SUSPENSION AND CLUSTERING OF HUMAN PLURIPOTENT STEM CELLS FOR DIFFERENTIATION INTO PANCREATIC ENDOCRINE CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[01] This application is a continuation-in-part of U.S. Application No. 13/998,974 (filed December 30, 2013), which claims priority to U.S. Provisional Application 61/747,799 (filed on December 31, 2012) and U.S. Provisional Application 61/962,158 (filed on November 1, 2013), which are incorporated by reference in their entireties.

FIELD OF THE INVENTION

[02] The present invention is in the field of cell differentiation including preparing embryonic stem cells and other pluripotent cells that maintain pluripotency in aggregated cell cluster for differentiation to endoderm progenitor cells, pancreatic endocrine cells, mesoderm cells or ectoderm cells. In one aspect, the invention discloses a method of generating clusters of pluripotent stem cells and maintaining them in suspension culture for differentiation to pancreatic endoderm, pancreatic endocrine precursor cells, and single-hormone pancreatic endocrine cells.

BACKGROUND

[03] Advances in cell-replacement therapy for Type I diabetes mellitus and a shortage of transplantable islets of Langerhans have focused interest on developing sources of insulin-producing cells, or β cells, appropriate for engraftment. One approach is the generation of functional β cells from pluripotent stem cells, such as, embryonic stem cells.

[04] In vertebrate embryonic development, a pluripotent cell gives rise to a group of cells comprising three germ layers (ectoderm, mesoderm, and endoderm) in a process known as gastrulation. Tissues such as, thyroid, thymus, pancreas, gut, and liver, will develop from the endoderm, via an intermediate stage. The intermediate stage in this process is the formation of definitive endoderm.

[05] By the end of gastrulation, the endoderm is partitioned into anterior-posterior domains that can be recognized by the expression of a panel of factors that uniquely mark anterior, mid, and posterior regions of the endoderm. For example, HHEX, and SOX2

identify the anterior region while CDX1, 2, and 4 identify the posterior region of the endoderm.

[06] Migration of endoderm tissue brings the endoderm into close proximity with different mesodermal tissues that help in regionalization of the gut tube. This is accomplished by a plethora of secreted factors, such as FGFs, Wnts, TGF-ßs, retinoic acid ("RA"), and BMP ligands and their antagonists. For example, FGF4 and BMP are reported to promote CDX2 expression in the presumptive hindgut endoderm and repress expression of the anterior genes HHEX and SOX2 (2000 *Development*, 127:1563–1567). WNT signaling has also been shown to work in parallel to FGF signaling to promote hindgut development and inhibit foregut fate (2007 *Development*, 134:2207–2217). Lastly, secreted retinoic acid by mesenchyme regulates the foregut-hindgut boundary (2002 *Curr Biol*, 12:1215–1220).

[07] The level of expression of specific transcription factors may be used to designate the identity of a tissue. During transformation of the definitive endoderm into a primitive gut tube, the gut tube becomes regionalized into broad domains that can be observed at the molecular level by restricted gene expression patterns. For example, the regionalized pancreas domain in the gut tube shows a very high expression of PDX1 and very low expression of CDX2 and SOX2. PDX1, NKX6.1, PTF1A, and NKX2.2 are highly expressed in pancreatic tissue; and expression of CDX2 is high in intestine tissue.

[08] Formation of the pancreas arises from the differentiation of definitive endoderm into pancreatic endoderm. Dorsal and ventral pancreatic domains arise from the foregut epithelium. Foregut also gives rise to the esophagus, trachea, lungs, thyroid, stomach, liver, pancreas, and bile duct system.

[09] Cells of the pancreatic endoderm express the pancreatic-duodenal homeobox gene PDX1. In the absence of PDX1, the pancreas fails to develop beyond the formation of ventral and dorsal buds. Thus, PDX1 expression marks a critical step in pancreatic organogenesis. The mature pancreas contains both, exocrine tissue and endocrine tissue arising from the differentiation of pancreatic endoderm.

