markers characteristic of the definitive endoderm lineage is a definitive endoderm cell.

[0062] Markers characteristic of the pancreatic endoderm lineage are selected from the group consisting of PDX1, NKX6.1, HNF1 beta, PTF1 alpha, HNF6, HNF4 alpha, SOX9, HB9 and PROX1. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endoderm lineage. In one aspect of the present invention, a cell expressing markers characteristic of the pancreatic endoderm lineage is a pancreatic endoderm cell.

[0063] Markers characteristic of the pancreatic endocrine lineage are selected from the group consisting of NGN3, NEUROD, ISL1, PDX1, NKX6.1, PAX4 and PTF-1 alpha. In one embodiment, a pancreatic endocrine cell is capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide. Suitable for use in the present invention is a cell that expresses at least one of the markers characteristic of the pancreatic endocrine lineage. In one aspect of the present invention, a cell expressing markers characteristic of the pancreatic endocrine lineage is a pancreatic endocrine cell. The pancreatic endocrine cell may be a pancreatic hormone-expressing cell. Alternatively, the pancreatic endocrine cell may be a pancreatic hormone-secreting cell.

[0064] In one aspect of the present invention, the pancreatic endocrine cell is a cell expressing markers characteristic of the β cell lineage. A cell expressing markers characteristic of the β cell lineage expresses PDX1 and at least one of the following transcription factors: NGN3, NKX2.2, NKX6.1, NEUROD, ISL1, HNF3 beta, MAFA, PAX4, and PAX6. In one aspect of the present invention, a cell expressing markers characteristic of the β cell lineage is a β cell.

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

[0065] In one aspect of the present invention, populations of pluripotent stem cells may be differentiated into populations of cells expressing markers characteristic of the definitive endoderm lineage by culturing the pluripotent stem cells in a medium wherein the concentration of glucose does not exceed 10.5 mM. In one embodiment,

Dulbecco's Medium (IMDM); McCoy's 5A Modified Medium; MCDB Media; Medium 199; Minimum Essential Medium Eagle (EMEM); NCTC Medium; Nutrient Mixture, Ham's F-10; Nutrient Mixture, Ham's F-12; Nutrient Mixture Ham's F-12 Kaighn's Modification (F12K); RPMI-1640; Serum-Free/Protein Free Hybridoma Medium; Waymouth Medium MB; Williams Medium E and various proprietary media. See http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/media-expert/glucose.html.

[0089] The amount of glucose in cell culture formulations varies. While the MCDB media series contain glucose in the range from 3.9 to 10 mM, most media contain from 1 g/L (5.5 mM) to as high as 10 g/L (55 mM) glucose, with RPMI-1640 set at 11 mM glucose. Concentrations of glucose above 10 mM are analogous to a diabetic condition within the cell culture system. This is important because the same processes that can affect cells and molecules *in vivo* can occur *in vitro*. The consequence of growing cells under conditions that are essentially diabetic is that cells and cell products are modified by the processes of glycation and glyoxidation and can be damaged by glucose mediated oxidative and carbonyl stress. See http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/media-expert/glucose.html.

[0090] One medium that is currently used for generating definitive endoderm is Iscove's Modified Dulbecco's Medium (IMDM) which contains 25mM glucose (Kubo *et al*; April 1, 2004, Development 131, 1651-1662), RPMI with 11mM glucose (D'Amour *et al* Nat Biotechnol. 2005 Dec; 23(12):1534-41), or DMEM-F12 with 17.5mM glucose. Each of these media is above the 10 mM glucose concentration analogous to a diabetic condition. Consequently, to reduce stress on the cells that might be induced by high glucose in the culture medium, it was attempted to find a glucose concentration lower than 10mM for differentiation of human embryonic stem cells to cells expressing markers characteristic of the definitive endoderm lineage. One such medium with a glucose concentration below 10mM is MCDB-131 which contains a base glucose concentration of 5.5mM.

