human cells, in at least about 30% of the human cells, in at least about 35% of the human cells, in at least about 40% of the human cells, in at least about 45% of the human cells, in at least about 50% of the human cells, in at least about 55% of the human cells, in at least about 60% of the human cells, in at least about 65% of the human cells, in at least about 70% of the human cells, in at least about 75% of the human cells, in at least about 80% of the human cells, in at least about 85% of the human cells, in at least about 90% of the human cells, in at least about 95% of the human cells or in greater than 95% of the human cells. In embodiments where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0166] It will be appreciated that some embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising human cells, such as human definitive endoderm cells, wherein the expression of one or more markers selected from the group consisting of GATA4, MIXL1, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 is greater than the expression of the OCT4, SPARC, AFP, TM and/or SOX7 markers in from at least about 5% to greater than at least about 95% of the human cells. In embodiments where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0167] Still other embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising human cells, such as human definitive endoderm cells, wherein the expression both the SOX17 and the CXCR4 marker is greater than the expression of the OCT4, SPARC, AFP, TM and/or SOX7 marker in at least about 5% of the human cells. In other embodiments, the expression of both the SOX17 and the CXCR4 marker is greater than the expression of the OCT4, SPARC, AFP, TM and/or SOX7 marker in at least about 10% of the human cells, in at least about 15% of the human cells, in at least about 20% of the human cells, in at least about 25% of the human cells, in at least about 30% of the human cells, in at least about 35% of the human cells, in at least about 40% of the human cells, in at least about 45% of the human cells, in at least about 50% of the human cells, in at least about 55% of the human cells, in at least about 60% of the human cells, in at least about 65% of the human cells, in at least about 70% of the human cells, in at least about 75% of the human cells, in at least about 80% of the human cells, in at least about 85% of the human cells, in at least about 90% of the human cells, in at least about 95% of the human cells or in greater than 95% of the human cells. In embodiments where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0168] It will be appreciated that some embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising human cells, such as human definitive endoderm cells, wherein the expression of the GATA4, MIXL1, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 markers is greater than the expression of the OCT4, SPARC, AFP, TM and/or SOX7 markers in from at least about 5% to greater than at least about 95% of the human cells. In embodiments where the cell cultures or cell populations comprise human feeder cells, the above percentages are calculated without respect to the human feeder cells in the cell cultures or cell populations.

[0169] Additional embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising mammalian endodermal cells, such as human endoderm cells, wherein the expression of either the SOX17 or the CXCR4 marker is greater than the expression of the OCT4, SPARC, AFP, TM and/or SOX7 marker in at least about 5% of the endodermal cells. In other embodiments, the expression of either the SOX17 or the CXCR4

marker is greater than the expression of the OCT4, SPARC, AFP, TM and/or SOX7 marker in at least about 10% of the endodermal cells, in at least about 15% of the endodermal cells, in at least about 20% of the endodermal cells, in at least about 25% of the endodermal cells, in at least about 30% of the endodermal cells, in at least about 35% of the endodermal cells, in at least about 40% of the endodermal cells, in at least about 45% of the endodermal cells, in at least about 50% of the endodermal cells, in at least about 55% of the endodermal cells, in at least about 60% of the endodermal cells, in at least about 65% of the endodermal cells, in at least about 70% of the endodermal cells, in at least about 75% of the endodermal cells, in at least about 80% of the endodermal cells, in at least about 85% of the endodermal cells, in at least about 90% of the endodermal cells, in at least about 95% of the endodermal cells or in greater than 95% of the endodermal cells.

[0170] It will be appreciated that some embodiments described herein relate to compositions, such as cell cultures or cell populations comprising mammalian endodermal cells, wherein the expression of one or more markers selected from the group consisting of GATA4, MIXL1, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 is greater than the expression of the OCT4, SPARC, AFP, TM and/or SOX7 markers in from at least about 5% to greater than at least about 95% of the endodermal cells.

[0171] Still other embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising mammalian endodermal cells, such as human endodermal cells, wherein the expression of both the SOX17 and the CXCR4 marker is greater than the expression of the OCT4, SPARC, AFP, TM and/or SOX7 marker in at least about 5% of the endodermal cells. In other embodiments, the expression of both the SOX17 and the CXCR4 marker is greater than the expression of the OCT4, SPARC, AFP, TM and/or SOX7 marker in at least about 10% of the endodermal cells, in at least about 15% of the endodermal cells, in at least about 20% of the endodermal cells, in at least about 25% of the endodermal cells, in at least about 30% of the endodermal cells, in at least about 35% of the endodermal cells, in at least about 40% of the endodermal cells, in at least about 45% of the endodermal cells, in at least about 50% of the endodermal cells, in at least about 55% of the endodermal cells, in at least about 60% of the endodermal cells, in at least about 65% of the endodermal cells, in at least about 70% of the endodermal cells, in at least about 75% of the endodermal cells, in at least about 80% of the endodermal cells, in at least about 85% of the endodermal cells, in at least about 90% of the endodermal cells, in at least about 95% of the endodermal cells or in greater than 95% of the endodermal cells.

[0172] It will be appreciated that some embodiments described herein relate to compositions, such as cell cultures or cell populations comprising mammalian endodermal cells, wherein the expression of the GATA4, MIXL1, HNF3b, GSC, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 markers is greater than the expression of the OCT4, SPARC, AFP, TM

and/or SOX7 markers in from at least about 5% to greater than at least about 95% of the endodermal cells.

[0173] Using the methods described herein, compositions comprising definitive endoderm cells substantially free of other cell types can be produced. In some embodiments described herein, the definitive endoderm cell populations or cell cultures produced by the methods described herein are substantially free of cells that significantly express the OCT4, SOX7, AFP, SPARC, TM, ZIC1 or BRACH marker genes.

[0174] In one embodiment, a description of a definitive endoderm cell based on the expression of marker genes is, SOX17 high, MIXL1 high, AFP low, SPARC low, Thrombomodulin low, SOX7 low, CXCR4 high.

The *PDX1* Gene Expression During Development

[0175] PDX1 (also called STF-1, IDX-1 and IPF-1) is a transcription factor that is necessary for development of the pancreas and rostral duodenum. PDX1 is first expressed in the pancreatic endoderm, which arises from posterior foregut endoderm and will produce both the exocrine and endocrine cells, starting at E8.5 in the mouse. Later, PDX1 becomes restricted to beta-cells and some delta-cells of the endocrine pancreas. This expression pattern is maintained in the adult. PDX1 is also expressed in duodenal endoderm early in development, which is adjacent to the forming pancreas, then in the duodenal enterocytes and enteroendocrine cells, antral stomach and in the common bile, cystic and biliary ducts. This region of expression also becomes limited, at the time that pancreatic expression becomes restricted, to predominantly the rostral duodenum.

PDX1-Positive Cells and Processes Related Thereto

[0176] Embodiments of other differentiation processes described herein relate to novel, defined processes for the production of PDX1-positive endoderm cells, wherein the PDX1-positive endoderm cells are multipotent cells that can differentiate into cells, tissues or organs derived from the foregut/midgut region of the gut tube (PDX1-positive foregut/midgut endoderm). Some preferred embodiments relate to processes for the production of PDX1-positive foregut endoderm cells. In some embodiments, these PDX1-positive foregut endoderm cells are multipotent cells that can differentiate into cells, tissues or organs derived from the anterior portion of the gut tube (PDX1-positive foregut endoderm). Additional preferred embodiments relate to processes for the production of PDX1-positive endoderm cells of the posterior portion of the foregut. In some embodiments, these PDX1-positive endoderm cells are multipotent cells that can differentiate into cells, tissues or organs derived from the posterior portion of the foregut region of the gut tube.

[0177] The PDX1-positive foregut endoderm cells, such as those produced according to the methods described herein, can be used to produce fully differentiated insulin-producing β-cells. In some embodiments, PDX1-positive foregut endoderm cells are produced by differentiating definitive endoderm cells that do not substantially express PDX1 (PDX1-negative definitive endoderm cells; also referred to herein as definitive endoderm) so as to form PDX1-positive foregut endoderm cells. PDX1-negative definitive endoderm cells can be prepared by differentiating pluripotent cells, such as embryonic stem cells, as described herein or by any other known methods. A convenient and highly efficient method for producing PDX1-negative definitive endoderm from pluripotent cells is described previously herein and in US Patent No. 11/021,618, entitled DEFINITIVE ENDODERM, filed December 23, 2004.

[0178] Processes of producing PDX1-positive foregut endoderm cells provide a basis for efficient production of pancreatic tissues such as acinar cells, ductal cells and islet cells from pluripotent cells. In certain preferred embodiments, human PDX1-positive foregut endoderm cells are derived from human PDX1-negative definitive endoderm cells, which in turn, are derived from hESCs. These human PDX1-positive foregut endoderm cells can then be used to produce functional insulin-producing β-cells. To obtain useful quantities of insulin-producing β-cells, high efficiency of differentiation is desirable for each of the differentiation steps that occur prior to reaching the pancreatic islet/β-cell fate. Because differentiation of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells represents an early step towards the production of functional pancreatic islet/β-cells (as shown in Figure 1), high efficiency of differentiation at this step is particularly desirable.

[0179] In view of the desirability of efficient differentiation of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells, some aspects of the processes described herein relate to *in vitro* methodology that results in approximately 2-25% conversion of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells. Typically, such methods encompass the application of culture and growth factor conditions in a defined and temporally specified fashion. Further enrichment of the cell population for PDX1-positive foregut endoderm cells can be achieved by isolation and/or purification of the PDX1-positive foregut endoderm cells from other cells in the population by using a reagent that specifically binds to the PDX1-positive foregut endoderm cells. As an alternative, PDX1-positive foregut endoderm cells can be labeled with a reporter gene, such as green fluorescent protein (GFP), so as to enable the detection of PDX1 expression. Such fluorescently labeled cells can then be purified by fluorescent activated cell sorting (FACS). Further aspects of the present invention relate to cell cultures and enriched cell populations comprising PDX1-positive foregut endoderm cells as well as methods for identifying factors useful in the differentiation to and from PDX1-positive foregut endoderm.

[0180] In order to determine the amount of PDX1-positive foregut endoderm cells in a cell culture or cell population, a method of distinguishing this cell type from the other cells in the culture or in the population is desirable. Accordingly, certain embodiments described herein relate to cell markers whose presence, absence and/or relative expression levels are indicative of PDX1-positive foregut endoderm cells as well as methods for detecting and determining the expression of such markers.

[0181] In some embodiments described herein, the presence, absence and/or level of expression of a marker is determined by quantitative PCR (Q-PCR). For example, the amount of transcript produced by certain genetic markers, such as PDX1, SOX17, SOX7, SOX1, ZIC1, NFM, alpha-fetoprotein (AFP), homeobox A13 (HOXA13), homeobox C6 (HOXC6), and/or other markers described herein is determined by Q-PCR. In other embodiments, immunohistochemistry is used to detect the proteins expressed by the above-mentioned genes. In still other embodiments, Q-PCR and immunohistochemical techniques are both used to identify and determine the amount or relative proportions of such markers.

[0182] By using the differentiation and detection methods described herein, it is possible to identify PDX1-positive foregut endoderm cells, as well as determine the proportion of PDX1-positive foregut endoderm cells in a cell culture or cell population. For example, in some embodiments, the PDX1-positive foregut endoderm cells or cell populations that are produced express the PDX1 gene at a level of at least about 2 orders of magnitude greater than PDX1-negative cells or cell populations. In other embodiments, the PDX1-positive foregut endoderm cells and cell populations that are produced express the PDX1 gene at a level of more than 2 orders of magnitude greater than PDX1-negative cells or cell populations. In still other embodiments, the PDX1-positive foregut endoderm cells or cell populations that are produced express one or more of the markers selected from the group consisting of PDX1, SOX17, HOXA13 and HOXC6 at a level of about 2 or more than 2 orders of magnitude greater than PDX1-negative definitive endoderm cells or cell populations.

[0183] The compositions and methods described herein have several useful features. For example, the cell cultures and cell populations comprising PDX1-positive endoderm, as well as the methods for producing such cell cultures and cell populations, are useful for modeling the early stages of human development. Furthermore, the compositions and methods described herein can also serve for therapeutic intervention in disease states, such as diabetes mellitus. For example, since PDX1-positive foregut endoderm serves as the source for only a limited number of tissues, it can be used in the development of pure tissue or cell types.

Production of PDX1-Positve Foregut Endoderm from PDX1-Negative Definitive Endoderm

[0184] The PDX1-positive foregut endoderm cell cultures and populations comprising PDX1-positive foregut endoderm cells that are described herein are produced from

PDX1-negative definitive endoderm, which is generated from pluripotent cells as described above. A preferred method utilizes human embryonic stem cells as the starting material. In one embodiment, hESCs are first converted to PDX1-negative definitive endoderm cells, which are then converted to PDX1-positive foregut endoderm cells. It will be appreciated, however, that the starting materials for the production of PDX1-positive foregut endoderm is not limited to definitive endoderm cells produced using pluripotent cell differentiation methods. Rather, any PDX1-negative definitive endoderm cells can be used in the methods described herein regardless of their origin.

[0185] In some embodiments described herein, cell cultures or cell populations comprising PDX1-negative definitive endoderm cells can be used for further differentiation to cell cultures and/or enriched cell populations comprising PDX1-positive foregut endoderm cells. For example, a cell culture or cell population comprising human PDX1-negative, SOX17-positive definitive endoderm cells can be used. In some embodiments, the cell culture or cell population may also comprise differentiation factors, such as activins, nodals and/or BMPs, remaining from the previous differentiation step (that is, the step of differentiating pluripotent cells to definitive endoderm cells). In other embodiments, factors utilized in the previous differentiation step are removed from the cell culture or cell population prior to the addition of factors used for the differentiation of the PDX1-negative, SOX17-positive definitive endoderm cells to PDX1-positive foregut endoderm cells. In other embodiments, cell populations enriched for PDX1-negative, SOX17-positive definitive endoderm cells are used as a source for the production of PDX1-positive foregut endoderm cells.

[0186] PDX1-negative definitive endoderm cells in culture are differentiated to PDX1-positive endoderm cells by providing to a cell culture comprising PDX1-negative, SOX17-positive definitive endoderm cells a differentiation factor that promotes differentiation of the cells to PDX1-positive foregut endoderm cells (foregut differentiation factor). In some embodiments of the present invention, the foregut differentiation factor is retinoid, such as retinoic acid (RA). In some embodiments, the retinoid is used in conjunction with a fibroblast growth factor, such as FGF-4 or FGF-10. In other embodiments, the retinoid is used in conjunction with a member of the TGFβ superfamily of growth factors and/or a conditioned medium.

[0187] As defined above, the phrase "conditioned medium" refers to a medium that is altered as compared to a base medium. For example, the conditioning of a medium may cause molecules, such as nutrients and/or growth factors, to be added to or depleted from the original levels found in the base medium. In some embodiments, a medium is conditioned by allowing cells of certain types to be grown or maintained in the medium under certain conditions for a certain period of time. For example, a medium can be conditioned by allowing hESCs to be expanded, differentiated or maintained in a medium of defined composition at a defined temperature for a defined number of hours. As will be appreciated by those of skill in the art,

numerous combinations of cells, media types, durations and environmental conditions can be used to produce nearly an infinite array of conditioned media. In some embodiments of the present invention, a medium is conditioned by allowing differentiated pluripotent cells to be grown or maintained in a medium comprising about 1% to about 20% serum concentration. In other embodiments, a medium is conditioned by allowing differentiated pluripotent cells to be grown or maintained in a medium comprising about 1 ng/ml to about 1000 ng/ml activin A. In still other embodiments, a medium is conditioned allowing differentiated pluripotent cells to be grown or maintained in a medium comprising about 1 ng/ml to about 1000 ng/ml BMP4. In a preferred embodiment, a conditioned medium is prepared by allowing differentiated hESCs to be grown or maintained for 24 hours in a medium, such as RPMI, comprising about 25 ng/ml activin A and about 2 μM RA.

[0188] In some embodiments described herein, the cells used to condition the medium, which is used to enhance the differentiation of PDX1-negative definitive endoderm to PDX1-positive foregut endoderm, are cells that are differentiated from pluripotent cells, such as hESCs, over about a 5 day time period in a medium such as RPMI comprising about 0% to about 20% serum and/or one or more growth/differentiation factors of the TGFβ superfamily. Differentiation factors, such as activin A and BMP4 are supplied at concentrations ranging from about 1 ng/ml to about 1000 ng/ml. In certain embodiments of the present invention, the cells used to condition the medium are differentiated from hESCs over about a 5 day period in low serum RPMI. According to some embodiments, low serum RPMI refers to a low serum containing medium, wherein the serum concentration is gradually increased over a defined time period. For example, in one embodiment, low serum RPMI comprises a concentration of about 0.2% fetal bovine serum (FBS) on the first day of cell growth, about 0.5% FBS on the second day of cell growth and about 2% FBS on the third through fifth day of cell growth. In another embodiment, low serum RPMI comprises a concentration of about 0% on day one, about 0.2% on day two and about 2% on days 3-6. In certain preferred embodiments, low serum RPMI is supplemented with one or more differentiation factors, such as activin A and BMP4. In addition to its use in preparing cells used to condition media, low serum RPMI can be used as a medium for the differentiation of PDX1-positive foregut endoderm cells from PDX1-negative definitive endoderm cells.

[0189] It will be appreciated by those of ordinary skill in the art that conditioned media can be prepared from media other than RPMI provided that such media do not interfere with the growth or maintenance of PDX1-positive foregut endoderm cells. It will also be appreciated that the cells used to condition the medium can be of various types. In embodiments where freshly differentiated cells are used to condition a medium, such cells can be differentiated in a medium other than RPMI provided that the medium does not inhibit the growth or

maintenance of such cells. Furthermore, a skilled artisan will appreciate that neither the duration of conditioning nor the duration of preparation of cells used for conditioning is required to be 24 hours or 5 days, respectively, as other time periods will be sufficient to achieve the effects reported herein.

[0190] In general, the use of a retinoid in combination with a fibroblast growth factor, a member of the TGFβ superfamily of growth factors, a conditioned medium or a combination of any of these foregut differentiation factors causes greater differentiation of PDX1-negative definitive endoderm to PDX1-positive foregut endoderm than the use of a retinoid alone. In a preferred embodiment, RA and FGF-10 are both provided to the PDX1-negative definitive endoderm cell culture. In another preferred embodiment, PDX1-negative definitive endoderm cells are differentiated in a culture comprising a conditioned medium, activin A, activin B and RA.