[010] D'Amour *et al.* describes the production of enriched cultures of human embryonic stem cell-derived definitive endoderm in the presence of a high concentration of activin and low serum (*Nature Biotechnol* 2005, 23:1534-1541; U.S. Patent No. 7,704,738). Transplanting these cells under the kidney capsule of mice reportedly resulted in differentiation into more mature cells with characteristics of endodermal tissue (U.S. Patent No. 7,704,738). Human embryonic stem cell derived definitive endoderm cells can be further differentiated into PDX1 positive cells after addition of FGF10 and retinoic acid (U.S. Patent

App. Pub. No. 2005/0266554A1). Subsequent transplantation of these pancreatic precursor cells in the fat pad of immune deficient mice resulted in the formation of functional pancreatic endocrine cells following a 3-4 month maturation phase (U.S. Patent No. 7,993,920 and U.S. Patent No. 7,534,608).

[011] Fisk *et al.* report a system for producing pancreatic islet cells from human embryonic stem cells (U.S. Patent No. 7,033,831). Small molecule inhibitors have also been used for induction of pancreatic endocrine precursor cells. For example, small molecule inhibitors of TGF-ß receptor and BMP receptors (*Development* 2011, 138:861-871; *Diabetes* 2011, 60:239-247) have been used to significantly enhance the number of pancreatic endocrine cells. In addition, small molecule activators have also been used to generate definitive endoderm cells or pancreatic precursor cells (*Curr Opin Cell Biol* 2009, 21:727-732; *Nature Chem Biol* 2009, 5:258-265).

[012] Great strides have been made in improving protocols for culturing progenitor cells such as pluripotent stem cells. PCT Publication No. WO2007/026353 (Amit *et al.*) discloses maintaining human embryonic stem cells in an undifferentiated state in a two-dimensional culture system. Ludwig *et al.*, 2006 (*Nature Biotechnology*, 24: 185-7) discloses a TeSR1 defined medium for culturing human embryonic stem cells on a matrix. U.S. Patent App. Pub. No. 2007/0155013 (Akaike *et al.*) discloses a method of growing pluripotent stem cells in suspension using a carrier that adheres to the pluripotent stem cells, and U.S. Patent App. Pub. No. 2009/0029462 (Beardsley *et al.*) discloses methods of expanding pluripotent stem cells in suspension using microcarriers or cell encapsulation. PCT Publication No. WO 2008/015682 (Amit *et al.*) discloses a method of expanding and maintaining human embryonic stem cells in a suspension culture under culturing conditions devoid of substrate adherence.

[013] U.S. Patent App. Pub. No. 2008/0159994 (Mantalaris *et al.*) discloses a method of culturing human embryonic stem cells encapsulated within alginate beads in a three-dimensional culture system.

[014] Despite these advances, a need still remains for a method to successfully culture pluripotent stem cells in a three-dimensional culture system that may differentiate to functional endocrine cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[015] The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended figures. For the purpose of illustrating the invention, the figures demonstrate embodiments of the present invention. It should be understood, however, that the invention is not limited to the precise arrangements, examples, and instrumentalities shown.

[016] Figure 1a shows micrographs of Dispase®-treated cells of the human embryonic stem ("hES") cell line H1 immediately after lifting (left hand panel) and after 24 hours in non-adherent static culture (right hand panel) according to Example 1. The cells after lifting (left hand panel) resembled fragments of monolayer with an average fragment diameter of about 20-30 microns each fragment consisting of clumps of cells. After 24 hours in non-adherent static culture, the cells assumed a cluster-like configuration.

[017] Figure 1b shows the results of flow cytometry for CD9, SSEA4, CXCR4, TRA-1-60 and TRA-1-81 for the Dispase®-treated cells of the human embryonic stem cell line H1 after culturing for 4 days in a 125 mL spinner flask containing 25 mL mTeSR®1 media according to Example 1. The cells exhibited high expression for markers of pluripotency (CD9, SSEA4, TRA-1-60 and TRA-1-81) with almost no expression of CXCR4, a marker for differentiation.