[0091] Cells of the human embryonic stem cell line H1 at passage 41 (p41) were lifted by TrypLE (Catalog# 12604-0*13, I*nvitrogen, CA) and seeded as single cells at a density of 100,000 cells/cm^2 on MATRIGEL® coated dishes (1:30 dilution) in MEF-CM

(mouse embryonic fibroblast conditioned media) supplemented with 20 ng/ml of FGF2 (Catalog# 100-18B, PeproTec^{h}, NJ) and 10 μM of Y-27632 (a Rho Kinase Inhibitor, Catalog# Y0503, Sigma, MO). The media was changed 24 and 48 hours post seeding with fresh MEF-CM with 20 ng/ml of FGF2. The cultures were differentiated into cells expressing markers characteristic of the definitive endoderm lineage 72 hrs post as follows:

a. MCDB-131 (Catalog# 10372-019, Invitrogen, CA) medium supplemented with 2% fatty acid-free BSA (Catalog# 68700, Proliant, IA), 0.0025 g/ml sodium bicarbonate (Catalog# S3187, Sigma, MO), 1X GlutaMax™ (Catalog # 35050-079, Invitrogen, Ca) 100 ng/ml activin A (R*&D Sy*stems, MN), 20 ng/ml WNT-3a (Catalog# 1324-WN-^{0}02, R&D Systems, MN), and either 0, 5, 10, 15, 20, or 25mM of glucose (Catalog# G8769, Sigma, MO) for one day, then

b. MCDB-131 medium supplemented with 2% BSA, sodium bicarbonate, Glutamax, 100 ng/ml activin A, and either 0, 5, 10, 15, 20, or 25mM of glucose for an additional three days.

[0092] At day 4, samples were collected for FACS and gene expression analysis using real-time PCR, and counted by ViaCount® (Guava®, Millipore, Billerica, MA). Consistent with results from Example 1, differentiation of pluripotent stem cells to cells expressing markers characteristic of the definitive endoderm lineage resulted in the robust expression of markers associated with the definitive endoderm lineage (Figure 2A). When the glucose concentration in the media was supplemented with 0, 5, 10, 15, 20, or 25 mM glucose (final concentration: 5.5, 10.5, 15.5, 20.5, 25.5, or 30.5 mM glucose respectively), a modest increase in cell number was observed in samples treated with additional 10mM glucose (15.5mM final glucose concentration) as shown in Figure 2B. A modest increase in CXCR4 expression for cells supplemented with additional 5mM glucose (10.5mM final glucose concentration) was also observed, as shown in Figure 2A. However, these increases in cell number and CXCR4 were offset by a reduction in total cell viability (Figure 2B).

[0093] At the basal level of glucose (5.5mM), almost every cell in the culture was SOX17 positive, and the cells were dispersed in the culture dish in a uniform pattern (Figure 3

A&B). As the glucose concentration increased, the cells maintained a high expression of SOX17, however the cells were observed to cluster. These clustered cells were subsequently less evenly dispersed on the culture surface than populations of cells cultured in the basal level of glucose. This effect correlated with a slight increase in expression of CD9 and OCT4- cellular markers for undifferentiated cells, and SOX7- a cellular marker for extraembryonic ectoderm, and a decrease in the gene expression of pancreatic pancreas homeobox 1 (MNX1) also known as Homeobox HB9 (HLXB9) in the clustered cells (Figure 4).

[0094] Similar glucose related effects on differentiation were also observed in cultures differentiated with DMEM containing either 5.5mM (low) or 25mM (high) glucose concentration (Cat#s 10567-014 and 21063-029, Invitrogen, CA). As described above, for controls, cells were seeded as single cells, cultured 3 days in MEF conditioned media and differentiated in MCDB-131 with 5.5mM or 25mM glucose supplemented media, or in DMEM high or low glucose media supplemented with 2% fatty acid free BSA, 100 ng/ml activin A, and 20 ng/ml WNT-3a on the first day, and 2% fatty acid free BSA and 100 ng/ml activin A for the next three days with daily media change.

[0095] Similar to results with MCDB-131 media, where elevated glucose inhibits definitive endoderm formation as compared to low glucose media treated cells, it was observed that a high glucose concentration in DMEM reduced hES cell differentiation. By flow cytometry, following differentiation to definitive endoderm, 88.6% of cells were positive for CXCR4 in media containing 5.5mM glucose versus 80% CXCR4 positive cells in media containing 25mM glucose. Additionally, markers of differentiation to definitive endoderm as measured by qRT-PCR (SOX17) were decreased while markers of undifferentiated cells (OCT4) or alternative differentiation fates (CDX2) were increased (Figure 5) in cells fed media containing high glucose versus those fed low glucose media. This effect was due at least in part to the pH of the media as over the four day differentiation, media pH dropped after 48 hours of differentiation day. Furthermore, the higher the starting and ending pH of culture media (8.1>pH>7.6) (Figure 6) during definitive endoderm formation, the more complete the conversion to definitive endoderm.

g/ml sodium bicarbonate supplemented with 2% BSA, Glutamax, and 100 ng/ml activin A each day for an additional three days.