[0191] With respect to some of the embodiments of differentiation processes described herein, the above-mentioned foregut differentiation factors are provided to the cells so that these factors are present in the cell culture or cell population at concentrations sufficient to promote differentiation of at least a portion of the PDX1-negative definitive endoderm cell culture or cell population to PDX1-positive foregut endoderm cells. As defined previously, when used in connection with cell cultures and/or cell populations, the term "portion" means any non-zero amount of the cell culture or cell population, which ranges from a single cell to the entirety of the cell culture or cells population.

[0192] In some embodiments of processes described herein, a retinoid is provided to the cells of a cell culture such that it is present at a concentration of at least about 0.01 μM, at least about 0.02 μM, at least about 0.04 μM, at least about 0.08 μM, at least about 0.1 μM, at least about 0.2 μM, at least about 0.3 μM, at least about 0.4 μM, at least about 0.5 μM, at least about 0.6 μM, at least about 0.7 μM, at least about 0.8 μM, at least about 0.9 μM, at least about 1 μM, at least about 1.1 μM, at least about 1.2 μM, at least about 1.3 μM, at least about 1.4 μM, at least about 1.5 μM, at least about 1.6 μM, at least about 1.7 μM, at least about 1.8 μM, at least about 1.9 μM, at least about 2 μM, at least about 2.1 μM, at least about 2.2 μM, at least about 2.3 μM, at least about 2.4 μM, at least about 2.5 μM, at least about 2.6 μM, at least about 2.7 μM, at least about 2.8 μM, at least about 2.9 μM, at least about 3 μM, at least about 3.5 μM, at least about 4 μM, at least about 4.5 μM, at least about 5 μM, at least about 10 μM, at least about 20 μM, at least about 30 μM, at least about 40 μM or at least about 50 μM. In a preferred embodiment, the retinoid is retinoic acid.

[0193] In other embodiments of the processes described herein, one or more differentiation factors of the fibroblast growth factor family are present in the cell culture. For example, in some embodiments, FGF-4 can be present in the cell culture at a concentration of at

least about 10 ng/ml, at least about 25 ng/ml, at least about 50 ng/ml, at least about 75 ng/ml, at least about 100 ng/ml, at least about 200 ng/ml, at least about 300 ng/ml, at least about 400 ng/ml, at least about 500 ng/ml, or at least about 1000 ng/ml. In further embodiments, FGF-10 is present in the cell culture at a concentration of at least about 10 ng/ml, at least about 25 ng/ml, at least about 50 ng/ml, at least about 75 ng/ml, at least about 100 ng/ml, at least about 200 ng/ml, at least about 300 ng/ml, at least about 400 ng/ml, at least about 500 ng/ml, or at least about 1000 ng/ml. In some embodiments, either FGF-4 or FGF-10, but not both, is provided to the cell culture along with RA. In a preferred embodiment, RA is present in the cell culture at 1 μM and FGF-10 is present at a concentration of 50 ng/ml.

[0194] In some embodiments of the processes described herein, growth factors of the TGFβ superfamily and/or a conditioned medium are present in the cell culture. These differentiation factors can be used in combination with RA and/or other mid-foregut differentiation factors including, but not limited to, FGF-4 and FGF-10. For example, in some embodiments, activin A and/or activin B can be present in the cell culture at a concentration of at least about 5 ng/ml, at least about 10 ng/ml, at least about 25 ng/ml, at least about 50 ng/ml, at least about 75 ng/ml, at least about 100 ng/ml, at least about 200 ng/ml, at least about 300 ng/ml, at least about 400 ng/ml, at least about 500 ng/ml, or at least about 1000 ng/ml. In further embodiments, a conditioned medium is present in the cell culture at a concentration of at least about 1%, at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 100% of the total medium. In some embodiments, activin A, activin B and a conditioned medium are provided to the cell culture along with RA. In a preferred embodiment, PDX1-negative definitive endoderm cells are differentiated to PDX1-positive foregut endoderm cells in cultures comprising about 1 μM RA, about 25 ng/ml activin A and low serum RPMI medium that has been conditioned for about 24 hours by differentiated hESCs, wherein the differentiated hESCs have been differentiated for about 5 days in low serum RPMI comprising about 100 ng/ml activin A. In another preferred embodiment, activin B and/or FGF-10 are also present in the culture at 25 ng/ml and 50 ng/ml, respectively.

[0195] In certain embodiments of the processes described herein, the above-mentioned foregut differentiation factors are removed from the cell culture subsequent to their addition. For example, the foregut differentiation factors can be removed within about one day, about two days, about three days, about four days, about five days, about six days, about seven days, about eight days, about nine days or about ten days after their addition.

[0196] Cultures of PDX1-positive foregut endoderm cells can be grown in a medium containing reduced serum. Serum concentrations can range from about 0.05% (v/v) to about 20% (v/v). In some embodiments, PDX1-positive foregut endoderm cells are grown with serum

replacement. For example, in certain embodiments, the serum concentration of the medium can be less than about 0.05% (v/v), less than about 0.1% (v/v), less than about 0.2% (v/v), less than about 0.3% (v/v), less than about 0.4% (v/v), less than about 0.5% (v/v), less than about 0.6% (v/v), less than about 0.7% (v/v), less than about 0.8% (v/v), less than about 0.9% (v/v), less than about 1% (v/v), less than about 2% (v/v), less than about 3% (v/v), less than about 4% (v/v), less than about 5% (v/v), less than about 6% (v/v), less than about 7% (v/v), less than about 8% (v/v), less than about 9% (v/v), less than about 10% (v/v), less than about 15% (v/v) or less than about 20% (v/v). In some embodiments, PDX1-positive foregut endoderm cells are grown without serum. In other embodiments, PDX1-positive foregut endoderm cells are grown with serum replacement.

[0197] In still other embodiments, PDX1-positive foregut endoderm cells are grown in the presence of B27. In such embodiments, B27 can be provided to the culture medium in concentrations ranging from about 0.1% (v/v) to about 20% (v/v) or in concentrations greater than about 20% (v/v). In certain embodiments, the concentration of B27 in the medium is about 0.1% (v/v), about 0.2% (v/v), about 0.3% (v/v), about 0.4% (v/v), about 0.5% (v/v), about 0.6% (v/v), about 0.7% (v/v), about 0.8% (v/v), about 0.9% (v/v), about 1% (v/v), about 2% (v/v), about 3% (v/v), about 4% (v/v), about 5% (v/v), about 6% (v/v), about 7% (v/v), about 8% (v/v), about 9% (v/v), about 10% (v/v), about 15% (v/v) or about 20% (v/v). Alternatively, the concentration of the added B27 supplement can be measured in terms of multiples of the strength of a commercially available B27 stock solution. For example, B27 is available from Invitrogen (Carlsbad, CA) as a 50X stock solution. Addition of a sufficient amount of this stock solution to a sufficient volume of growth medium produces a medium supplemented with the desired amount of B27. For example, the addition of 10 ml of 50X B27 stock solution to 90 ml of growth medium would produce a growth medium supplemented with 5X B27. The concentration of B27 supplement in the medium can be about 0.1X, about 0.2X, about 0.3X, about 0.4X, about 0.5X, about 0.6X, about 0.7X, about 0.8X, about 0.9X, about 1X, about 1.1X, about 1.2X, about 1.3X, about 1.4X, about 1.5X, about 1.6X, about 1.7X, about 1.8X, about 1.9X, about 2X, about 2.5X, about 3X, about 3.5X, about 4X, about 4.5X, about 5X, about 6X, about 7X, about 8X, about 9X, about 10X, about 11X, about 12X, about 13X, about 14X, about 15X, about 16X, about 17X, about 18X, about 19X, about 20X and greater than about 20X.

Monitoring the Differentiation of PDX1-Negative Definitive Endoderm to PDX1-Positive Endoderm

[0198] As with the differentiation of definitive endoderm cells from pluripotent cells, the progression of differentiation from PDX1-negative, SOX17-positive definitive endoderm to PDX1-positive foregut endoderm can be monitored by determining the expression of markers characteristic of these cell types. Such monitoring permits one to determine the amount

of time that is sufficient for the production of a desired amount of PDX1-positive foregut endoderm under various conditions, for example, one or more differentiation factor concentrations and environmental conditions. In preferred embodiments, the amount of time that is sufficient for the production of a desired amount of PDX1-positive foregut endoderm is determined by detecting the expression of PDX1. In some embodiments, the expression of certain markers is determined by detecting the presence or absence of the marker. Alternatively, the expression of certain markers can be determined by measuring the level at which the marker is present in the cells of the cell culture or cell population. In such embodiments, the measurement of marker expression can be qualitative or quantitative. As described above, a preferred method of quantitating the expression markers that are produced by marker genes is through the use of Q-PCR. In particular embodiments, Q-PCR is used to monitor the progression of cells of the PDX1-negative, SOX17-positive definitive endoderm culture to PDX1-positive foregut endoderm cells by quantitating expression of marker genes characteristic of PDX1-positive foregut endoderm and the lack of expression of marker genes characteristic of other cell types. Other methods which are known in the art can also be used to quantitate marker gene expression. For example, the expression of a marker gene product can be detected by using antibodies specific for the marker gene product of interest. In some embodiments, the expression of marker genes characteristic of PDX1-positive foregut endoderm as well as the lack of significant expression of marker genes characteristic of PDX1-negative definitive endoderm, hESCs and other cell types is determined.

[0199] As described further in the Examples below, PDX1 is a marker gene that is associated with PDX1-positive foregut endoderm. As such, in some embodiments of the processes described herein, the expression of PDX1 is determined. In other embodiments, the expression of other markers, which are expressed in PDX1-positive foregut endoderm, including, but not limited to, SOX17, HOXA13 and/or HOXC6 is also determined. Since PDX1 can also be expressed by certain other cell types (that is, visceral endoderm and certain neural ectoderm), some embodiments described herein relate to demonstrating the absence or substantial absence of marker gene expression that is associated with visceral endoderm and/or neural ectoderm. For example, in some embodiments, the expression of markers, which are expressed in visceral endoderm and/or neural cells, including, but not limited to, SOX7, AFP, SOX1, ZIC1 and/or NFM is determined.

[0200] In some embodiments, PDX1-positive foregut endoderm cell cultures produced by the methods described herein are substantially free of cells expressing the SOX7, AFP, SOX1, ZIC1 or NFM marker genes. In certain embodiments, the PDX1-positive foregut endoderm cell cultures produced by the processes described herein are substantially free of visceral endoderm, parietal endoderm and/or neural cells.

Enrichment, Isolation and/or Purification of PDX1-Positive Foregut Endoderm

[0201] With respect to additional aspects of the processes described herein, PDX1-positive foregut endoderm cells can be enriched, isolated and/or purified. In some embodiments, cell populations enriched for PDX1-positive foregut endoderm cells are produced by isolating such cells from cell cultures.

[0202] In some embodiments of the processes described herein, PDX1-positive foregut endoderm cells are fluorescently labeled then isolated from non-labeled cells by using a fluorescence activated cell sorter (FACS). In such embodiments, a nucleic acid encoding green fluorescent protein (GFP) or another nucleic acid encoding an expressible fluorescent marker gene is used to label PDX1-positive cells. For example, in some embodiments, at least one copy of a nucleic acid encoding GFP or a biologically active fragment thereof is introduced into a pluripotent cell, preferably a human embryonic stem cell, downstream of the PDX1 promoter such that the expression of the GFP gene product or biologically active fragment thereof is under control of the PDX1 promoter. In some embodiments, the entire coding region of the nucleic acid, which encodes PDX1, is replaced by a nucleic acid encoding GFP or a biologically active fragment thereof. In other embodiments, the nucleic acid encoding GFP or a biologically active fragment thereof is fused in frame with at least a portion of the nucleic acid encoding PDX1, thereby generating a fusion protein. In such embodiments, the fusion protein retains a fluorescent activity similar to GFP.

[0203] Fluorescently marked cells, such as the above-described pluripotent cells, are differentiated to definitive endoderm and then to PDX1-positive foregut endoderm as described previously above. Because PDX1-positive foregut endoderm cells express the fluorescent marker gene, whereas PDX1-negative cells do not, these two cell types can be separated. In some embodiments, cell suspensions comprising a mixture of fluorescently-labeled PDX1-positive cells and unlabeled PDX1-negative cells are sorted using a FACS. PDX1-positive cells are collected separately from PDX1-negative cells, thereby resulting in the isolation of such cell types. If desired, the isolated cell compositions can be further purified by additional rounds of sorting using the same or different markers that are specific for PDX1-positve foregut endoderm.

[0204] In addition to the procedures just described, PDX1-positive foregut endoderm cells may also be isolated by other techniques for cell isolation. Additionally, PDX1-positive foregut endoderm cells may also be enriched or isolated by methods of serial subculture in growth conditions which promote the selective survival or selective expansion of said PDX1-positive foregut endoderm cells.

[0205] It will be appreciated that the above-described enrichment, isolation and purification procedures can be used with such cultures at any stage of differentiation.

[0206] Using the methods described herein, enriched, isolated and/or purified populations of PDX1-positive foregut endoderm cells and/or tissues can be produced *in vitro* from

PDX1-negative, SOX17-positive definitive endoderm cell cultures or cell populations which have undergone at least some differentiation. In some embodiments, the cells undergo random differentiation. In a preferred embodiment, however, the cells are directed to differentiate primarily into PDX1-positive foregut endoderm cells. Some preferred enrichment, isolation and/or purification methods relate to the *in vitro* production of PDX1-positive foregut endoderm cells from human embryonic stem cells.

[0207] Using the methods described herein, cell populations or cell cultures can be enriched in PDX1-positive foregut endoderm cell content by at least about 2- to about 1000-fold as compared to untreated cell populations or cell cultures. In some embodiments, PDX1-positive foregut endoderm cells can be enriched by at least about 5- to about 500-fold as compared to untreated cell populations or cell cultures. In other embodiments, PDX1-positive foregut endoderm cells can be enriched from at least about 10- to about 200-fold as compared to untreated cell populations or cell cultures. In still other embodiments, PDX1-positive foregut endoderm cells can be enriched from at least about 20- to about 100-fold as compared to untreated cell populations or cell cultures. In yet other embodiments, PDX1-positive foregut endoderm cells can be enriched from at least about 40- to about 80-fold as compared to untreated cell populations or cell cultures. In certain embodiments, PDX1-positive foregut endoderm cells can be enriched from at least about 2- to about 20-fold as compared to untreated cell populations or cell cultures.

Compositions Comprising PDX1-Positive Foregut Endoderm

[0208] Some embodiments described herein relate to cell compositions, such as cell cultures or cell populations, comprising PDX1-positive endoderm cells, wherein the PDX1-positive endoderm cells are multipotent cells that can differentiate into cells, tissues or organs derived from the anterior portion of the gut tube (PDX1-positive foregut endoderm). In accordance with certain embodiments, the PDX1-positive foregut endoderm are mammalian cells, and in a preferred embodiment, these cells are human cells..

[0209] Other embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising cells of one or more cell types selected from the group consisting of hESCs, PDX1-negative definitive endoderm cells, PDX1-positive foregut endoderm cells and mesoderm cells. In some embodiments, hESCs comprise less than about 5%, less than about 4%, less than about 3%, less than about 2% or less than about 1% of the total cells in the culture. In other embodiments, PDX1-negative definitive endoderm cells comprise less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 12%, less than about 10%, less than

about 8%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2% or less than about 1% of the total cells in the culture. In yet other embodiments, mesoderm cells comprise less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 12%, less than about 10%, less than about 8%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2% or less than about 1% of the total cells in the culture.

[0210] Additional embodiments described herein relate to compositions, such as cell cultures or cell populations, produced by the processes described herein, which comprise PDX1-positive foregut endoderm as the majority cell type. In some embodiments, the processes described herein produce cell cultures and/or cell populations comprising at least about 99%, at least about 98%, at least about 97%, at least about 96%, at least about 95%, at least about 94%, at least about 93%, at least about 92%, at least about 91%, at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 55%, at least about 54%, at least about 53%, at least about 52% or at least about 51% PDX1-positive foregut endoderm cells. In preferred embodiments the cells of the cell cultures or cell populations comprise human cells. In other embodiments, the processes described herein produce cell cultures or cell populations comprising at least about 50%, at least about 45%, at least about 40%, at least about 35%, at least about 30%, at least about 25%, at least about 24%, at least about 23%, at least about 22%, at least about 21%, at least about 20%, at least about 19%, at least about 18%, at least about 17%, at least about 16%, at least about 15%, at least about 14%, at least about 13%, at least about 12%, at least about 11%, at least about 10%, at least about 9%, at least about 8%, at least about 7%, at least about 6%, at least about 5%, at least about 4%, at least about 3%, at least about 2% or at least about 1% PDX1-positive foregut endoderm cells. In preferred embodiments, the cells of the cell cultures or cell populations comprise human cells. In some embodiments, the percentage of PDX1-positive foregut endoderm cells in the cell cultures or populations is calculated without regard to the feeder cells remaining in the culture.

[0211] Still other embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising mixtures of PDX1-positive foregut endoderm cells and PDX1-negative definitive endoderm cells. For example, cell cultures or cell populations comprising at least about 5 PDX1-positive foregut endoderm cells for about every 95 PDX1-negative definitive endoderm cells can be produced. In other embodiments, cell cultures or cell populations comprising at least about 95 PDX1-positive foregut endoderm cells for about every 5 PDX1-negative definitive endoderm cells can be produced. Additionally, cell cultures or cell populations comprising other ratios of PDX1-positive foregut endoderm cells to PDX1-negative

definitive endoderm cells are contemplated. For example, compositions comprising at least about 1 PDX1-positive foregut endoderm cell for about every 1,000,000 PDX1-negative definitive endoderm cells, at least about 1 PDX1-positive foregut endoderm cell for about every 100,000 PDX1-negative definitive endoderm cells, at least about 1 PDX1-positive foregut endoderm cell for about every 10,000 PDX1-negative definitive endoderm cells, at least about 1 PDX1-positive foregut endoderm cell for about every 1000 PDX1-negative definitive endoderm cells, at least about 1 PDX1-positive foregut endoderm cell for about every 500 PDX1-negative definitive endoderm cells, at least about 1 PDX1-positive foregut endoderm cell for about every 100 PDX1-negative definitive endoderm cells, at least about 1 PDX1-positive foregut endoderm cell for about every 10 PDX1-negative definitive endoderm cells, at least about 1 PDX1-positive foregut endoderm cell for about every 5 PDX1-negative definitive endoderm cells, at least about 1 PDX1-positive foregut endoderm cell for about every 4 PDX1-negative definitive endoderm cells, at least about 1 PDX1-positive foregut endoderm cell for about every 2 PDX1-negative definitive endoderm cells, at least about 1 PDX-1 positive foregut endoderm cell for about every 1 PDX1-negative definitive endoderm cell, at least about 2 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell, at least about 4 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell, at least about 5 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell, at least about 10 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell, at least about 20 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell, at least about 50 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell, at least about 100 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell, at least about 1000 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell, at least about 10,000 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell, at least about 100,000 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell and at least about 1,000,000 PDX1-positive foregut endoderm cells for about every 1 PDX1-negative definitive endoderm cell are contemplated.

[0212] In some embodiments described herein, the PDX1-negative definitive endoderm cells from which PDX1-positive foregut endoderm cells are produced are derived from human pluripotent cells, such as human pluripotent stem cells. In certain embodiments, the human pluripotent cells are derived from a morula, the inner cell mass of an embryo or the gonadal ridges of an embryo. In certain other embodiments, the human pluripotent cells are derived from the gonadal or germ tissues of a multicellular structure that has developed past the embryonic stage.

[0213] Further embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising human cells, including human PDX1-positive foregut endoderm, wherein the expression of the PDX1 marker is greater than the expression of the AFP, SOX7, SOX1, ZIC1 and/or NFM marker in at least about 2% of the human cells. In other embodiments, the expression of the PDX1 marker is greater than the expression of the AFP, SOX7, SOX1, ZIC1 and/or NFM marker in at least about 5% of the human cells, in at least about 10% of the human cells, in at least about 15% of the human cells, in at least about 20% of the human cells, in at least about 25% of the human cells, in at least about 30% of the human cells, in at least about 35% of the human cells, in at least about 40% of the human cells, in at least about 45% of the human cells, in at least about 50% of the human cells, in at least about 55% of the human cells, in at least about 60% of the human cells, in at least about 65% of the human cells, in at least about 70% of the human cells, in at least about 75% of the human cells, in at least about 80% of the human cells, in at least about 85% of the human cells, in at least about 90% of the human cells, in at least about 95% of the human cells or in at least about 98% of the human cells. In some embodiments, the percentage of human cells in the cell cultures or populations, wherein the expression of PDX1 is greater than the expression of the AFP, SOX7, SOX1, ZIC1 and/or NFM marker, is calculated without regard to feeder cells.

[0214] It will be appreciated that some embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising human PDX1-positive foregut endoderm cells, wherein the expression of one or more markers selected from the group consisting of SOX17, HOXA13 and HOXC6 is greater than the expression of the AFP, SOX7, SOX1, ZIC1 and/or NFM marker in from at least about 2% to greater than at least about 98% of the human cells. In some embodiments, the expression of one or more markers selected from the group consisting of SOX17, HOXA13 and HOXC6 is greater than the expression of the AFP, SOX7, SOX1, ZIC1 and/or NFM marker in at least about 5% of the human cells, in at least about 10% of the human cells, in at least about 15% of the human cells, in at least about 20% of the human cells, in at least about 25% of the human cells, in at least about 30% of the human cells, in at least about 35% of the human cells, in at least about 40% of the human cells, in at least about 45% of the human cells, in at least about 50% of the human cells, in at least about 55% of the human cells, in at least about 60% of the human cells, in at least about 65% of the human cells, in at least about 70% of the human cells, in at least about 75% of the human cells, in at least about 80% of the human cells, in at least about 85% of the human cells, in at least about 90% of the human cells, in at least about 95% of the human cells or in at least about 98% of the human cells. In some embodiments, the percentage of human cells in the cell cultures or populations, wherein the expression of one or more markers selected from the group consisting of SOX17, HOXA13 and HOXC6 is greater than the expression of the AFP, SOX7, SOX1, ZIC1 and/or NFM marker, is calculated without regard to feeder cells.

[0215] Additional embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising mammalian endodermal cells, such as human endoderm cells, wherein the expression of the PDX1 marker is greater than the expression of the AFP, SOX7, SOX1, ZIC1 and/or NFM marker in at least about 2% of the endodermal cells. In other embodiments, the expression of the PDX1 marker is greater than the expression of the AFP, SOX7, SOX1, ZIC1 and/or NFM marker in at least about 5% of the endodermal cells, in at least about 10% of the endodermal cells, in at least about 15% of the endodermal cells, in at least about 20% of the endodermal cells, in at least about 25% of the endodermal cells, in at least about 30% of the endodermal cells, in at least about 35% of the endodermal cells, in at least about 40% of the endodermal cells, in at least about 45% of the endodermal cells, in at least about 50% of the endodermal cells, in at least about 55% of the endodermal cells, in at least about 60% of the endodermal cells, in at least about 65% of the endodermal cells, in at least about 70% of the endodermal cells, in at least about 75% of the endodermal cells, in at least about 80% of the endodermal cells, in at least about 85% of the endodermal cells, in at least about 90% of the endodermal cells, in at least about 95% of the endodermal cells or in at least about 98% of the endodermal cells.

[0216] Still other embodiments described herein relate to compositions, such as cell cultures or cell populations, comprising mammalian endodermal cells, such as human endodermal cells, wherein the expression of one or more markers selected from the group consisting of SOX17, HOXA13 and HOXC6 is greater than the expression of the AFP, SOX7, SOX1, ZIC1 and/or NFM marker in at least about 2% of the endodermal cells. In other embodiments, the expression of one or more markers selected from the group consisting of SOX17, HOXA13 and HOXC6 is greater than the expression of the AFP, SOX7, SOX1, ZIC1 and/or NFM marker in at least about 5% of the endodermal cells, in at least about 10% of the endodermal cells, in at least about 15% of the endodermal cells, in at least about 20% of the endodermal cells, in at least about 25% of the endodermal cells, in at least about 30% of the endodermal cells, in at least about 35% of the endodermal cells, in at least about 40% of the endodermal cells, in at least about 45% of the endodermal cells, in at least about 50% of the endodermal cells, in at least about 55% of the endodermal cells, in at least about 60% of the endodermal cells, in at least about 65% of the endodermal cells, in at least about 70% of the endodermal cells, in at least about 75% of the endodermal cells, in at least about 80% of the endodermal cells, in at least about 85% of the endodermal cells, in at least about 90% of the endodermal cells, in at least about 95% of the endodermal cells or at least about 98% of the endodermal cells.

[0217] Using the processes described herein, compositions comprising PDX1-positive foregut endoderm cells substantially free of other cell types can be produced. In some embodiments of the present invention, the PDX1-positive foregut endoderm cell populations or

cell cultures produced by the methods described herein are substantially free of cells that significantly express the AFP, SOX7, SOX1, ZIC1 and/or NFM marker genes.

[0218] In one embodiment, a description of a PDX1-positive foregut endoderm cell based on the expression of marker genes is, PDX1 high, AFP low, SOX7 low, SOX1 low, ZIC1 low and NFM low.

Increasing Expression of PDX1 in a SOX17-Positive Definitive Endoderm Cell

[0219] Some aspects of the processes described herein are related to methods of increasing the expression of the PDX1 gene product in cell cultures or cell populations comprising SOX17-positive definitive endoderm cells. In such embodiments, the SOX17-positive definitive endoderm cells are contacted with a differentiation factor in an amount that is sufficient to increase the expression of the PDX1 gene product. The SOX17-positive definitive endoderm cells that are contacted with the differentiation factor can be either PDX1-negative or PDX1-positive. In some embodiments, the differentiation factor can be a retinoid. In certain embodiments, SOX17-positive definitive endoderm cells are contacted with a retinoid at a concentration ranging from about 0.01 μM to about 50 μM. In a preferred embodiment, the retinoid is RA.

[0220] In other embodiments of the processes described herein, the expression of the PDX1 gene product in cell cultures or cell populations comprising SOX17-positive definitive endoderm cells is increased by contacting the SOX17-positive cells with a differentiation factor of the fibroblast growth factor family. Such differentiation factors can either be used alone or in conjunction with RA. In some embodiments, the SOX17-positive definitive endoderm cells are contacted with a fibroblast growth factor at a concentration ranging from about 10 ng/ml to about 1000 ng/ml. In a preferred embodiment, the FGF growth factor is FGF-10.

[0221] In some embodiments of the processes described herein, the expression of the PDX1 gene product in cell cultures or cell populations comprising SOX17-positive definitive endoderm cells is increased by contacting the SOX17-positive cells with B27. This differentiation factor can either be used alone or in conjunction with one or both of retinoid and FGF family differentiation factors. In some embodiments, the SOX17-positive definitive endoderm cells are contacted with B27 at a concentration ranging from about 0.1% (v/v) to about 20% (v/v). In a preferred embodiment, the SOX17-positive definitive endoderm cells are contacted with RA, FGF-10 and B27.

[0222] Methods for increasing the expression of the PDX1 gene product in cell cultures or cell populations comprising SOX17-positive definitive endoderm cells can be carried out in growth medium containing reduced or no serum. In some embodiments, serum concentrations range from about 0.05% (v/v) to about 20% (v/v). In some embodiments, the SOX17-positive cells are grown with serum replacement.

Identification of Factors Capable of Promoting the Differentiation of Definitive Endoderm Cells

[0223] Certain screening methods described herein relate to methods for identifying at least one differentiation factor that is capable of promoting the differentiation of definitive endoderm cells. In some embodiments of these methods, cell populations comprising definitive endoderm cells, such as human definitive endoderm cells, are obtained. The cell population is then provided with a candidate differentiation factor. At a first time point, which is prior to or at approximately the same time as providing the candidate differentiation factor, expression of a marker is determined. Alternatively, expression of the marker can be determined after providing the candidate differentiation factor. At a second time point, which is subsequent to the first time point and subsequent to the step of providing the candidate differentiation factor to the cell population, expression of the same marker is again determined. Whether the candidate differentiation factor is capable of promoting the differentiation of the definitive endoderm cells is determined by comparing expression of the marker at the first time point with the expression of the marker at the second time point. If expression of the marker at the second time point is increased or decreased as compared to expression of the marker at the first time point, then the candidate differentiation factor is capable of promoting the differentiation of definitive endoderm cells.

[0224] Some embodiments of the screening methods described herein utilize cell populations or cell cultures which comprise human definitive endoderm cells. For example, the cell population can be a substantially purified population of human definitive endoderm cells. Alternatively, the cell population can be an enriched population of human definitive endoderm cells, wherein at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97% or greater than at least about 97% of the human cells in the cell population are human definitive endoderm cells. In other embodiments described herein, the cell population comprises human cells wherein at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85% or greater than at least about 85% of the human cells are human definitive endoderm cells. In some embodiments, the cell population includes non-human cells such as non-human feeder cells. In other embodiments, the cell population includes human feeder cells. In such embodiments, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or greater than at least about 95% of the human cells, other than said feeder cells, are human definitive endoderm cells.

In some embodiments of the screening methods described herein, the cell populations further comprise PDX1-positive endoderm cells including, but not limited to, PDX1-positive foregut endoderm cells.

[0225] In embodiments of the screening methods described herein, the cell population is contacted or otherwise provided with a candidate (test) differentiation factor. The candidate differentiation factor can comprise any molecule that may have the potential to promote the differentiation of human definitive endoderm cells. In some embodiments described herein, the candidate differentiation factor comprises a molecule that is known to be a differentiation factor for one or more types of cells. In alternate embodiments, the candidate differentiation factor comprises a molecule that in not known to promote cell differentiation. In preferred embodiments, the candidate differentiation factor comprises molecule that is not known to promote the differentiation of human definitive endoderm cells.

[0226] In some embodiments of the screening methods described herein, the candidate differentiation factor comprises a small molecule. In preferred embodiments, a small molecule is a molecule having a molecular mass of about 10,000 amu or less. In some embodiments, the small molecule comprises a retinoid. In some embodiments, the small molecule comprises retinoic acid.

[0227] In other embodiments described herein, the candidate differentiation factor comprises a polypeptide. The polypeptide can be any polypeptide including, but not limited to, a glycoprotein, a lipoprotein, an extracellular matrix protein, a cytokine, a chemokine, a peptide hormone, an interleukin or a growth factor. Preferred polypeptides include growth factors. In some preferred embodiments, the candidate differentiation factors comprises one or more growth factors selected from the group consisting of FGF10, FGF4, FGF2 and Wnt3B.

[0228] In some embodiments of the screening methods described herein, the candidate differentiation factors comprise one or more growth factors selected from the group consisting of Amphiregulin, B-lymphocyte stimulator, IL-16, Thymopoietin, TRAIL/Apo-2, Pre B cell colony enhancing factor, Endothelial differentiation-related factor 1 (EDF1), Endothelial monocyte activating polypeptide II, Macrophage migration inhibitory factor (MIF), Natural killer cell enhancing factor (NKEFA), Bone mophogenetic protein 2, Bone mophogenetic protein 8 (osteogeneic protein 2), Bone morphogenic protein 6, Bone morphogenic protein 7, Connective tissue growth factor (CTGF), CGI-149 protein (neuroendocrine differentiation factor), Cytokine A3 (macrophage inflammatory protein 1-alpha), Gliablastoma cell differentiation-related protein (GBDR1), Hepatoma-derived growth factor, Neuromedin U-25 precursor, Vascular endothelial growth factor (VEGF), Vascular endothelial growth factor B (VEGF-B), T-cell specific RANTES precursor, thymic dendritic cell-derived factor 1, Transferrin, Interleukin-1 (IL 1), Interleukin-2 (IL 2), Interleukin-3 (IL 3), Interleukin-4 (IL 4), Interleukin-5 (IL 5), Interleukin-6 (IL 6), Interleukin-7 (IL 7), Interleukin-8 (IL 8), Interleukin-9 (IL 9), Interleukin-10 (IL 10), Interleukin-

11 (IL 11), Interleukin-12 (IL 12), Interleukin-13 (IL 13), Granulocyte-colony stimulating factor (G-CSF), Granulocyte macrophage colony stimulating factor (GM-CSF), Macrophage colony stimulating factor (M-CSF), Erythropoietin, Thrombopoietin, Vitamin D_3, Epidermal growth factor (EGF), Brain-derived neurotrophic factor, Leukemia inhibitory factor, Thyroid hormone, Basic fibroblast growth factor (bFGF), aFGF, FGF-4, FGF-6, Keratinocyte growth factor (KGF), Platelet-derived growth factor (PDGF), Platelet-derived growth factor-BB, beta nerve growth factor, activin A, Transforming growth factor beta 1 (TGF-β1), Interferon-α, Interferon-β, Interferon-γ, Tumor necrosis factor- α, Tumor necrosis factor- β, Burst promoting activity (BPA), Erythroid promoting activity (EPA), PGE_2, insulin growth factor-1 (IGF-1), IGF-II, Neutrophin growth factor (NGF), Neutrophin-3, Neutrophin 4/5, Ciliary neurotrophic factor, Glial-derived nexin, Dexamethasone, β-mercaptoethanol, Retinoic acid, Butylated hydroxyanisole, 5-azacytidine, Amphotericin B, Ascorbic acid, Ascrorbate, isobutylxanthine, indomethacin, β-glycerolphosphate, nicotinamide, DMSO, Thiazolidinediones, TWS119, oxytocin, vasopressin, melanocyte-stimulating hormone, corticortropin, lipotropin, thyrotropin, growth hormone, prolactin, luteinizing hormone, human chorionic gonadotropin, follicle stimulating hormone, corticotropin-releasing factor, gonadotropin-releasing factor, prolactin-releasing factor, prolactin-inhibiting factor, growth-hormone releasing factor, somatostatin, thyrotropin-releasing factor, calcitonin gene-related peptide, parathyroid hormone, glucagon-like peptide 1, glucose-dependent insulinotropic polypeptide, gastrin, secretin, cholecystokinin, motilin, vasoactive intestinal peptide, substance P, pancreatic polypeptide, peptide tyrosine tyrosine, neuropeptide tyrosine, , insulin, glucagon, placental lactogen, relaxin, angiotensin II, calctriol, atrial natriuretic peptide, and melatonin. thyroxine, triiodothyronine, calcitonin, estradiol, estrone, progesterone, testosterone, cortisol, corticosterone, aldosterone, epinephrine, norepinepherine, androstiene, calcitriol, collagen, Dexamethasone, β-mercaptoethanol, Retinoic acid, Butylated hydroxyanisole, 5-azacytidine, Amphotericin B, Ascorbic acid, Ascrorbate, isobutylxanthine, indomethacin, β-glycerolphosphate, nicotinamide, DMSO, Thiazolidinediones, and TWS119.

[0229] In some embodiments of the screening methods described herein, the candidate differentiation factor is provided to the cell population in one or more concentrations. In some embodiments, the candidate differentiation factor is provided to the cell population so that the concentration of the candidate differentiation factor in the medium surrounding the cells ranges from about 0.1 ng/ml to about 10 mg/ml. In some embodiments, the concentration of the candidate differentiation factor in the medium surrounding the cells ranges from about 1 ng/ml to about 1 mg/ml. In other embodiments, the concentration of the candidate differentiation factor in the medium surrounding the cells ranges from about 10 ng/ml to about 100 μg/ml. In still other embodiments, the concentration of the candidate differentiation factor in the medium surrounding the cells ranges from about 100 ng/ml to about 10 μg/ml. In preferred embodiments, the concentration of the candidate differentiation factor in the medium surrounding the cells is about

5 ng/ml, about 25 ng/ml, about 50 ng/ml, about 75 ng/ml, about 100 ng/ml, about 125 ng/ml, about 150 ng/ml, about 175 ng/ml, about 200 ng/ml, about 225 ng/ml, about 250 ng/ml, about 275 ng/ml, about 300 ng/ml, about 325 ng/ml, about 350 ng/ml, about 375 ng/ml, about 400 ng/ml, about 425 ng/ml, about 450 ng/ml, about 475 ng/ml, about 500 ng/ml, about 525 ng/ml, about 550 ng/ml, about 575 ng/ml, about 600 ng/ml, about 625 ng/ml, about 650 ng/ml, about 675 ng/ml, about 700 ng/ml, about 725 ng/ml, about 750 ng/ml, about 775 ng/ml, about 800 ng/ml, about 825 ng/ml, about 850 ng/ml, about 875 ng/ml, about 900 ng/ml, about 925 ng/ml, about 950 ng/ml, about 975 ng/ml, about 1 μg/ml, about 2 μg/ml, about 3 μg/ml, about 4 μg/ml, about 5 μg/ml, about 6 μg/ml, about 7 μg/ml, about 8 μg/ml, about 9 μg/ml, about 10 μg/ml, about 11 μg/ml, about 12 μg/ml, about 13 μg/ml, about 14 μg/ml, about 15 μg/ml, about 16 μg/ml, about 17 μg/ml, about 18 μg/ml, about 19 μg/ml, about 20 μg/ml, about 25 μg/ml, about 50 μg/ml, about 75 μg/ml, about 100 μg/ml, about 125 μg/ml, about 150 μg/ml, about 175 μg/ml, about 200 μg/ml, about 250 μg/ml, about 300 μg/ml, about 350 μg/ml, about 400 μg/ml, about 450 μg/ml, about 500 μg/ml, about 550 μg/ml, about 600 μg/ml, about 650 μg/ml, about 700 μg/ml, about 750 μg/ml, about 800 μg/ml, about 850 μg/ml, about 900 μg/ml, about 950 μg/ml, about 1000 μg/ml or greater than about 1000 μg/ml.