[0098] At day 4, samples were collected for FACS and gene expression analysis using real-time PCR, and counted by ViaCount® (Guava®, Millipore, Billerica, MA). As shown in Example 2, a relatively more acidic pH of differentiation media (<7.6pH) can reduce CXCR4 expression due to less directed differentiation and increased alternative differentiation.

[0099] In order to test if this effect was due to pH, cells were differentiated in basal MCDB-131 that contains the published concentration of 1gram/liter of sodium bicarbonate and we differentiated cells in media supplemented to the bicarbonate concentration of DMEM, which is 3.7grams/liter. It was observed that differentiation, as measured by the increased expression of the cellular surface markers CXCR4 and decreased expression of CD9, was improved by the use of a buffering agent. MCDB-131 media with 3.7g/l of Sodium Bicarbonate for a buffer had significantly higher CXCR4 expression and lower CD9 expression levels versus cells differentiated in MCDB-131 that contained only the base level of Bicarbonate (1g/l) (Figure 7A and B). This is due in part to the fact that MCDB-131 media has a pH level of 7.5, and addition of 2.7g/l of Sodium Bicarbonate raises the pH to 7.6.

[0100] Furthermore, at the end of differentiation, the media (containing the pH color sensor phenol red) from cultures grown in undifferentiated media were significantly more yellow and acidic than cultures with supplemental sodium bicarbonate buffered media which remained red in color. These results indicate that increasing media pH to 7.6 or higher promotes more efficient definitive endoderm differentiation from pluripotent stem cells, and raising and stabilizing media pH could be achieved by alternatives to bicarbonate buffering including, but not limited to, increasing incubator CO_2 levels and other soluble buffer systems like HEPES, or phosphate.

Example 4

The Role of RPMI-1640 or MCDB-131 Media and the TGF-Beta superfamily members Activin A and GDF-8 in the Differentiation of Human Pluripotent Stem Cells to Cells Expressing Markers Characteristic of the Definitive Endoderm Lineage

Invitrogen, Ca) and 100 ng/ml GDF-8 (R&D Systems, MN) for four days, or,

d. RPMI-1640 (Catalog #22400-105, Invitrogen, CA), was supplemented with 2% fatty acid-free BSA (Catalog# 68700, Proliant, IA), and 100 ng/ml activin A (R&D Systems, MN) plus 20 ng/ml WNT-3a (Catalog# 1324-WN-002, R&D Systems, MN), for one day, then RPMI-1640 medium supplemented with 2% BSA, and 100 ng/ml activin A each day for an additional three days.

e. RPMI-1640 (Catalog #22400-105, Invitrogen, CA), was supplemented with 2% fatty acid-free BSA (Catalog# 68700, Proliant, IA), and 100 ng/ml GDF-8 (R&D Systems, MN) plus 2.5μM of the GSK3B inhibitor 14-Prop-2-en-1-yl-3,5,7,14,17,23,27-heptaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacosa-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one for one day, then RPMI-1640 medium supplemented with 2% BSA, and 100 ng/ml GDF-8 each day for an additional three days.

[0103] At day 4, samples were collected for FACS analysis and qRT-PCR. In Table 2, the percentage of cells expressing CXCR4, CD9, and CD99 (an additional marker of differentiation) are summarized in Table 2. Differentiation, as measured by the increased expression of the cellular surface marker CXCR4 was improved by the use of MCDB-131 media compared to RPMI-1640, and expression of CXCR4 was further increased by using GDF-8 in combination with GSK3B inhibitor ("MCX"), compared to cells treated with activin A and Wnt3a. Similar results, showing improved differentiation with the use of MCDB-131 media compared to RPMI-1640, and by using GDF-8 in combination with a GSK3B inhibitor compared to cells treated with activin A and Wnt3a were observed by qRT-PCR for the gene MNX-1 (Figure 8). Furthermore, these data correlated with decreased expression of CD9, a cellular marker for undifferentiated cells, as measured by flow cytometry (Table 2) or OCT4 and CD9, as measured by qRT-PCR (Figure 8). These data indicate that H1 human embryonic stem cells differentiate more efficiently in the presence of MCDB-131 medium than RPMI-1640 medium, and that differentiation in MCBD-131 can be

further improved by differentiating the cells in the presence of GDF-8 and a GSK3B inhibitor versus differentiation with activin A and Wnt3a.