[0230] In certain embodiments of the screening methods described herein, the cell population is provided with a candidate differentiation factor which comprises any molecule other than foregut differentiation factor. For example, in some embodiments, the cell population is provided with a candidate differentiation factor which comprises any molecule other than a retinoid, a member of the TGFβ superfamily of growth factors, FGF10 or FGF4. In some embodiments, the cell population is provided with a candidate differentiation factor which comprises any molecule other than retinoic acid.

[0231] In some embodiments, steps of the screening methods described herein comprise determining expression of at least one marker at a first time point and a second time point. In some of these embodiments, the first time point can be prior to or at approximately the same time as providing the cell population with the candidate differentiation factor. Alternatively, in some embodiments, the first time point is subsequent to providing the cell population with the candidate differentiation factor. In some embodiments, expression of a plurality of markers is determined at a first time point.

[0232] In addition to determining expression of at least one marker at a first time point, some embodiments of the screening methods described herein contemplate determining expression of at least one marker at a second time point, which is subsequent to the first time point and which is subsequent to providing the cell population with the candidate differentiation factor. In such embodiments, expression of the same marker is determined at both the first and second time points. In some embodiments, expression of a plurality of markers is determined at both the first and second time points. In such embodiments, expression of the same plurality of

markers is determined at both the first and second time points. In some embodiments, marker expression is determined at a plurality of time points, each of which is subsequent to the first time point, and each of which is subsequent to providing the cell population with the candidate differentiation factor. In certain embodiments, marker expression is determined by Q-PCR. In other embodiments, marker expression is determined by immunocytochemistry.

[0233] In certain embodiments of the screening methods described herein, the marker having its expression is determined at the first and second time points is a marker that is associated with the differentiation of human definitive endoderm cells to cells which are the precursors of cells which make up tissues and/or organs that are derived from the gut tube. In some embodiments, the tissues and/or organs that are derived from the gut tube comprise terminally differentiated cells. In some embodiments, the marker is indicative of pancreatic cells or pancreatic precursor cells. In preferred embodiments, the marker is pancreatic-duodenal homeobox factor-1 (PDX1). In other embodiments, the marker is homeobox A13 (HOXA13) or homeobox C6 (HOXC6). Additionally, in other embodiments, the marker is indicative of liver cells or liver precursor cells. In certain preferred embodiments, the marker is albumin, hepatocyte specific antigen (HSA) or prospero-related homeobox 1 (PROX1). In other embodiments, the marker is indicative of lung or lung precursor cells. In some preferred embodiments, the marker is thyroid transcription factor 1 (TITF1). In yet other embodiments, the marker is indicative of intestinal or intestinal precursor cells. In additional preferred embodiments, the marker is villin, glucose transporter-2 (GLUT2), apolipoprotein A1 (APOA1), vascular cell adhesion molecule-1 (VACM1), von Willebrand factor (VWF), CXC-type chemokine receptor 4 (CXCR4) or caudal type homeobox transcription factor 2 (CDX2). In still other embodiments, the marker is indicative of stomach or stomach precursor cells. In additional preferred embodiments, the marker is VCAM1, VWF or CXCR4. In other embodiments, the marker is indicative of thyroid or thyroid precursor cells. In such embodiments, the marker is TITF1. In still other embodiments, the marker is indicative of thymus or thymus precursor cells.

[0234] In some embodiments of the screening methods described herein, sufficient time is allowed to pass between providing the cell population with the candidate differentiation factor and determining marker expression at the second time point. Sufficient time between providing the cell population with the candidate differentiation factor and determining expression of the marker at the second time point can be as little as from about 1 hour to as much as about 10 days. In some embodiments, the expression of at least one marker is determined multiple times subsequent to providing the cell population with the candidate differentiation factor. In some embodiments, sufficient time is at least about 1 hour, at least about 6 hours, at least about 12 hours, at least about 18 hours, at least about 24 hours, at least about 30 hours, at least about 36 hours, at least about 42 hours, at least about 48 hours, at least about 54 hours, at least about 60 hours, at least about 66 hours, at least about 72 hours, at least about 78 hours, at least about 84

hours, at least about 90 hours, at least about 96 hours, at least about 102 hours, at least about 108 hours, at least about 114 hours, at least about 120 hours, at least about 126 hours, at least about 132 hours, at least about 138 hours, at least about 144 hours, at least about 150 hours, at least about 156 hours, at least about 162 hours, at least about 168 hours, at least about 174 hours, at least about 180 hours, at least about 186 hours, at least about 192 hours, at least about 198 hours, at least about 204 hours, at least about 210 hours, at least about 216 hours, at least about 222 hours, at least about 228 hours, at least about 234 hours or at least about 240 hours.

[0235] In some embodiments of the methods described herein, it is further determined whether the expression of the marker at the second time point has increased or decreased as compared to the expression of this marker at the first time point. An increase or decrease in the expression of the at least one marker indicates that the candidate differentiation factor is capable of promoting the differentiation of the definitive endoderm cells. Similarly, if expression of a plurality of markers is determined, it is further determined whether the expression of the plurality of markers at the second time point has increased or decreased as compared to the expression of this plurality of markers at the first time point. An increase or decrease in marker expression can be determined by measuring or otherwise evaluating the amount, level or activity of the marker in the cell population at the first and second time points. Such determination can be relative to other markers, for example housekeeping gene expression, or absolute. In certain embodiments, wherein marker expression is increased at the second time point as compared with the first time point, the amount of increase is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold or more than at least about 100-fold. In some embodiments, the amount of increase is less than 2-fold. In embodiments where marker expression is decreased at the second time point as compared with the first time point, the amount of decrease is at least about 2-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold or more than at least about 100-fold. In some embodiments, the amount of decrease is less than 2-fold.

[0236] In some embodiments of the screening methods described herein, after providing the cell population with a candidate differentiation factor, the human definitive endoderm cells differentiate into one or more cell types of the definitive endoderm lineage. In some embodiments, after providing the cell population with a candidate differentiation factor, the human definitive endoderm cells differentiate into cells that are derived from the gut tube. Such cells include, but are not limited to, cells of the pancreas, liver, lungs, stomach, intestine, thyroid, thymus, pharynx, gallbladder and urinary bladder as well as precursors of such cells.

Additionally, these cells can further develop into higher order structures such as tissues and/or organs.

[0237] It will be appreciated that screening methods similar to those described above can be used to identify one or more differentiation factors capable of promoting the differentiation of human PDX1-positive endoderm cells in a cell population which comprises human PDX1-positive endoderm cells. In certain embodiments, the human PDX1-positive endoderm cells are PDX1-positive foregut/midgut endoderm cells. In preferred embodiments, the human PDX1-positive endoderm cells are PDX1-positive foregut endoderm cells. In other preferred embodiments, the human PDX1-positive endoderm cells are PDX1-positive endoderm cells of the posterior portion of the foregut. In especially preferred embodiments, the human PDX1-positive foregut endoderm cells are multipotent cells that can differentiate into cells, tissues or organs derived from the anterior portion of the gut tube.

Identification of Factors Capable of Promoting the Differentiation of PDX1-Negative Definitive Endoderm Cells to PDX1-Positive Foregut Endoderm Cells

[0238] Aspects of the screening methods described herein relate to methods of identifying one or more differentiation factors capable of promoting the differentiation of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells. In such methods, a cell culture or cell population comprising PDX1-negative definitive endoderm cells is obtained and the expression of PDX1 in the cell culture or cell population is determined. After determining the expression of PDX1, the cells of the cell culture or cell population are contacted with a candidate differentiation factor. In some embodiments, the expression of PDX1 is determined at the time of contacting or shortly after contacting the cells with a candidate differentiation factor. PDX1 expression is then determined at one or more times after contacting the cells with the candidate differentiation factor. If the expression of PDX1 has increased after contact with the candidate differentiation factor as compared to PDX1 expression prior to contact with the candidate differentiation factor, the candidate differentiation factor is identified as capable of promoting the differentiation of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells.

[0239] In some embodiments, the above-described methods of identifying factors capable of promoting the differentiation of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells also include determining the expression of the HOXA13 gene and/or the HOXC6 gene in the cell culture or cell population. In such embodiments, the expression of HOXA13 and/or HOXC6 is determined both before and after the cells are contacted with the candidate differentiation factor. If the expression of PDX1 and HOXA13 has increased after contact with the candidate differentiation factor as compared to PDX1 and HOXA13 expression prior to contact with the candidate differentiation factor, the candidate differentiation

factor is identified as capable of promoting the differentiation of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells. Similarly, if the expression of PDX1 and HOXC6 has increased after contact with the candidate differentiation factor as compared to PDX1 and HOXC6 expression prior to contact with the candidate differentiation factor, the candidate differentiation factor is identified as capable of promoting the differentiation of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells. In a preferred embodiment, a candidate differentiation factor is identified as being capable of promoting the differentiation of PDX1-negative definitive endoderm cells to PDX1-positive foregut endoderm cells by determining the expression of PDX1, HOXA13 and HOXC6 both before and after contacting the cells of the cell culture or cell population with the candidate differentiation factor. In preferred embodiments, the expression of PDX1, HOXA13 and/or HOXC6 is determined Q-PCR.

[0240] It will be appreciated that in some embodiments, the expression of one or more of PDX1, HOXA13 and HOXC6 can be determined at the time of contacting or shortly after contacting the cells of the cell cultures or cell populations with a candidate differentiation factor rather than prior to contacting the cells with a candidate differentiation factor. In such embodiments, the expression of one or more of PDX1, HOXA13 and HOXC6 at the time of contacting or shortly after contacting the cells with a candidate differentiation factor is compared to the expression of one or more of PDX1, HOXA13 and HOXC6 at one or more times after contacting the cells with a candidate differentiation factor.

[0241] In some embodiments of the above-described methods, the one or more times at which PDX1 expression is determined after contacting the cells with the candidate differentiation factor can range from about 1 hour to about 10 days. For example, PDX1 expression can be determined about 1 hour after contacting the cells with the candidate differentiation factor, about 2 hours after contacting the cells with the candidate differentiation factor, about 4 hours after contacting the cells with the candidate differentiation factor, about 6 hours after contacting the cells with the candidate differentiation factor, about 8 hours after contacting the cells with the candidate differentiation factor, about 10 hours after contacting the cells with the candidate differentiation factor, about 12 hours after contacting the cells with the candidate differentiation factor, about 16 hours after contacting the cells with the candidate differentiation factor, about 24 hours after contacting the cells with the candidate differentiation factor, about 2 days after contacting the cells with the candidate differentiation factor, about 3 days after contacting the cells with the candidate differentiation factor, about 4 days after contacting the cells with the candidate differentiation factor, about 5 days after contacting the cells with the candidate differentiation factor, about 6 days after contacting the cells with the candidate differentiation factor, about 7 days after contacting the cells with the candidate differentiation factor, about 8 days after contacting the cells with the candidate differentiation

factor, about 9 days after contacting the cells with the candidate differentiation factor, about 10 days after contacting the cells with the candidate differentiation factor or more than 10 days after contacting the cells with the candidate differentiation factor.

[0242] Candidate differentiation factors for use in the methods described herein can be selected from compounds, such as polypeptides and small molecules. For example, candidate polypeptides can include, but are not limited to, growth factors, cytokines, chemokines, extracellular matrix proteins, and synthetic peptides. In a preferred embodiment, the growth factor is from the FGF family, for example FGF-10. Candidate small molecules include, but are not limited to, compounds generated from combinatorial chemical synthesis and natural products, such as steroids, isoprenoids, terpenoids, phenylpropanoids, alkaloids and flavinoids. It will be appreciated by those of ordinary skill in the art that thousands of classes of natural and synthetic small molecules are available and that the small molecules contemplated for use in the methods described herein are not limited to the classes exemplified above. Typically, small molecules will have a molecular weight less than 10,000 amu. In a preferred embodiment, the small molecule is a retinoid, for example RA.

Identification of Factors Capable of Promoting the Differentiation of PDX1-Positive Foregut Endoderm Cells

[0243] Other aspects of the screening methods described herein relate to methods of identifying one or more differentiation factors capable of promoting the differentiation of PDX1-positive foregut endoderm cells. In such methods, a cell culture or cell population comprising PDX1-positive foregut endoderm cells is obtained and the expression of a marker in the cell culture or cell population is determined. After determining the expression of the marker, the cells of the cell culture or cell population are contacted with a candidate differentiation factor. In some embodiments, the expression of the marker is determined at the time of contacting or shortly after contacting the cells with a candidate differentiation factor. The expression of the same marker is then determined at one or more times after contacting the cells with the candidate differentiation factor. If the expression of the marker has increased or decreased after contact with the candidate differentiation factor as compared to the marker expression prior to contact with the candidate differentiation factor, the candidate differentiation factor is identified as capable of promoting the differentiation of PDX1-positive foregut endoderm cells. In preferred embodiments, expression of the marker is determined by Q-PCR.

[0244] In some embodiments of the above-described methods, the one or more times at which the marker expression is determined after contacting the cells with the candidate differentiation factor can range from about 1 hour to about 10 days. For example, marker expression can be determined about 1 hour after contacting the cells with the candidate differentiation factor, about 2 hours after contacting the cells with the candidate differentiation

factor, about 4 hours after contacting the cells with the candidate differentiation factor, about 6 hours after contacting the cells with the candidate differentiation factor, about 8 hours after contacting the cells with the candidate differentiation factor, about 10 hours after contacting the cells with the candidate differentiation factor, about 12 hours after contacting the cells with the candidate differentiation factor, about 16 hours after contacting the cells with the candidate differentiation factor, about 24 hours after contacting the cells with the candidate differentiation factor, about 2 days after contacting the cells with the candidate differentiation factor, about 3 days after contacting the cells with the candidate differentiation factor, about 4 days after contacting the cells with the candidate differentiation factor, about 5 days after contacting the cells with the candidate differentiation factor, about 6 days after contacting the cells with the candidate differentiation factor, about 7 days after contacting the cells with the candidate differentiation factor, about 8 days after contacting the cells with the candidate differentiation factor, about 9 days after contacting the cells with the candidate differentiation factor, about 10 days after contacting the cells with the candidate differentiation factor or more than 10 days after contacting the cells with the candidate differentiation factor.

[0245] As described previously, candidate differentiation factors for use in the methods described herein can be selected from compounds such as polypeptides and small molecules.

[0246] Although each of the methods disclosed herein have been described with respect to PDX1-positive foregut endoderm cells, it will be appreciated that in certain embodiments, these methods can be used to produce compositions comprising the PDX1-positive foregut/midgut endoderm cells that are described herein and/or the PDX1-positive endoderm cells of the posterior portion of the foregut that are described herein. Furthermore, any of the PDX1-positive endoderm cell types disclosed in this specification can be utilized in the screening methods described herein.

[0247] Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only, and are not intended to be limiting.

EXAMPLES

[0248] Many of the examples below describe the use of pluripotent human cells. Methods of producing pluripotent human cells are well known in the art and have been described numerous scientific publications, including U.S. Patent Nos. 5,453,357, 5,670,372, 5,690,926, 6,090,622, 6,200,806 and 6,251,671 as well as U.S. Patent Application Publication No. 2004/0229350.

EXAMPLE 1

Human ES cells

[0249] For our studies of endoderm development we employed human embryonic stem cells, which are pluripotent and can divide seemingly indefinitely in culture while maintaining a normal karyotype. ES cells were derived from the 5-day-old embryo inner cell mass using either immunological or mechanical methods for isolation. In particular, the human embryonic stem cell line hESCyt-25 was derived from a supernumerary frozen embryo from an *in vitro* fertilization cycle following informed consent by the patient. Upon thawing the hatched blastocyst was plated on mouse embryonic fibroblasts (MEF), in ES medium (DMEM, 20% FBS, non essential amino acids, beta-mercaptoethanol, ITS supplement). The embryo adhered to the culture dish and after approximately two weeks, regions of undifferentiated hESCs were transferred to new dishes with MEFs. Transfer was accomplished with mechanical cutting and a brief digestion with dispase, followed by mechanical removal of the cell clusters, washing and re-plating. Since derivation, hESCyt-25 has been serially passaged over 100 times. We employed the hESCyt-25 human embryonic stem cell line as our starting material for the production of definitive endoderm.

[0250] It will be appreciated by those of skill in the art that stem cells or other pluripotent cells can also be used as starting material for the differentiation procedures described herein. For example, cells obtained from embryonic gonadal ridges, which can be isolated by methods known in the art, can be used as pluripotent cellular starting material.

EXAMPLE 2

hESCyt-25 Characterization

[0251] The human embryonic stem cell line, hESCyt-25 has maintained a normal morphology, karyotype, growth and self-renewal properties over 18 months in culture. This cell line displays strong immunoreactivity for the OCT4, SSEA-4 and TRA-1-60 antigens, all of which, are characteristic of undifferentiated hESCs and displays alkaline phosphatase activity as well as a morphology identical to other established hESC lines. Furthermore, the human stem cell line, hESCyt-25, also readily forms embryoid bodies (EBs) when cultured in suspension. As a demonstration of its pluripotent nature, hESCyT-25 differentiates into various cell types that represent the three principal germ layers. Ectoderm production was demonstrated by Q-PCR for ZIC1 as well as immunocytochemistry (ICC) for nestin and more mature neuronal markers. Immunocytochemical staining for β-III tubulin was observed in clusters of elongated cells, characteristic of early neurons. Previously, we treated EBs in suspension with retinoic acid, to induce differentiation of pluripotent stem cells to visceral endoderm (VE), an extra-embryonic lineage. Treated cells expressed high levels of α-fetoprotein (AFP) and SOX7, two markers of

VE, by 54 hours of treatment. Cells differentiated in monolayer expressed AFP in sporadic patches as demonstrated by immunocytochemical staining. As will be described below, the hESCyT-25 cell line was also capable of forming definitive endoderm, as validated by real-time quantitative polymerase chain reaction (Q-PCR) and immunocytochemistry for SOX17, in the absence of AFP expression. To demonstrate differentiation to mesoderm, differentiating EBs were analyzed for Brachyury gene expression at several time points. Brachyury expression increased progressively over the course of the experiment. In view of the foregoing, the hESCyT-25 line is pluripotent as shown by the ability to form cells representing the three germ layers.

EXAMPLE 3

Production of SOX17 Antibody

[0252] A primary obstacle to the identification of definitive endoderm in hESC cultures is the lack of appropriate tools. We therefore undertook the production of an antibody raised against human SOX17 protein.

[0253] The marker SOX17 is expressed throughout the definitive endoderm as it forms during gastrulation and its expression is maintained in the gut tube (although levels of expression vary along the A-P axis) until around the onset of organogenesis. SOX17 is also expressed in a subset of extra-embryonic endoderm cells. No expression of this protein has been observed in mesoderm or ectoderm. It has now been discovered that SOX17 is an appropriate marker for the definitive endoderm lineage when used in conjunction with markers to exclude extra-embryonic lineages.

[0254] As described in detail herein, the SOX17 antibody was utilized to specifically examine effects of various treatments and differentiation procedures aimed at the production of SOX17 positive definitive endoderm cells. Other antibodies reactive to AFP, SPARC and Thrombomodulin were also employed to rule out the production of visceral and parietal endoderm (extra-embryonic endoderm).

[0255] In order to produce an antibody against SOX17, a portion of the human SOX17 cDNA (SEQ ID NO: 1) corresponding to amino acids 172-414 (SEQ ID NO: 2) in the carboxyterminal end of the SOX17 protein (Figure 2) was used for genetic immunization in rats at the antibody production company, GENOVAC (Freiberg, Germany), according to procedures developed there. Procedures for genetic immunization can be found in US Patent Nos. 5,830,876, 5,817,637, 6,165,993 and 6,261,281 as well as International Patent Application Publication Nos. WO00/29442 and WO99/13915.

[0256] Other suitable methods for genetic immunization are also described in the non-patent literature. For example, Barry et al. describe the production of monoclonal antibodies by genetic immunization in *Biotechniques* 16: 616-620, 1994. Specific examples of genetic immunization methods to produce antibodies against specific proteins can be found, for example,

in Costaglia *et al.*, (1998) Genetic immunization against the human thyrotropin receptor causes thyroiditis and allows production of monoclonal antibodies recognizing the native receptor, *J. Immunol.* 160: 1458-1465; Kilpatrick *et al* (1998) Gene gun delivered DNA-based immunizations mediate rapid production of murine monoclonal antibodies to the Flt-3 receptor, *Hybridoma* 17: 569-576; Schmolke *et al.*, (1998) Identification of hepatitis G virus particles in human serum by E2-specific monoclonal antibodies generated by DNA immunization, *J. Virol.* 72: 4541-4545; Krasemann et al., (1999) Generation of monoclonal antibodies against proteins with an unconventional nucleic acid-based immunization strategy, *J. Biotechnol.* 73: 119-129; and Ulivieri *et al.*, (1996) Generation of a monoclonal antibody to a defined portion of the *Heliobacter pylori* vacuolating cytotoxin by DNA immunization, *J. Biotechnol.* 51: 191-194.

[0257] SOX7 and SOX18 are the closest Sox family relatives to SOX17 as depicted in the relational dendrogram shown in Figure 3. We employed the human SOX7 polypeptide as a negative control to demonstrate that the SOX17 antibody produced by genetic immunization is specific for SOX17 and does not react with its closest family member. In particular, SOX7 and other proteins were expressed in human fibroblasts, and then, analyzed for cross reactivity with the SOX17 antibody by Western blot and ICC. For example, the following methods were utilized for the production of the SOX17, SOX7 and EGFP expression vectors, their transfection into human fibroblasts and analysis by Western blot. Expression vectors employed for the production of SOX17, SOX7, and EGFP were pCMV6 (OriGene Technologies, Inc., Rockville, MD), pCMV-SPORT6 (Invitrogen, Carlsbad, CA) and pEGFP-N1 (Clonetech, Palo Alto, CA), respectively. For protein production, telomerase immortalized MDX human fibroblasts were transiently transfected with supercoiled DNA in the presence of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Total cellular lysates were collected 36 hours post-transfection in 50 mM TRIS-HCl (pH 8), 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, containing a cocktail of protease inhibitors (Roche Diagnostics Corporation, Indianapolis, IN). Western blot analysis of 100 μg of cellular proteins, separated by SDS-PAGE on NuPAGE (4-12 % gradient polyacrylamide, Invitrogen, Carlsbad, CA), and transferred by electro-blotting onto PDVF membranes (Hercules, CA), were probed with a 1/1000 dilution of the rat SOX17 anti-serum in 10 mM TRIS-HCl (pH 8), 150 mM NaCl, 10% BSA, 0.05 % Tween-20 (Sigma, St. Louis, MO), followed by Alkaline Phosphatase conjugated anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and revealed through Vector Black Alkaline Phosphatase staining (Vector Laboratories, Burlingame, CA). The proteins size standard used was wide range color markers (Sigma, St. Louis, MO).

[0258] In Figure 4, protein extracts made from human fibroblast cells that were transiently transfected with SOX17, SOX7 or EGFP cDNA's were probed on Western blots with the SOX17 antibody. Only the protein extract from hSOX17 transfected cells produced a band of ~51Kda which closely matched the predicted 46 Kda molecular weight of the human SOX17

protein. There was no reactivity of the SOX17 antibody to extracts made from either human SOX7 or EGFP transfected cells. Furthermore, the SOX17 antibody clearly labeled the nuclei of human fibroblast cells transfected with the hSOX17 expression construct but did not label cells transfected with EGFP alone. As such, the SOX17 antibody exhibits specificity by ICC.

EXAMPLE 4

Validation of SOX17 Antibody as a Marker of Definitive Endoderm

[0259] Partially differentiated hESCs were co-labeled with SOX17 and AFP antibodies to demonstrate that the SOX17 antibody is specific for human SOX17 protein and furthermore marks definitive endoderm. It has been demonstrated that SOX17, SOX7 (which is a closely related member of the SOX gene family subgroup F (Figure 3)) and AFP are each expressed in visceral endoderm. However, AFP and SOX7 are not expressed in definitive endoderm cells at levels detectable by ICC, and thus, they can be employed as negative markers for bonifide definitive endoderm cells. It was shown that SOX17 antibody labels populations of cells that exist as discrete groupings of cells or are intermingled with AFP positive cells. In particular, Figure 5A shows that small numbers of SOX17 cells were co-labeled with AFP; however, regions were also found where there were little or no AFP^+ cells in the field of $SOX17^+$ cells (Figure 5B). Similarly, since parietal endoderm has been reported to express SOX17, antibody co-labeling with SOX17 together with the parietal markers SPARC and/or Thrombomodulin (TM) can be used to identify the $SOX17^+$ cells that are parietal endoderm. As shown in Figures 6A-C, Thrombomodulin and SOX17 co-labeled parietal endoderm cells were produced by random differentiation of hES cells.

[0260] In view of the above cell labeling experiments, the identity of a definitive endoderm cell can be established by the marker profile $SOX17^{hi}/AFP^{lo}/[TM^{lo}$ or $SPARC^{lo}]$. In other words, the expression of the SOX17 marker is greater than the expression of the AFP marker, which is characteristic of visceral endoderm, and the TM or SPARC markers, which are characteristic of parietal endoderm. Accordingly, those cells positive for SOX17 but negative for AFP and negative for TM or SPARC are definitive endoderm.

[0261] As a further evidence of the specificity of the $SOX17^{hi}/AFP^{lo}/TM^{lo}/SPARC^{lo}$ marker profile as predictive of definitive endoderm, SOX17 and AFP gene expression was quantitatively compared to the relative number of antibody labeled cells. As shown in Figure 7A, hESCs treated with retinoic acid (visceral endoderm inducer), or activin A (definitive endoderm inducer), resulted in a 10-fold difference in the level of SOX17 mRNA expression. This result mirrored the 10-fold difference in SOX17 antibody-labeled cell number (Figure 7B). Furthermore, as shown in Figure 8A, activin A treatment of hESCs suppressed AFP gene expression by 6.8-fold in comparison to no treatment. This was visually reflected by a dramatic decrease in the number of AFP labeled cells in these cultures as shown in Figures 8B-C. To

quantify this further, it was demonstrated that this approximately 7-fold decrease in AFP gene expression was the result of a similar 7-fold decrease in AFP antibody-labeled cell number as measured by flow cytometry (Figures 9A-B). This result is extremely significant in that it indicates that quantitative changes in gene expression as seen by Q-PCR mirror changes in cell type specification as observed by antibody staining.

[0262] Incubation of hESCs in the presence of Nodal family members (Nodal, activin A and activin B - NAA) resulted in a significant increase in SOX17 antibody-labeled cells over time. By 5 days of continuous activin treatment greater than 50% of the cells were labeled with SOX17 (Figures 10A-F). There were few or no cells labeled with AFP after 5 days of activin treatment.

[0263] In summary, the antibody produced against the carboxy-terminal 242 amino acids of the human SOX17 protein identified human SOX17 protein on Western blots but did not recognize SOX7, it's closest Sox family relative. The SOX17 antibody recognized a subset of cells in differentiating hESC cultures that were primarily $SOX17^{+}/AFP^{lo/-}$ (greater than 95% of labeled cells) as well as a small percentage (< 5%) of cells that co-label for SOX17 and AFP (visceral endoderm). Treatment of hESC cultures with activins resulted in a marked elevation of SOX17 gene expression as well as SOX17 labeled cells and dramatically suppressed the expression of AFP mRNA and the number of cells labeled with AFP antibody.

EXAMPLE 5

Q-PCR Gene Expression Assay

[0264] In the following experiments, real-time quantitative RT-PCR (Q-PCR) was the primary assay used for screening the effects of various treatments on hESC differentiation. In particular, real-time measurements of gene expression were analyzed for multiple marker genes at multiple time points by Q-PCR. Marker genes characteristic of the desired as well as undesired cell types were evaluated to gain a better understanding of the overall dynamics of the cellular populations. The strength of Q-PCR analysis includes its extreme sensitivity and relative ease of developing the necessary markers, as the genome sequence is readily available. Furthermore, the extremely high sensitivity of Q-PCR permits detection of gene expression from a relatively small number of cells within a much larger population. In addition, the ability to detect very low levels of gene expression provides indications for "differentiation bias" within the population. The bias towards a particular differentiation pathway, prior to the overt differentiation of those cellular phenotypes, is unrecognizable using immunocytochemical techniques. For this reason, Q-PCR provides a method of analysis that is at least complementary and potentially much superior to immunocytochemical techniques for screening the success of differentiation treatments. Additionally, Q-PCR provides a mechanism by which to evaluate the success of a differentiation protocol in a quantitative format at semi-high throughput scales of analysis.

[0265] The approach taken here was to perform relative quantitation using SYBR Green chemistry on a Rotor Gene 3000 instrument (Corbett Research) and a two-step RT-PCR format. Such an approach allowed for the banking of cDNA samples for analysis of additional marker genes in the future, thus avoiding variability in the reverse transcription efficiency between samples.

[0266] Primers were designed to lie over exon-exon boundaries or span introns of at least 800 bp when possible, as this has been empirically determined to eliminate amplification from contaminating genomic DNA. When marker genes were employed that do not contain introns or they possess pseudogenes, DNase I treatment of RNA samples was performed.

[0267] We routinely used Q-PCR to measure the gene expression of multiple markers of target and non-target cell types in order to provide a broad profile description of gene expression in cell samples. The markers relevant for the early phases of hESC differentiation (specifically ectoderm, mesoderm, definitive endoderm and extra-embryonic endoderm) and for which validated primer sets are available are provided below in Table 1. The human specificity of these primer sets has also been demonstrated. This is an important fact since the hESCs were often grown on mouse feeder layers. Most typically, triplicate samples were taken for each condition and independently analyzed in duplicate to assess the biological variability associated with each quantitative determination.

[0268] To generate PCR template, total RNA was isolated using RNeasy (Qiagen) and quantitated using RiboGreen (Molecular Probes). Reverse transcription from 350-500 ng of total RNA was carried out using the iScript reverse transcriptase kit (BioRad), which contains a mix of oligo-dT and random primers. Each 20 μL reaction was subsequently diluted up to 100 μL total volume and 3 μL was used in each 10 μL Q-PCR reaction containing 400 nM forward and reverse primers and 5 μL 2X SYBR Green master mix (Qiagen). Two step cycling parameters were used employing a 5 second denature at 85-94°C (specifically selected according to the melting temp of the amplicon for each primer set) followed by a 45 second anneal/extend at 60°C. Fluorescence data was collected during the last 15 seconds of each extension phase. A three point, 10-fold dilution series was used to generate the standard curve for each run and cycle thresholds (Ct's) were converted to quantitative values based on this standard curve. The quantitated values for each sample were normalized to housekeeping gene performance and then average and standard deviations were calculated for triplicate samples. At the conclusion of PCR cycling, a melt curve analysis was performed to ascertain the specificity of the reaction. A single specific product was indicated by a single peak at the T_m appropriate for that PCR amplicon. In addition, reactions performed without reverse transcriptase served as the negative control and do not amplify.

[0269] A first step in establishing the Q-PCR methodology was validation of appropriate housekeeping genes (HGs) in the experimental system. Since the HG was used to normalize across samples for the RNA input, RNA integrity and RT efficiency, it was of value that the HG exhibited a constant level of expression over time in all sample types in order for the normalization to be meaningful. We measured the expression levels of *Cyclophilin G, hypoxanthine phosphoribosyltransferase 1 (HPRT), beta-2-microglobulin, hydroxymethylbiane synthase (HMBS), TATA-binding protein (TBP), and glucoronidase beta (GUS)* in differentiating hESCs. Our results indicated that *beta-2-microglobulin* expression levels increased over the course of differentiation and therefore we excluded the use of this gene for normalization. The other genes exhibited consistent expression levels over time as well as across treatments. We routinely used both Cyclophilin G and GUS to calculate a normalization factor for all samples. The use of multiple HGs simultaneously reduces the variability inherent to the normalization process and increases the reliability of the relative gene expression values.

[0270] After obtaining genes for use in normalization, Q-PCR was then utilized to determine the relative gene expression levels of many marker genes across samples receiving different experimental treatments. The marker genes employed have been chosen because they exhibit enrichment in specific populations representative of the early germ layers and in particular have focused on sets of genes that are differentially expressed in definitive endoderm and extra-embryonic endoderm. These genes as well as their relative enrichment profiles are highlighted in Table 1.

TABLE 1

Germ Layer	Gene	Expression Domains
Endoderm	SOX17	definitive, visceral and parietal endoderm
	MIXL1	endoderm and mesoderm
	GATA4	definitive and primitive endoderm
	HNF3b	definitive endoderm and primitive endoderm, mesoderm, neural plate
	GSC	endoderm and mesoderm
Extra-embryonic	SOX7	visceral endoderm
	AFP	visceral endoderm, liver
	SPARC	parietal endoderm
	TM	parietal endoderm/trophectoderm
Ectoderm	ZIC1	neural tube, neural progenitors
Mesoderm	BRACH	nascent mesoderm

[0271] Since many genes are expressed in more than one germ layer it is useful to quantitatively compare expression levels of many genes within the same experiment. SOX17 is expressed in definitive endoderm and to a smaller extent in visceral and parietal endoderm. SOX7

and AFP are expressed in visceral endoderm at this early developmental time point. SPARC and TM are expressed in parietal endoderm and Brachyury is expressed in early mesoderm.

[0272] Definitive endoderm cells were predicted to express high levels of SOX17 mRNA and low levels of AFP and SOX7 (visceral endoderm), SPARC (parietal endoderm) and Brachyury (mesoderm). In addition, ZIC1 was used here to further rule out induction of early ectoderm. Finally, GATA4 and HNF3b were expressed in both definitive and extra-embryonic endoderm, and thus, correlate with SOX17 expression in definitive endoderm (Table 1). A representative experiment is shown in Figures 11-14 which demonstrates how the marker genes described in Table 1 correlate with each other among the various samples, thus highlighting specific patterns of differentiation to definitive endoderm and extra-embryonic endoderm as well as to mesodermal and neural cell types.

[0273] In view of the above data it is clear that increasing doses of activin resulted in increasing SOX17 gene expression. Further this SOX17 expression predominantly represented definitive endoderm as opposed to extra-embryonic endoderm. This conclusion stems from the observation that SOX17 gene expression was inversely correlated with AFP, SOX7, and SPARC gene expression.

EXAMPLE 6

Directed Differentiation of Human ES Cells to Definitive Endoderm

[0274] Human ES cell cultures randomly differentiate if cultured under conditions that do not actively maintain their undifferentiated state. This heterogeneous differentiation results in production of extra-embryonic endoderm cells comprised of both parietal and visceral endoderm (AFP, SPARC and SOX7 expression) as well as early ectodermal and mesodermal derivatives as marked by ZIC1 and Nestin (ectoderm) and Brachyury (mesoderm) expression. Definitive endoderm cell appearance has not been examined or specified for lack of specific antibody markers in ES cell cultures. As such, and by default, early definitive endoderm production in ES cell cultures has not been well studied. Since satisfactory antibody reagents for definitive endoderm cells have been unavailable, most of the characterization has focused on ectoderm and extra-embryonic endoderm. Overall, there are significantly greater numbers of extra-embryonic and neurectodermal cell types in comparison to $SOX17^{hi}$ definitive endoderm cells in randomly differentiated ES cell cultures.

[0275] As undifferentiated hESC colonies expand on a bed of fibroblast feeders, the cells at the edges of the colony take on alternative morphologies that are distinct from those cells residing within the interior of the colony. Many of these outer edge cells can be distinguished by their less uniform, larger cell body morphology and by the expression of higher levels of OCT4. It has been described that as ES cells begin to differentiate they alter the levels of OCT4 expression up or down relative to undifferentiated ES cells. Alteration of OCT4 levels above or

below the undifferentiated threshold may signify the initial stages of differentiation away from the pluripotent state.