Table 2.

Media Treatment	CD184	CD9	CD99
RPMI+AA+Wnt	77.8	20.9	77.8
RPMI+GDF8+GSK3B inhibitor	81.6	13.8	83.4
MCDB131+AA+Wnt	81.2	21.1	60.0
MCDB131+GDF8+ GSK3B inhibitor	87.1	14.3	50.9
MCDB131+GDF8	43.2	31.2	23.7

[0104] Publications cited throughout this document are hereby incorporated by reference in their entirety. Although the various aspects of the invention have been illustrated above by reference to examples and preferred embodiments, it will be appreciated that the scope of the invention is defined not by the foregoing description but by the following claims properly construed under principles of patent law.

Claims

1. An isolated population of cells obtained by differentiating pluripotent stem cells in medium, wherein the glucose concentration in the medium does not exceed 10.5 mM, wherein greater than 80% of the cells in the population express markers characteristic of the definitive endoderm lineage, wherein the cells are obtained by differentiating pluripotent stem cells in serum-free medium in which the glucose concentration does not exceed 10.5 mM.

2. The isolated population of cells of claim 1, wherein the cells expressing markers characteristic of the definitive endoderm lineage are definitive endoderm cells.

3. The isolated population of cells of claim 2, wherein the definitive endoderm cells express at least two of the following markers: CXCR4, CD9 or CD99.

4. The isolated population of cells of claim 1, wherein the cells are obtained by differentiating pluripotent stem cells seeded as clusters in serum-free medium in which the glucose concentration does not exceed 10.5 mM.

5. The isolated population of cells of claims 1, 2 or 4, wherein the cells are obtained by differentiating the pluripotent stem cells in a medium containing activin A and a Wnt ligand.

6. The isolated population of cells of claims 1, 2 or 4, wherein the cells are obtained by differentiating pluripotent stem cells in a medium containing GDF-8 and at least one other factor is selected from the group consisting of: an aniline-pyridinotriazine, a cyclic aniline-pyridinotriazine, N-{[1-(Phenylmethyl)azepan-4-yl]methyl}-2-pyridin-3-ylacetamide, 4-{[4-(4-{[2-(Pyridin-2-ylamino)ethyl]amino}-1,3,5-triazin-2-yl)pyridin-2-yl]oxy}butan-1-ol, 3-({3-[4-({2-[Methyl(pyridin-2-yl)amino]ethyl}amino)-1,3,5-triazin-2-yl]pyridin-2-yl}amino)propan-1-ol, N~4~-[2-(3-Fluorophenyl)ethyl]-N~2~-[3-(4-methylpiperazin-1-yl)propyl]pyrido[2,3-d]pyrimidine-2,4-diamine, 1-Methyl-N-[(4-pyridin-3-yl-2-{[3-(trifluoromethyl)phenyl]amino}-1,3-thiazol-5-yl)methyl]piperidine-4-carboxamide, 1,1-Dimethylethyl {2-[4-({5-[3-(3-hydroxypropyl)phenyl]-4H-1,2,4-triazol-3-yl}amino)phenyl]ethyl}carbamate, 1,1-Dimethylethyl {[3-({5-[5-(3-hydroxypropyl)-2-(methyloxy)phenyl]-1,3-oxazol-2-yl}amino)phenyl]methyl}carbamate, 1-({5-[6-({4-[(4-Methylpiperazin-1-yl)sulfonyl]phenyl}amino)pyrazin-2-yl]thiophen-2-yl}methyl)piperidin-4-ol, 1-({4-[6-({4-[(4-

Methylpiperazin-1-yl)sulfonyl]phenyl}amino)pyrazin-2-yl]thiophen-2-yl}methyl)piperidine-4-carboxamide, and 2-{[4-(1-Methylethyl)phenyl]amino}-N-(2-thiophen-2-ylethyl)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxamide.