[0276] When undifferentiated colonies were examined by SOX17 immunocytochemistry, occasionally small 10-15-cell clusters of SOX17-positive cells were detected at random locations on the periphery and at the junctions between undifferentiated hESC colonies. As noted above, these scattered pockets of outer colony edges appeared to be some of the first cells to differentiate away from the classical ES cell morphology as the colony expanded in size and became more crowded. Younger, smaller fully undifferentiated colonies (< 1mm; 4-5 days old) showed no SOX17 positive cells within or at the edges of the colonies while older, larger colonies (1-2 mm diameter, > 5days old) had sporadic isolated patches of SOX17 positive, AFP negative cells at the periphery of some colonies or in regions interior to the edge that did not display the classical hESC morphology described previously. Given that this was the first development of an effective SOX17 antibody, definitive endoderm cells generated in such early "undifferentiated" ES cell cultures have never been previously demonstrated.

[0277] Based on negative correlations of SOX17 and SPARC gene expression levels by Q-PCR, the vast majority of these SOX17 positive, AFP negative cells will be negative for parietal endoderm markers by antibody co-labeling. This was specifically demonstrated for TM-expressing parietal endoderm cells as shown in Figures 15A-B. Exposure to Nodal factors activin A and B resulted in a dramatic decrease in the intensity of TM expression and the number of TM positive cells. By triple labeling using SOX17, AFP and TM antibodies on an activin treated culture, clusters of SOX17 positive cells that were also negative for AFP and TM were observed (Figures 16A-D). These are the first cellular demonstrations of SOX17 positive definitive endoderm cells in differentiating hESC cultures (Figures 16A-D and 17).

[0278] With the SOX17 antibody and Q-PCR tools described above we have explored a number of procedures capable of efficiently programming hESCs to become $SOX17^{hi}/AFP^{lo}$ / $SPARC/TM^{lo}$ definitive endoderm cells. We applied a variety of differentiation protocols aimed at increasing the number and proliferative capacity of these cells as measured at the population level by Q-PCR for SOX17 gene expression and at the level of individual cells by antibody labeling of SOX17 protein.

[0279] We were the first to analyze and describe the effect of TGFβ family growth factors, such as Nodal/activin/BMP, for use in creating definitive endoderm cells from embryonic stem cells in *in vitro* cell cultures. In typical experiments, activin A, activin B, BMP or combinations of these growth factors were added to cultures of undifferentiated human stem cell line hESCyt-25 to begin the differentiation process.

[0280] As shown in Figure 19, addition of activin A at 100 ng/ml resulted in a 19-fold induction of SOX17 gene expression vs. undifferentiated hESCs by day 4 of differentiation. Adding activin B, a second member of the activin family, together with activin A, resulted in a

37-fold induction over undifferentiated hESCs by day 4 of combined activin treatment. Finally, adding a third member of the TGFβ family from the Nodal/Activin and BMP subgroups, BMP4, together with activin A and activin B, increased the fold induction to 57 times that of undifferentiated hESCs (Figure 19). When SOX17 induction with activins and BMP was compared to no factor medium controls 5-, 10-, and 15-fold inductions resulted at the 4-day time point. By five days of triple treatment with activins A, B and BMP, SOX17 was induced more than 70 times higher than hESCs. These data indicate that higher doses and longer treatment times of the Nodal/activin TGFβ family members results in increased expression of SOX17.

[0281] Nodal and related molecules activin A, B and BMP facilitate the expression of SOX17 and definitive endoderm formation *in vivo* or *in vitro*. Furthermore, addition of BMP results in an improved SOX17 induction possibly through the further induction of Cripto, the Nodal co-receptor.

[0282] We have demonstrated that the combination of activins A and B together with BMP4 result in additive increases in SOX17 induction and hence definitive endoderm formation. BMP4 addition for prolonged periods (>4 days), in combination with activin A and B may induce SOX17 in parietal and visceral endoderm as well as definitive endoderm. In some embodiments of the present invention, it is therefore valuable to remove BMP4 from the treatment within 4 days of addition.

[0283] To determine the effect of TGFβ factor treatment at the individual cell level, a time course of TGFβ factor addition was examined using SOX17 antibody labeling. As previously shown in Figures 10A-F, there was a dramatic increase in the relative number of SOX17 labeled cells over time. The relative quantification (Figure 20) shows more than a 20-fold increase in SOX17-labeled cells. This result indicates that both the numbers of cells as well SOX17 gene expression level are increasing with time of TGFβ factor exposure. As shown in Figure 21, after four days of exposure to Nodal, activin A, activin B and BMP4, the level of SOX17 induction reached 168-fold over undifferentiated hESCs. Figure 22 shows that the relative number of SOX17-positive cells was also dose responsive. activin A doses of 100 ng/ml or more were capable of potently inducing SOX17 gene expression and cell number.

[0284] In addition to the TGFβ family members, the Wnt family of molecules may play a role in specification and/or maintenance of definitive endoderm. The use of Wnt molecules was also beneficial for the differentiation of hESCs to definitive endoderm as indicated by the increased SOX17 gene expression in samples that were treated with activins plus Wnt3a over that of activins alone (Figure 23).

[0285] All of the experiments described above were performed using a tissue culture medium containing 10% serum with added factors. Surprisingly, we discovered that the concentration of serum had an effect on the level of SOX17 expression in the presence of added

activins as shown in Figures 24A-C. When serum levels were reduced from 10% to 2%, SOX17 expression tripled in the presence of activins A and B.

[0286] Finally, we demonstrated that activin induced SOX17$^+$ cells divide in culture as depicted in Figures 25A-D. The arrows show cells labeled with SOX17/PCNA/DAPI that are in mitosis as evidenced by the PCNA/DAPI-labeled mitotic plate pattern and the phase contrast mitotic profile.

EXAMPLE 7

Chemokine receptor 4 (CXCR4) expression correlates with markers for definitive endoderm and not markers for mesoderm, ectoderm or visceral endoderm

[0287] As described above, hESCs can be induced to differentiate to the definitive endoderm germ layer by the application of cytokines of the TGFβ family and more specifically of the activin/nodal subfamily. Additionally, we have shown that the proportion of fetal bovine serum (FBS) in the differentiation culture medium effects the efficiency of definitive endoderm differentiation from hESCs. This effect is such that at a given concentration of activin A in the medium, higher levels of FBS will inhibit maximal differentiation to definitive endoderm. In the absence of exogenous activin A, differentiation of hESCs to the definitive endoderm lineage is very inefficient and the FBS concentration has much milder effects on the differentiation process of hESCs.

[0288] In these experiments, hESCs were differentiated by growing in RPMI medium (Invitrogen, Carlsbad, CA; cat# 61870-036) supplemented with 0.5%, 2.0% or 10% FBS and either with or without 100 ng/ml activin A for 6 days. In addition, a gradient of FBS ranging from 0.5% to 2.0% over the first three days of differentiation was also used in conjunction with 100 ng/ml of activin A. After the 6 days, replicate samples were collected from each culture condition and analyzed for relative gene expression by real-time quantitative PCR. The remaining cells were fixed for immunofluorescent detection of SOX17 protein.

[0289] The expression levels of CXCR4 varied dramatically across the 7 culture conditions used (Figure 26). In general, CXCR4 expression was high in activin A treated cultures (A100) and low in those which did not receive exogenous activin A (NF). In addition, among the A100 treated cultures, CXCR4 expression was highest when FBS concentration was lowest. There was a remarkable decrease in CXCR4 level in the 10% FBS condition such that the relative expression was more in line with the conditions that did not receive activin A (NF).

[0290] As described above, expression of the SOX17, GSC, MIXL1, and HNF3β genes is consistent with the characterization of a cell as definitive endoderm. The relative expression of these four genes across the 7 differentiation conditions mirrors that of CXCR4 (Figures 27A-D). This demonstrates that CXCR4 is also a marker of definitive endoderm.

[0291] Ectoderm and mesoderm lineages can be distinguished from definitive endoderm by their expression of various markers. Early mesoderm expresses the genes Brachyury and MOX1 while nascent neuro-ectoderm expresses SOX1 and ZIC1. Figures 28A-D demonstrate that the cultures which did not receive exogenous activin A were preferentially enriched for mesoderm and ectoderm gene expression and that among the activin A treated cultures, the 10% FBS condition also had increased levels of mesoderm and ectoderm marker expression. These patterns of expression were inverse to that of CXCR4 and indicated that CXCR4 was not highly expressed in mesoderm or ectoderm derived from hESCs at this developmental time period.

[0292] Early during mammalian development, differentiation to extra-embryonic lineages also occurs. Of particular relevance here is the differentiation of visceral endoderm that shares the expression of many genes in common with definitive endoderm, including SOX17. To distinguish definitive endoderm from extra-embryonic visceral endoderm one should examine a marker that is distinct between these two. SOX7 represents a marker that is expressed in the visceral endoderm but not in the definitive endoderm lineage. Thus, culture conditions that exhibit robust SOX17 gene expression in the absence of SOX7 expression are likely to contain definitive and not visceral endoderm. It is shown in Figure 28E that SOX7 was highly expressed in cultures that did not receive activin A, SOX7 also exhibited increased expression even in the presence of activin A when FBS was included at 10%. This pattern is the inverse of the CXCR4 expression pattern and suggests that CXCR4 is not highly expressed in visceral endoderm.

[0293] The relative number of SOX17 immunoreactive ($SOX17^+$) cells present in each of the differentiation conditions mentioned above was also determined. When hESCs were differentiated in the presence of high dose activin A and low FBS concentration (0.5% - 2.0%) $SOX17^+$ cells were ubiquitously distributed throughout the culture. When high dose activin A was used but FBS was included at 10% (v/v), the $SOX17^+$ cells appeared at much lower frequency and always appeared in isolated clusters rather than evenly distributed throughout the culture (Figures 29A and C as well as B and E). A further decrease in $SOX17^+$ cells was seen when no exogenous activin A was used. Under these conditions the $SOX17^+$ cells also appeared in clusters and these clusters were smaller and much more rare than those found in the high activin A, low FBS treatment (Figure 29 C and F). These results demonstrate that the CXCR4 expression patterns not only correspond to definitive endoderm gene expression but also to the number of definitive endoderm cells in each condition.

EXAMPLE 8

Differentiation conditions that enrich for definitive endoderm increase the proportion of CXCR4 positive cells

[0294] The dose of activin A also effects the efficiency at which definitive endoderm can be derived from hESCs. This example demonstrates that increasing the dose of activin A increases the proportion of $CXCR4^+$ cells in the culture.

[0295] hESCs were differentiated in RPMI media supplemented with 0.5%-2% FBS (increased from 0.5% to 1.0% to 2.0% over the first 3 days of differentiation) and either 0, 10, or 100 ng/ml of activin A. After 7 days of differentiation the cells were dissociated in PBS without Ca^{2+}/Mg^{2+} containing 2% FBS and 2 mM (EDTA) for 5 minutes at room temperature. The cells were filtered through 35 μm nylon filters, counted and pelleted. Pellets were resuspended in a small volume of 50% human serum/50% normal donkey serum and incubated for 2 minutes on ice to block non-specific antibody binding sites. To this, 1 μl of mouse anti-CXCR4 antibody (Abcam, cat# ab10403-100) was added per 50 μl (containing approximately 10^5 cells) and labeling proceeded for 45 minutes on ice. Cells were washed by adding 5 ml of PBS containing 2% human serum (buffer) and pelleted. A second wash with 5 ml of buffer was completed then cells were resuspended in 50 μl buffer per 10^5 cells. Secondary antibody (FITC conjugated donkey anti-mouse; Jackson ImmunoResearch, cat# 715-096-151) was added at 5 μg/ml final concentration and allowed to label for 30 minutes followed by two washes in buffer as above. Cells were resuspended at $5x10^6$ cells/ml in buffer and analyzed and sorted using a FACS Vantage (Beckton Dickenson) by the staff at the flow cytometry core facility (The Scripps Research Institute). Cells were collected directly into RLT lysis buffer (Qiagen) for subsequent isolation of total RNA for gene expression analysis by real-time quantitative PCR.

[0296] The number of $CXCR4^+$ cells as determined by flow cytometry were observed to increase dramatically as the dose of activin A was increased in the differentiation culture media (Figures 30A-C). The $CXCR4^+$ cells were those falling within the R4 gate and this gate was set using a secondary antibody-only control for which 0.2% of events were located in the R4 gate. The dramatically increased numbers of $CXCR4^+$ cells correlates with a robust increase in definitive endoderm gene expression as activin A dose is increased (Figures 31A-D).

EXAMPLE 9

Isolation of CXCR4 positive cells enriches for definitive endoderm gene expression and depletes cells expressing markers of mesoderm, ectoderm and visceral endoderm

[0297] The $CXCR4^+$ and $CXCR4^-$ cells identified in Example 8 above were collected and analyzed for relative gene expression and the gene expression of the parent populations was determined simultaneously.

[0298] The relative levels of CXCR4 gene expression was dramatically increased with increasing dose of activin A (Figure 32). This correlated very well with the activin A dose-dependent increase of $CXCR4^+$ cells (Figures 30A-C). It is also clear that isolation of the $CXCR4^+$ cells from each population accounted for nearly all of the CXCR4 gene expression in that population. This demonstrates the efficiency of the FACS method for collecting these cells.

[0299] Gene expression analysis revealed that the $CXCR4^+$ cells contain not only the majority of the CXCR4 gene expression, but they also contained gene expression for other markers of definitive endoderm. As shown in Figures 31A-D, the $CXCR4^+$ cells were further enriched over the parent A100 population for SOX17, GSC, HNF3B, and MIXL1. In addition, the $CXCR4^-$ fraction contained very little gene expression for these definitive endoderm markers. Moreover, the $CXCR4^+$ and $CXCR4^-$ populations displayed the inverse pattern of gene expression for markers of mesoderm, ectoderm and extra-embryonic endoderm. Figures 33A-D shows that the $CXCR4^+$ cells were depleted for gene expression of Brachyury, MOX1, ZIC1, and SOX7 relative to the A100 parent population. This A100 parent population was already low in expression of these markers relative to the low dose or no activin A conditions. These results show that the isolation of $CXCR4^+$ cells from hESCs differentiated in the presence of high activin A yields a population that is highly enriched for and substantially pure definitive endoderm.

EXAMPLE 10

Quantitation of Definitive Endoderm Cells in a Cell Population Using CXCR4

[0300] To confirm the quantitation of the proportion of definitive endoderm cells present in a cell culture or cell population as determined previously herein and as determined in United States Provisional Patent Application No. 60/532,004, entitled DEFINITIVE ENDODERM, filed December 23, 2003, cells expressing CXCR4 and other markers of definitive endoderm were analyzed by FACS.

[0301] Using the methods such as those described in the above Examples, hESCs were differentiated to produce definitive endoderm. In particular, to increase the yield and purity in differentiating cell cultures, the serum concentration of the medium was controlled as follows: 0.2% FBS on day1, 1.0% FBS on day 2 and 2.0% FBS on days 3-6. Differentiated cultures were sorted by FACS using three cell surface epitopes, E-Cadherin, CXCR4, and Thrombomodulin. Sorted cell populations were then analyzed by Q-PCR to determine relative expression levels of markers for definitive and extraembryonic-endoderm as well as other cell types. CXCR4 sorted cells taken from optimally differentiated cultures resulted in the isolation of definitive endoderm cells that were >98% pure.

[0302] Table 2 shows the results of a marker analysis for a definitive endoderm culture that was differentiated from hESCs using the methods described herein.

Table 2

Composition of Definitive Endoderm Cultures

Marker(s)	Percent of culture	Percent Definitive Endoderm	Percent Extraembryonic endoderm	Percent hES cells
SOX17	70-80	100		
Thrombomodulin	<2	0	75	
AFP	<1	0	25	
CXCR4	70-80	100	0	
ECAD	10	0		100
other (ECAD neg.)	10-20			
Total	100	100	100	100

[0303] In particular, Table 2 indicates that CXCR4 and SOX17 positive cells (endoderm) comprised from 70%-80% of the cells in the cell culture. Of these SOX17-expressing cells, less than 2% expressed TM (parietal endoderm) and less than 1% expressed AFP (visceral endoderm). After subtracting the proportion of TM-positive and AFP-positive cells (combined parietal and visceral endoderm; 3% total) from the proportion of SOX17/CXCR4 positive cells, it can be seen that about 67% to about 77% of the cell culture was definitive endoderm. Approximately 10% of the cells were positive for E-Cadherin (ECAD), which is a marker for hESCs, and about 10-20% of the cells were of other cell types.

[0304] We have discovered that the purity of definitive endoderm in the differentiating cell cultures that are obtained prior to FACS separation can be improved as compared to the above-described low serum procedure by maintaining the FBS concentration at $\leq$0.5% throughout the 5-6 day differentiation procedure. However, maintaining the cell culture at $\leq$0.5% throughout the 5-6 day differentiation procedure also results in a reduced number of total definitive endoderm cells that are produced.

[0305] Definitive endoderm cells produced by methods described herein have been maintained and expanded in culture in the presence of activin for greater than 50 days without appreciable differentiation. In such cases, SOX17, CXCR4, MIXL1, GATA4, HNF3β expression is maintained over the culture period. Additionally, TM, SPARC, OCT4, AFP, SOX7, ZIC1 and BRACH were not detected in these cultures. It is likely that such cells can be maintained and expanded in culture for substantially longer than 50 days without appreciable differentiation.

EXAMPLE 11

Additional Markers of Definitive Endoderm Cells

[0306] In the following experiment, RNA was isolated from purified definitive endoderm and human embryonic stem cell populations. Gene expression was then analyzed by

gene chip analysis of the RNA from each purified population. Q-PCR was also performed to further investigate the potential of genes expressed in definitive endoderm, but not in embryonic stem cells, as a marker for definitive endoderm.

[0307] Human embryonic stem cells (hESCs) were maintained in DMEM/F12 media supplemented with 20% KnockOut Serum Replacement, 4 ng/ml recombinant human basic fibroblast growth factor (bFGF), 0.1 mM 2-mercaptoethanol, L-glutamine, non-essential amino acids and penicillin/streptomycin. hESCs were differentiated to definitive endoderm by culturing for 5 days in RPMI media supplemented with 100 ng/ml of recombinant human activin A, fetal bovine serum (FBS), and penicillin/streptomycin. The concentration of FBS was varied each day as follows: 0.1% (first day), 0.2% (second day), 2% (days 3-5).

[0308] Cells were isolated by fluorescence activated cell sorting (FACS) in order to obtain purified populations of hESCs and definitive endoderm for gene expression analysis. Immuno-purification was achieved for hESCs using SSEA4 antigen (R&D Systems, cat# FAB1435P) and for definitive endoderm using CXCR4 (R&D Systems, cat# FAB170P). Cells were dissociated using trypsin/EDTA (Invitrogen, cat# 25300-054), washed in phosphate buffered saline (PBS) containing 2% human serum and resuspended in 100% human serum on ice for 10 minutes to block non-specific binding. Staining was carried out for 30 minutes on ice by adding 200 µl of phycoerythrin-conjugated antibody to 5 x 10^6 cells in 800 µl human serum. Cells were washed twice with 8 ml of PBS buffer and resuspended in 1 ml of the same. FACS isolation was carried out by the core facility of The Scripps Research Institute using a FACS Vantage (BD Biosciences). Cells were collected directly into RLT lysis buffer and RNA was isolated by RNeasy according to the manufacturers instructions (Qiagen).

[0309] Purified RNA was submitted in duplicate to Expression Analysis (Durham, NC) for generation of the expression profile data using the Affymetrix platform and U133 Plus 2.0 high-density oligonucleotide arrays. Data presented is a group comparison that identifies genes differentially expressed between the two populations, hESCs and definitive endoderm. Genes that exhibited a robust upward change in expression level over that found in hESCs were selected as new candidate markers that are highly characteristic of definitive endoderm. Select genes were assayed by Q-PCR, as described above, to verify the gene expression changes found on the gene chip and also to investigate the expression pattern of these genes during a time course of hESC differentiation.

[0310] Figures 34A-M show the gene expression results for certain markers. Results are displayed for cell cultures analyzed 1, 3 and 5 days after the addition of 100 ng/ml activin A, CXCR4-expressing definitive endoderm cells purified at the end of the five day differentiation procedure (CXDE), and in purified hESCs. A comparison of Figures 34C and G-M demonstrates that the six marker genes, FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1, exhibit an expression pattern that is almost identical to each other and which is also identical to the pattern

of expression of CXCR4 and the ratio of SOX17/SOX7. As described previously, SOX17 is expressed in both the definitive endoderm as well as in the SOX7-expressing extra-embryonic endoderm. Since SOX7 is not expressed in the definitive endoderm, the ratio of SOX17/SOX7 provides a reliable estimate of definitive endoderm contribution to the SOX17 expression witnessed in the population as a whole. The similarity of panels G-L and M to panel C indicates that FGF17, VWF, CALCR, FOXQ1, CMKOR1 and CRIP1 are likely markers of definitive endoderm and that they are not significantly expressed in extra-embryonic endoderm cells.

[0311] It will be appreciated that the Q-PCR results described herein can be further confirmed by ICC.

EXAMPLE 12

Retinoic Acid and FGF-10 Induces PDX1 Specifically in Definitive Endoderm Cultures

[0312] The following experiment demonstrates that RA and FGF-10 induces the expression of PDX1 in definitive endoderm cells.

[0313] Human embryonic stem cells were cultured with or without activins for four days. On day four, 1 μM RA and 50 ng/ml FGF-10 were added to the cell culture. Forty-eight hours after the RA/FGF-10 addition, the expression of the PDX1 marker gene and other marker genes not specific to foregut endoderm were quantitated by Q-PCR.

[0314] The application of RA to definitive endoderm cells caused a robust increase in PDX1 gene expression (see Figure 35) without increasing the expression of visceral endoderm (SOX7, AFP), neural (SOX1, ZIC1), or neuronal (NFM) gene expression markers (see Figure 36A-F). PDX1 gene expression was induced to levels approximately 500-fold higher than observed in definitive endoderm after 48 hours exposure to 1 μM RA and 50 ng/ml FGF-10. Furthermore, these results show that substantial PDX1 induction occurred only in cell cultures which had been previously differentiated to definitive endoderm (SOX17) as indicated by the 160-fold higher PDX1 expression found in the activin treated cell cultures relative to those cultures that received no activin prior to RA application.

EXAMPLE 13

FGF-10 Provides Additional Increase in PDX1 Expression Over RA Alone

[0315] This Example shows that the combination of RA and FGF-10 induces PDX1 expression to a greater extent than RA alone.

[0316] As in the previous Example, hESCs were cultured with or without activins for four days. On day four, the cells were treated with one of the following: 1 μM RA alone; 1 μM RA in combination with either FGF-4 or FGF-10; or 1 μM RA in combination with both FGF-4 and FGF-10. The expression of PDX1, SOX7 and NFM were quantitated by Q-PCR ninety six hours after RA or RA/FGF.

[0317] The treatment of hESC cultures with activin followed by retinoic acid induced a 60-fold increase in PDX1 gene expression. The addition of FGF-4 to the RA treatment induced slightly more PDX1 (approximately 3-fold over RA alone). However, by adding FGF-10 and retinoic acid together, the induction of PDX1 was further enhanced 60-fold over RA alone (see Figure 37A). This very robust PDX1 induction was greater than 1400-fold higher than with no activin or RA/FGF treatment. Interestingly, addition of FGF-4 and FGF-10 simultaneously abolished the beneficial effect of the FGF-10, producing only the modest PDX1 increase attributed to FGF-4 addition.

[0318] Addition of RA/FGF-4 or RA/FGF-10 combinations did not increase the expression of marker genes not associated with foregut endoderm when compared to cells not exposed to RA/FGF combinations (see Figure 37B-C).

EXAMPLE 14

Retinoic Acid Dose Affects Anterior-Posterior (A-P) Position *In Vitro*

[0319] To determine whether the dose of RA affects A-P position in *in vitro* cell cultures, the following experiment was performed.

[0320] Human embryonic stem cells were cultured with or without activins for four days. On day four, FGF-10 at 50 ng/ml was added to the culture in combination with RA at 0.04 μM, 0.2 μM or 1.0 μM. The expression of the PDX1 marker gene as well as other markers not specific for foregut endoderm were quantitated by Q-PCR.

[0321] The addition of retinoic acid at various doses, in combination with FGF-10 at 50 ng/ml, induced differential gene expression patterns that correlate with specific anterior-posterior positional patterns. The highest dose of RA (1 μM) preferentially induced expression of anterior endoderm marker (HOXA3) and also produced the most robust increase in PDX1 (Figure 38A-B). The middle dose of RA (0.2 μM) induced midgut endoderm markers (CDX1, HOXC6) (see Figure 38C and 41E), while the lowest dose of RA (0.04 μM) preferentially induced a marker of hindgut endoderm (HOXA13) (see Figure 38D). The RA dose had essentially no effect on the relative expression of either neural (SOX1) or neuronal (NFM) markers (see Figure 38F-G). This example highlights the use of RA as a morphogen *in vitro* and in particular as a morphogen of endoderm derivatives of differentiating hESCs.

EXAMPLE 15

Use of B27 Supplement Enhances Expression of PDX1

[0322] PDX1 expression in definitive endoderm can be influenced by the use of a number of factors and cell growth/differentiation conditions. In the following experiment, we show that the use of B27 supplement enhances the expression of PDX1 in definitive endoderm cells.

[0323] Human embryonic stem cells were induced to differentiate to definitive endoderm by treatment of undifferentiated hES cells grown on mouse embryonic fibroblast feeders with high dose activin A (100-200 ng/ml in 0.5-2 % FBS/DMEM/F12) for 4 days. The no activin A control received 0.5-2 % FBS/DMEM/F12 with no added activin A. At four days, cultures received either no activin A in 2% FBS (none), and in 2% serum replacement (SR), or 50 ng/ml activin A together with 2 μM RA and 50 ng/ml FGF-10 in 2% FBS/DMEM/F12 (none, +FBS, +B27) and similarly in 2% Serum replacement (SR). B27 supplement, (Gibco/BRL), was added as a 1/50 dilution directly into 2%FBS/DMEM/F12 (+B27). Duplicate cell samples where taken for each point, and total RNA was isolated and subjected to Q-PCR as previously described.

[0324] Figure 39A-E shows that serum-free supplement B27 provided an additional benefit for induction of PDX1 gene expression without inducing an increase in the expression of markers genes not specific for foregut endoderm as compared to such marker gene expression in cells grown without serum.

EXAMPLE 16

Use of Activin B to Enhance Induction of PDX1

[0325] This Example shows that the use of activin B enhances the differentiation of PDX1-negative cells to PDX1-positive cells in *in vitro* cell culture.

[0326] Human embryonic stem cells were induced to differentiate to definitive endoderm by treatment of undifferentiated hESCs grown on mouse embryonic fibroblast feeders with high dose activin A (50 ng/ml) in low serum/RPMI for 6 days. The FBS dose was 0% on day one, 0.2% on day two and 2% on days 3-6. The negative control for definitive endoderm production (NF) received 2% FBS/RPMI with no added activin A. In order to induce PDX1 expression, each of the cultures received retinoic acid at 2 μM in 2% FBS/RPMI on day 6. The cultures treated with activin A on days one through five were provided with different dosing combinations of activin A and activin B or remained in activin A alone at 50ng/ml. The no activin A control culture (NF) was provided neither activin A nor activin B. This RA/activin treatment was carried out for 3 days at which time PDX1 gene expression was measured by Q-PCR from duplicate cell samples.

[0327] Figure 40A shows that the addition of activin B at doses ranging from 10-50 ng/ml (a10, a25 and a50) in the presence of 25 ng/ml (A25) or 50 ng/ml (A50) of activin A increased the PDX1 expression at least 2-fold over the culture that received only activin A at 50 ng/ml. The increase in PDX1 as a result of activin B addition was without increase in HNF6 expression (see Figure 40B), which is a marker for liver as well as pancreas at this time in development. This result suggests that the proportion of cells differentiating to pancreas had been increased relative to liver.

EXAMPLE 17

Use of Serum Dose to Enhance Induction of PDX1

[0328] The expression of PDX1 in definitive endoderm cells is influenced by the amount of serum present in the cell culture throughout the differentiation process. The following experiment shows that the level of serum in a culture during the differentiation of hESCs to PDX1-negative definitive endoderm has an effect on the expression of PDX1 during further differentiation of these cells to PDX1-positive endoderm.

[0329] Human embryonic stem cells were induced to differentiate to definitive endoderm by treatment of undifferentiated hESCs grown on mouse embryonic fibroblast feeders with high dose activin A (100 ng/ml) in low serum/RPMI for 5 days. The FBS dose was 0.1% on day one, 0.5% on day two and either 0.5%, 2% or 10% on days 3-5. The no activin A control (NF) received the same daily FBS/RPMI dosing, but with no added activin A. PDX1 expression was induced beginning at day 6 by the addition of RA. During days 6-7, cultures received retinoic acid at 2 μM in 0.5% FBS/RPMI, 1 μM on day 8 and 0.2 μM on day 9-11. The activin A was lowered to 50 ng/ml during retinoic acid treatment and was left absent from the no activin A control (NF).

[0330] Figure 41A shows that the FBS dosing during the 3 day period of definitive endoderm induction (days 3, 4 and 5) had a lasting ability to change the induction of PDX1 gene expression during the retinoic acid treatment. This was without significant alteration in the expression pattern of ZIC1 (Figure 41B) or SOX7 (Figure 41C) gene expression.

EXAMPLE 18

Use of Conditioned Medium to Enhance Induction of PDX1

[0331] Other factors and growth conditions which influence the expression of PDX1 in definitive endoderm cells were also studied. The following experiment shows the effect of conditioned media on the differentiation of PDX1-negative definitive endoderm cells to PDX1-positive endoderm cells.

[0332] Human embryonic stem cells were induced to differentiate to definitive endoderm by treatment of undifferentiated hESCs grown on mouse embryonic fibroblast feeders with high dose activin A (100 ng/ml) in low serum/RPMI for 5 days. The FBS dose was 0.2% on day one, 0.5% on day two and 2% on days 3-5.

[0333] The definitive endoderm cultures generated by 5 days of activin A treatment were then induced to differentiate to PDX1 expressing endoderm by the addition of RA in 2% FBS/RPMI containing activin A at 25 ng/ml for four days. The RA was 2 μM for the first two days of addition, 1 μM on the third day and 0.5 μM on the fourth day. This base medium for PDX1 induction was provided fresh (2A25R) or after conditioning for 24 hours by one of four

different cell populations. Conditioned media (CM) were generated from either mouse embryonic fibroblasts (MEFCM) or from hESCs that were first differentiated for 5 days by one of three conditions; *i*) 3% FBS/RPMI (CM2), or *ii*) activin A (CM3) or *iii*) bone morphogenic protein 4 (BMP4) (CM4). Activin A or BMP4 factors were provided at 100 ng/ml under the same FBS dosing regimen described above (0.2%, 0.5%, 2%). These three different differentiation paradigms yield three very different populations of human cells by which the PDX1 induction media can be conditioned. The 3% FBS without added growth factor (NF) yields a heterogeneous population composed in large part of extraembryonic endoderm, ectoderm and mesoderm cells. The activin A treated culture (A100) yields a large proportion of definitive endoderm and the BMP4 treated culture (B100) yields primarily trophectoderm and some extraembryonic endoderm.

[0334] Figure 42A shows that PDX1 was induced equivalently in fresh and conditioned media over the first two days of RA treatment. However, by the third day PDX1 expression had started to decrease in fresh media and MEF conditioned media treatments. The differentiated hESCs produced conditioned media that resulted in maintenance or further increases in the PDX1 gene expression at levels 3 to 4-fold greater than fresh media. The effect of maintaining high PDX1 expression in hESC-conditioned media was further amplified on day four of RA treatment achieving levels 6 to 7-fold higher than in fresh media. Figure 42B shows that the conditioned media treatments resulted in much lower levels of CDX1 gene expression, a gene not expressed in the region of PDX1 expressing endoderm. This indicates that the overall purity of PDX1-expressing endoderm was much enhanced by treating definitive endoderm with conditioned media generated from differentiated hESC cultures.

[0335] Figure 43 shows that PDX1 gene expression exhibited a positive dose response to the amount of conditioned media applied to the definitive endoderm cells. Total volume of media added to each plate was 5 ml and the indicated volume (see Figure 43) of conditioned media was diluted into fresh media (A25R). It is of note that just 1 ml of conditioned media added into 4 ml of fresh media was still able to induce and maintain higher PDX1 expression levels than 5 ml of fresh media alone. This suggests that the beneficial effect of conditioned media for induction of PDX1 expressing endoderm is dependent on the release of some substance or substances from the cells into the conditioned media and that this substance(s) dose dependently enhances production of PDX1-expressing endoderm.

EXAMPLE 19

Validation of Antibodies Which Bind to PDX1

[0336] Antibodies that bind to PDX1 are useful tools for monitoring the induction of PDX1 expression in a cell population. This Example shows that rabbit polyclonal and IgY antibodies to PDX1 can be used to detect the presence of this protein.

[0337] In a first experiment, IgY anti-PDX1 (IgY α-PDX1) antibody binding to PDX1 in cell lysates was validated by Western blot analysis. In this analysis, the binding of IgY α-PDX1 antibody to 50 μg of total cell lysate from MDX12 human fibroblasts or MDX12 cells transfected 24 hrs previously with a PDX1 expression vector was compared. The cell lysates separated by SDS-PAGE, transferred to a membrane by electroblotting, and then probed with the IgY α-PDX1 primary antiserum followed by alkaline phosphatase conjugated rabbit anti-IgY (Rb α-IgY) secondary antibodies. Different dilutions of primary and secondary antibodies were applied to separate strips of the membrane in the following combinations: A (500x dilution of primary, 10,000x dilution of secondary), B (2,000x, 10,000x), C (500x, 40,000x), D (2,000x, 40,000), E (8,000x, 40,000x).

[0338] Binding was detected in cells transfected with the PDX1 expression vector (PDX1-positive) at all of the tested antibody combinations. Binding was only observed in untransfected (PDX1-negative) fibroblasts when using the highest concentrations of both primary and secondary antibody together (combination A). Such non-specific binding was characterized by the detection of an additional band at a molecular weight slightly higher than PDX1 in both the transfected and untransfected fibroblasts.

[0339] In a second experiment, the binding of polyclonal rabbit anti-PDX1 (Rb α-PDX1) antibody to PDX1 was tested by immunocytochemistry. To produce a PDX1 expressing cell for such experiments, MS1-V cells (ATCC # CRL-2460) were transiently transfected with an expression vector of PDX1-EGFP (constructed using pEGFP-N1, Clontech). Transfected cells were then labeled with Rb α-PDX1 and α-EGFP antisera. Transfected cells were visualized by both EGFP fluorescence as well as α-EGFP immunocytochemistry through the use of a Cy5 conjugated secondary antibody. PDX1 immunofluorescence was visualized through the use of an α-Rb Cy3-conjugated secondary antibody.

[0340] Binding of the Rb α-PDX1 and the α-EGPF antibodies co-localized with GPF expression.

EXAMPLE 20

Immunocytochemistry of Human Pancreatic Tissue

[0341] This Example shows that antibodies having specificity for PDX1 can be used to identify human PDX1-positive cells by immunocytochemistry.

[0342] In a first experiment, paraffin embedded sections of human pancreas were stained for insulin with guinea pig anti-insulin (Gp α-Ins) primary antibody at a 1/200 dilution followed by dog anti-guinea pig (D α-Gp) secondary antibody conjugated to Cy2 at a 1/100 dilution. In a second experiment, the same paraffin embedded sections of human pancreas were stained for PDX1 with IgY α-PDX1 primary antibody at a 1/4000 dilution followed Rb α-IgY

secondary antibody conjugated to AF555 at a 1/300 dilution. The images collected from the first and second experiments where then merged. In a third experiment, cells that were stained with IgY α-PDX1 antibodies were also stained with DAPI.

[0343] Analysis of the human pancreatic sections revealed the presence of strong staining of islets of Langerhans. Although the strongest PDX1 signal appeared in islets (insulin-positive), weak staining was also seen in acinar tissue (insulin-negative). DAPI and PDX1 co-staining shows that PDX1 was mostly but not exclusively localized to the nucleus.

EXAMPLE 21

Immunoprecipitation of PDX1 from Retinoic Acid Treated Cells

[0344] To further confirm PDX1 expression in definitive endoderm cells that have been differentiated in the presence of RA and the lack of PDX1 in definitive endoderm cells that have not been differentiated with RA, a rabbit anti-PDX1 (Rb α-PDX1) antibody was used to immunoprecipitate PDX1 from both RA differentiated and undifferentiated definitive endoderm cells. Immunoprecipitated RA was detected by Western blot analysis using IgY α-PDX1 antibody.