7. The isolated population of cells of claim 6, wherein the at least one other factor is 14-Prop-2-en-1-yl-3,5,7,14,17,23,27-heptaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacosa-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one.

8. The isolated population of cells of any one of claims 1 to 7, wherein the population is obtained differentiating pluripotent stem cells in medium, wherein the glucose concentration in the medium does not exceed 5.5 mM.

9. A cell culture containing the population of cells of any one of claims 1 to 8.

10. A method for generating a population of cells wherein greater than 80% of the cells in the population express markers characteristic of the definitive endoderm lineage comprising differentiating a population of pluripotent stem cells to a population of cells wherein greater than 80% of the cells in the population express markers characteristic of the definitive endoderm lineage in medium containing activin A and a Wnt ligand, wherein the concentration of glucose does not exceed 10.5 mM.

11. A method for generating a population of cells wherein greater than 80% of the cells in the population express markers characteristic of the definitive endoderm lineage comprising differentiating a population of pluripotent stem cells to a population of cells wherein greater than 80% of the cells in the population express markers characteristic of the definitive endoderm lineage in medium containing GPF-8 and at least one other factor, wherein the concentration of glucose does not exceed 10.5 mM, and wherein the at least one other factor is selected from the group consisting of: an aniline-pyridinotriazine, a cyclic aniline-pyridinotriazine, N-{[1-(Phenylmethyl)azepan-4-yl]methyl}-2-pyridin-3-ylacetamide, 4-{[4-(4-{[2-(Pyridin-2-ylamino)ethyl]amino}-1,3,5-triazin-2-yl)pyridin-2-yl]oxy}butan-1-ol, 3-({3-[4-({2-[Methyl(pyridin-2-yl)amino]ethyl}amino)-1,3,5-triazin-2-yl]pyridin-2-yl}amino)propan-1-ol, N~4~-[2-(3-Fluorophenyl)ethyl]-N~2~-[3-(4-methylpiperazin-1-yl)propyl]pyrido[2,3-d]pyrimidine-2,4-diamine, 1-Methyl-N-[(4-pyridin-3-yl-2-{[3-(trifluoromethyl)phenyl]amino}-

1,3-thiazol-5-yl)methyl]piperidine-4-carboxamide, 1,1-Dimethylethyl {2-[4-({5-[3-(3-hydroxypropyl)phenyl]-4H-1,2,4-triazol-3-yl}amino)phenyl]ethyl}carbamate, 1,1-Dimethylethyl {[3-({5-[5-(3-hydroxypropyl)-2-(methyloxy)phenyl]-1,3-oxazol-2-yl}amino)phenyl]methyl}carbamate, 1-({5-[6-({4-[(4-Methylpiperazin-1-yl)sulfonyl]phenyl}amino)pyrazin-2-yl]thiophen-2-yl}methyl)piperidin-4-ol, 1-({4-[6-({4-[(4-Methylpiperazin-1-yl)sulfonyl]phenyl}amino)pyrazin-2-yl]thiophen-2-yl}methyl)piperidine-4-carboxamide, and 2-{[4-(1-Methylethyl)phenyl]amino}-N-(2-thiophen-2-ylethyl)-7,8-dihydropyrido[4,3-d]pyrimidine-6(5H)-carboxamide.

12. The method of claim 11, wherein the at least one other factor is 14-Prop-2-en-1-yl-3,5,7,14,17,23,27-heptaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacosa-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one.

13. The method of any of claims 10 to 12, wherein the cells expressing markers characteristic of the definitive endoderm lineage are definitive endoderm cells.

14. A method of differentiating pluripotent stem cells into definitive endoderm cells comprising culturing pluripotent stem cells in a medium supplemented with (1) activin A and a Wnt ligand or (2) GDF-8 and 14-Prop-2-en-1-yl-3,5,7,14,17,23,27-heptaazatetracyclo[19.3.1.1~2,6~.1~8,12~]heptacosa-1(25),2(27),3,5,8(26),9,11,21,23-nonaen-16-one, wherein the concentration of glucose does not exceed 10.5 mM.

15. The method of any one of claims 10 to 14, wherein the concentration of glucose does not exceed 5.5 mM.

16. The method of any one of claims 10 to 15, wherein the method further comprises culturing the pluripotent stem cells prior to differentiation.

17. An isolated cell population obtained by the method of any of claims 10 to 16.