[0345] To obtain undifferentiated and differentiated definitive endoderm cell lysates for immunoprecipitation, hESCs were treated for 5 days with activin A at 100 ng/ml in low serum (definitive endoderm) followed by treatment with activin A at 50 ng/ml and 2 μM all-trans RA for two days, 1 μM for one day and 0.2 μM for one day (PDX1-positive foregut endoderm). As a positive control cell lysates were also prepared from MS1-V cells (ATCC # CRL-2460) transfected with a PDX1 expression vector. PDX1 was immunoprecipitated by adding Rb α-PDX1 and rabbit-specific secondary antibodies to each lysate. The precipitate was harvested by centrifugation. Immunoprecipitates were dissolved in SDS-containing buffer then loaded onto a polyacrylamide gel. After separation, the proteins were transferred to a membrane by electroblotting, and then probed with the IgY α-PDX1 primary antibody followed by labeled Rb α-IgY secondary antibodies.

[0346] Immunoprecipitates collected from the MS1-V positive control cells as well as those from day 8 (lane d8, three days after the start of RA treatment) and day 9 (lane d9, four days after the start of RA) cells were positive for PDX1 protein (Figure 44). Precipitates obtained from undifferentiated definitive endoderm cells (that is, day 5 cells treated with activin A – designated (A) in Figure 44) and undifferentiated hESCs (that is, untreated day 5 cells – designated as (NF) in Figure 44) were negative for PDX1.

EXAMPLE 22

Generation of PDX1 promoter-EGFP transgenic hESC lines

[0347] In order to use the PDX1 marker for cell isolation, we genetically tagged PDX1-positive foregut endoderm cells with an expressible reporter gene. This Example describes the construction of a vector comprising a reporter cassette which comprises a reporter gene under the control of the PDX1 regulatory region. This Example also describes the preparation of a cell, such as a human embryonic stem cell, transfected with this vector as well as a cell having this reporter cassette integrated into its genome.

[0348] PDX1-expressing definitive endoderm cell lines genetically tagged with a reporter gene were constructed by placing a GFP reporter gene under the control of the regulatory region (promoter) of the PDX1 gene. First, a plasmid construct in which EGFP expression is driven by the human PDX1 gene promoter was generated by replacing the CMV promoter of vector pEGFP-N1 (Clontech) with the human PDX1 control region (Genbank Accession No. AF192496), which comprises a nucleotide sequence ranging from about 4.4 kilobase pairs (kb) upstream to about 85 base pairs (bp) downstream of the PDX1 transcription start site. This region contains the characterized regulatory elements of the PDX1 gene, and it is sufficient to confer the normal PDX1 expression pattern in transgenic mice. In the resulting vector, expression of EFGP is driven by the PDX1 promoter. In some experiments, this vector can be transfected into hESCs.

[0349] The PDX1 promoter/EGFP cassette was excised from the above vector, and then subcloned into a selection vector containing the neomycin phosphotransferase gene under control of the phosphoglycerate kinase-1 promoter. The selection cassette was flanked by flp recombinase recognition sites to allow removal of the cassette. This selection vector was linearized, and then introduced into hESCs using standard lipofection methods. Following 10-14 days of selection in G418, undifferentiated transgenic hESC clones were isolated and expanded.

EXAMPLE 23

Isolation of PDX1-Positive Foregut Endoderm

[0350] The following Example demonstrates that hESCs comprising the PDX1 promoter/EGFP cassette can be differentiated into PDX1-positive endoderm cells and then subsequently isolated by fluorescence-activated cell sorting (FACS).

[0351] PDX1 promoter/EGFP transgenic hESCs were differentiated for 5 days in activin A-containing media followed by two days in media comprising activin A and RA. The differentiated cells were then harvested by trypsin digestion and sorted on a Becton Dickinson FACS Diva directly into RNA lysis buffer or PBS. A sample of single live cells was taken without gating for EGFP (Live) and single live cells were gated into EGFP positive (GFP) and GFP negative (Neg) populations. In one experiment, the EGFP positive fraction was separated into two equally sized populations according to fluorescence intensity (Hi and Lo).

[0352] Following sorting, cell populations were analyzed by both Q-PCR and immunocytochemistry. For Q-PCR analysis, RNA was prepared using Qiagen RNeasy columns and then converted to cDNA. Q-PCR was conducted as described previously. For immunocytochemistry analysis, cells were sorted into PBS, fixed for 10 minutes in 4% paraformaldehyde, and adhered to glass slides using a Cytospin centrifuge. Primary antibodies to Cytokeratin19 (KRT19) were from Chemicon; to Hepatocyte nuclear factor 3 beta (HNF3β) from Santa Cruz; to Glucose Transporter 2 (GLUT2) from R&D systems. Appropriate secondary antibodies conjugated to FITC (green) or Rhodamine (Red) were used to detect binding of the primary antibodies.

[0353] A typical FACS sort of differentiated cells is shown in Figure 45. The percent isolated PDX1-positive cells in this example was approximately 7%, which varied depending on the differentiation efficiency from about 1% to about 20%.

[0354] Sorted cells were further subjected to Q-PCR analysis. Differentiated cells showed a correlation of EGFP fluorescence with endogenous PDX1 gene expression. Compared to non-fluorescing cells, the EGFP positive cells showed a greater than 20-fold increase in PDX1 expression levels (Figure 46). The separation of high and low EGFP intensity cells indicated that EGFP expression level correlated with PDX1 expression level (Figure 47). In addition to PDX1 marker analysis, sorted cells were subjected to Q-PCR analysis of several genes that are expressed in pancreatic endoderm. Products of each of these marker genes (NKX2.2, GLUT2, KRT19, HNF4α and HNF3β) were all enriched in the EGFP positive fraction (Figures 48A-E). In contrast, the neural markers ZIC1 and GFAP were not enriched in sorted EGFP expressing cells (Figures 49A and B).

[0355] By immunocytochemistry, virtually all the isolated PDX1-positive cells were seen to express KRT19 and GLUT2. This result is expected for cells of the pancreatic endoderm lineage. Many of these cells were also HNF3β positive by antibody staining.

EXAMPLE 24

Transplantation of Human Definitive Endoderm Cells under Mouse Kidney Capsule

[0356] To demonstrate that the human definitive endoderm cells produced using the methods described herein are capable of responding to differentiation factors so as to produce cells that are derived from the gut tube, such human definitive endoderm cells were subjected to an *in vivo* differentiation protocol.

[0357] Human definitive endoderm cells were produced as described in the foregoing Examples. Such cells were harvested and transplanted under the kidney capsule of immunocompromised mice using standard procedures. After three weeks, the mice were

sacrificed and the transplanted tissue was removed, sectioned and subjected to histological and immunocytochemical analysis.

[0358] Figures 50A-D show that after three weeks post-transplantation, the human definitive endoderm cells differentiated into cells and cellular structures derived from the gut tube. In particular, Figure 50A shows hematoxylin and eosin stained sections of transplanted human definitive endoderm tissue that has differentiated into gut-tube-like structures. Figure 50B shows a transplanted human definitive endoderm section immunostained with antibody to hepatocyte specific antigen (HSA). This result indicates that the human definitive endoderm cells are capable of differentiating into liver or liver precursor cells. Figures 50C and 50D show a transplanted human definitive endoderm section immunostained with antibody to villin and antibody to caudal type homeobox transcription factor 2 (CDX2), respectively. These results indicate that the human definitive endoderm cells are capable of differentiating into intestinal cells or intestinal cell precursors.

EXAMPLE 25

Identification of Differentiation Factors Capable of Promoting the Differentiation of Human Definitive Endoderm Cells *In Vitro*

[0359] To exemplify the differentiation factor screening methods described herein, populations of human definitive endoderm cells produced using the methods described herein were separately provided with several candidate differentiation factors while determining the normalized expression levels of certain marker gene products at various time points.

[0360] Human definitive endoderm cells were produced as described in the foregoing Examples. In brief, hESCs cells were grown in the presence of 100 ng/ml activin A in low serum RPMI medium for four days, wherein the fetal bovine serum (FBS) concentration on day 1 was 0%, on day 2 was 0.2% and on days 3-4 was 2%. After formation of definitive endoderm, beginning on day 5 and ending on day 10, cell populations maintained in individual plates in RPMI containing 0.2% FBS were treated with one of: Wnt3B at 20 ng/ml, FGF2 at 5 ng/ml or FGF2 at 100 ng/ml. The expression of marker gene products for albumin, PROX1 and TITF1 were quantitated using Q-PCR.

[0361] Figure 51A shows that expression of the albumin gene product (a marker for liver precursors and liver cells) substantially increased on days 9 and 10 in response to FGF2 at 5 ng/ml as compared to expression in definitive endoderm cells on day 4 prior to treatment with this differentiation factor. Expression of the albumin gene product was also increased in response to 20 ng/ml Wnt3B on days 9 and 10 as compared to expression in untreated definitive endoderm cells, however, the increase was not as large as that observed for the 5 ng/ml FGF2 treatment. Of particular significance is the observation that the expression of the albumin gene product was not increased on days 9 and 10 in response to FGF2 at 100 ng/ml as compared to expression in

definitive endoderm cells on day 4. Similar results were seen with the PROX1 marker (a second marker for liver precursors and liver cells) as shown in Figure 51B. Figure 51C shows that in cell populations provided with 100 ng/ml FGF2, expression of the TITF1 marker gene substantially increased on days 7, 9 and 10 as compared to expression in definitive endoderm cells on day 4 prior to treatment with this differentiation factor, but FGF2 at 5 ng/ml had very little effect on expression of this gene product as compared to untreated definitive endoderm. Taken together, the results shown in Figures 51A-C indicate that the concentration at which the candidate differentiation factor is provided to the cell population can affect the differentiation fate of definitive endoderm cells *in vitro*.

EXAMPLE 26

Marker Upregulation and Downregulation in Response to Candidate Differentiation Factors

[0362] To further exemplify the differentiation factor screening methods described herein, populations of human definitive endoderm cells were screened with candidate differentiation factors using procedures similar to those described in Example 25.

[0363] Human definitive endoderm cells were produced as described in the foregoing Examples. In brief, hESCs cells were grown in the presence of 100 ng/ml activin A in low serum RPMI medium for four days, wherein the fetal bovine serum (FBS) concentration on day 1 was 0%, on day 2 was 0.2% and on days 3-4 was 2%. After formation of definitive endoderm, beginning on day 5 and ending on day 10, cell populations maintained in individual plates in RPMI containing 0.2% FBS were treated with one of: Wnt3A at 20-50 ng/ml, FGF2 at 5 ng/ml or FGF2 at 100 ng/ml. On day 5 post definitive endoderm formation (day 9 after the start of the differentiation from hESCs), BMP4 was added to all the cultures at a concentration of 50 ng/ml. The expression of marker gene products (mRNAs) for alpha fetoprotein (AFP), cytochrome P450 7A (CYP7A), tyrosine aminotransferase (TAT), hepatocyte nuclear factor 4a (HNF4a), CXC-type chemokine receptor 4 (CXCR4), von Willebrand factor (VWF), vascular cell adhesion molecule-1 (VACM1), apolipoprotein A1 (APOA1), glucose transporter-2 (GLUT2), alpha-1-antitrypsin (AAT), glukokinase (GLUKO), and human hematopoietically expressed homeobox (hHEX) were quantitated using Q-PCR.

[0364] Figures 52A-B show that expression of the AFP gene product (a marker for liver precursors and liver cells) and AAT substantially increased on days 9 and 10 in response to FGF2 at 5 ng/ml and BMP4 at 50 ng/ml as compared to expression in definitive endoderm cells on day 4. Expression of AFP and AAT mRNAs was not substantially increased by higher concentration of FGF2 (100 ng/ml) even in the presence of BMP4 (Figures 51A-B days 9 and 10). In contrast to the above results, the expression of GLUKO, hHEX and TAT mRNAs was substantially upregulated in the presence of FGF2 at 100 ng/ml and BMP4 at 50 ng/ml on days 9 and 10 as compared to expression in definitive endoderm cells on day 4. In the case of GLUKO, neither Wnt3A nor FGF2 at 5 ng/ml with or without BMP4 caused an increase in the expression

of this marker (Figure 52C). FGF2 at 5 ng/ml did, however, cause an increase in expression of hHEX in the presence of BMP to an extent greater than or equal to the increase caused by FGF2 at 100 ng/ml in the presence of BMP (Figure 52D). Expression of TAT on days 9 and 10 as compared to expression in definitive endoderm cells was increased by each of the factors tested (Figure 52E). Additionally, certain cell markers were expressed at an increased level as compared to definitive endoderm cells in the presence of Wnt3A, but not in response to FGF/BMP combinations. In particular, the expression of hNF4a mRNA significantly increased on days 9 and 10 in response to the combination of Wnt3A and BMP4 (Figure 52F). Furthermore, CYP7A showed a marginal increase on response to Wnt3A/BMP4 on day 10 (Figure 52G).

[0365] Several markers that are known to be expressed in a number of different cells types were also observed. Specifically the markers APOA1, GLUT2, VCAM1, VWF and CXCR4 were examined. Previously the expression of each of these markers has been correlated with specific cell types as follows: The markers APOA1 and GLUT2 are highly expressed in the liver and moderately expressed in the duodenum and small intestine. The marker VCAM1 is expressed at a high level in the liver, expressed at a moderate level in the stomach, duodenum, and small intestine, and expressed at lower but significant levels in the lung and pancreas. In contrast, the markers VWF and CXCR4 are expressed at high levels in the lung but only at low levels in liver. Both VWF and CXCR4 are also expressed at moderate to high levels in the stomach, pancreas, duodenum, and small intestine.

[0366] Expression of each of the above-described markers was monitored in definitive endoderm cell cultures contacted with combinations of Wnt3A, FGF2 and BMP4. Consistent with the above results, Figures 52H-J show that GLUT2, APOA1 and VCAM1 mRNA expression was increased in response to the combination of FGF2 at 5 ng/ml and BMP4 on days 9 and 10 as compared to the expression in definitive endoderm. The mRNA expression for these markers was not substantially increased in response to the combination of FGF2 at 100 ng/ml an BMP4. In the case of the APOA1 and VCAM1 marker mRNAs, the largest increase in expression on days 9 and 10 was mediated by the combination of Wnt3A and BMP4 (Figures 52I-J).

[0367] In addition to the foregoing, the expression of certain mRNAs was decreased as compared to the expression in definitive endoderm. For example, as compared to the expression in definitive endoderm, both VWF and CXCR4 mRNA expression was decreased after contact with Wnt3A in the presence and in the absence of BMP4 as well as after contact with FGF2 at 5 ng/ml in the presence and in the absence of BMP4 (Figures 52K-L). Contact with FGF2 at 100 ng/ml, both in the absence and and in the presence of BMP4, greatly slowed the rate of decrease of these two markers (Figures 52K-L). In fact, expression of CXCR4 was substantially maintained even on day 10 (Figure 52L).

[0368] The methods, compositions, and devices described herein are presently representative of preferred embodiments and are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the disclosure. Accordingly, it will be apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

[0369] As used in the claims below and throughout this disclosure, by the phrase "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

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[0370] Numerous literature and patent references have been cited in the present patent application.

[0371] For some references, the complete citation is in the body of the text. For other references the citation in the body of the text is by author and year, the complete citation being as follows:

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WHAT IS CLAIMED IS:

1. A method of identifying a factor that promotes the differentiation of human definitive endoderm cells, said method comprising the steps of:

obtaining a human cell population comprising human definitive endoderm cells, wherein said human definitive endoderm cells comprise at least 10% of the human cells in said cell population;

providing a candidate differentiation factor to said cell population;

determining expression of a marker of cell differentiated from definitive endoderm in said cell population at a first time point;

determining expression of the same marker in said cell population at a second time point, wherein said second time point is subsequent to said first time point and wherein said second time point is subsequent to providing said cell population with said candidate differentiation factor; and

determining if expression of the marker in said cell population at said second time point is decreased as compared to the expression of the marker in said definitive endoderm cell population at said first time point, wherein a decrease in expression of said marker in said cell population indicates that said candidate differentiation factor promotes the differentiation of said human definitive endoderm cells.

2. The method of claim 1, wherein human feeder cells are present in said cell population and wherein at least 10% of the human cells other than said feeder cells are definitive endoderm cells.

3. The method of claim 1, wherein said human definitive endoderm cells comprise at least 90% of the human cells in said cell population.

4. The method of claim 1, wherein said human feeder cells are present in said cell population and wherein at least 90% of the human cells other than said feeder cells are definitive endoderm cells.

5. The method of claim 1, wherein expression of said marker is determined by quantitative polymerase chain reaction (Q-PCR).

6. The method of claim 1, wherein expression of said marker is determined by immunocytochemistry.

7. The method of claim 1, wherein said candidate differentiation factor comprises a small molecule.

8. The method of claim 1, wherein said candidate differentiation factor comprises a retinoid.

9. The method of claim 1, wherein said candidate differentiation factor comprises retinoic acid.

10. The method of claim 1, wherein said candidate differentiation factor comprises a polypeptide.

11. The method of claim 1, wherein said candidate differentiation factor comprises a growth factor.

12. The method of claim 1, wherein said candidate differentiation factor comprises FGF-10.

13. The method of claim 1, wherein said candidate differentiation factor comprises FGF-2.

14. The method of claim 1, wherein said candidate differentiation factor comprises Wnt3B.

15. The method of claim 1, wherein said candidate differentiation factor is not a foregut differentiation factor.

16. The method of claim 1, wherein said candidate differentiation factor is not a retinoid.

17. The method of claim 1, wherein said candidate differentiation factor is not retinoic acid.

18. The method of claim 1, wherein said candidate differentiation factor is provided to said cell population at a concentration of between 0.1 ng/ml to 10 mg/ml.

19. The method of claim 1, wherein said candidate differentiation factor is provided to said cell population at a concentration of between 1 ng/ml to 1 mg/ml

20. The method of claim 1, wherein said candidate differentiation factor is provided to said cell population at a concentration of between 10 ng/ml to 100 μg/ml.

21. The method of claim 1, wherein said candidate differentiation factor is provided to said cell population at a concentration of between 100 ng/ml to 10 μg/ml.

22. The method of claim 1, wherein said candidate differentiation factor is provided to said cell population at a concentration of 1 μg/ml.

23. The method of claim 1, wherein said candidate differentiation factor is provided to said cell population at a concentration of 100 ng/ml.

SEQUENCE LISTING

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Kevin Allen D'Amour
Alan D. Agulnick
Susan Eliazer
Emmanuel E. Baetge

<120> METHODS OF IDENTIFYING FACTORS FOR DIFFERENTIATING DEFINITIVE ENDODERM

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