[018] Figure 1c shows micrographs of the Dispase®-treated cells of the human embryonic stem cell line H1 after 72 and 96 hours of differentiation at the end of stage 1. Visible in Figure 1c are loose cell aggregates after 72 hours at 4X magnification (left hand panel), 96 hours at 4X magnification (center panel) and 96 hours at 10X magnification (right hand panel).

[019] Figure 1d shows flow cytometry results for the Dispase®-treated cells of the human embryonic stem cell line H1 at the end of stage 1 differentiation for the markers CD9, CD184 (CXCR4) and CD99 (*see* Example 1). As shown in Figure 1d, expression of CD9, a marker for pluripotency, was nearly eliminated, while the expression of markers of definitive endoderm differentiation CXCR4 (CD184) and CD99 were quite high.

[020] Figure 1e shows quantitative reverse transcription polymerase chain reaction (qRT-PCR) results for expression of selected genes associated with pluripotency and genes associated with definitive endoderm for the Dispase®-treated cells of the human embryonic stem cell line H1 at the end of stage 1 compared to undifferentiated H1 (WA01) hES cells (*see* Example 1). The cells at the end of stage 1 showed a dramatic decrease in the

expression of pluripotency genes (CD9, NANOG, and POU5F1/OCT4) and a large increase in genes associated with definitive endoderm (CXCR4, CERBERUS (CER1), GSC, FOXA2, GATA4, GATA6, MNX1, and SOX17) versus undifferentiated WA01 hES cells.

[021] Figure 1f shows micrographs of the Dispase®-treated cells of the human embryonic stem cell line H1 as the cells further differentiated from definitive endoderm toward the pancreatic endoderm (*see* Example 1). Clear morphological changes to cells and cell clusters are visible as differentiation progresses from stage 2, day 1 (top left hand panel) to stage 2, day 3 (top right hand panel) to stage 3, day 4 (lower left hand panel) and stage 4, day 1 (lower right hand panel).

[022] Figure 2a shows flow cytometry data for EDTA-treated cells of the human embryonic stem cell line H1 after 2 days of culture in stirred suspension culture post-EDTA treatment, and prior to transition to differentiation culture, for markers associated with pluripotency and differentiation according to Example 2. The data showed high expression for the markers of pluripotency (CD9, SSEA4, TRA-1-60, and TRA-1-81) with almost no expression of a marker for differentiation (CXCR4).

[023] Figure 2b shows micrographs of the EDTA-treated cells of the human embryonic stem cell line H1 differentiated into stage 1, day 3 cells grown in spinner flask and stage 2 day 2, stage 4 day 1 and stage 4 day 3 cells grown in spinner flasks or Erlenmeyer flasks according to Example 2. Suspension differentiated cultures formed substantially uniform and homogenous populations of cells in spherical aggregates.

[024] Figure 2c shows flow cytometry data for the EDTA-treated cells of the human embryonic stem cell line H1 at the end of stage 1 for cell surface markers of pluripotency and endoderm differentiation. As visible in Figure 2c, expression of CD9, a marker for pluripotency, was nearly eliminated while expression for CXCR4 (CD184), a marker for definitive endoderm differentiation was quite high.

[025] Figure 2d shows qRT-PCR results for expression of selected genes associated with pluripotency and genes associated with definitive endoderm for the EDTA-treated cells of the human embryonic stem cell line H1 at the end of stage 1 compared to undifferentiated H1 (WA01) hES cells (*see* Example 2). Figure 2d shows a decrease in the expression of pluripotency genes (CD9, Nanog, and POU5F1/OCT4) and a large increase in genes associated with definitive endoderm (CXCR4, CERBERUS ("CER1"), FOXA2, GATA4, GATA6, MNX1, and SOX17).

[026] Figure 2e shows flow cytometry data for markers indicative of differentiation (NKX6.1, CDX2, SOX2, and Chromagranin) for the EDTA-treated cells of the human

embryonic stem cell line H1 which were differentiated from stage 1 to pancreatic endoderm cells by suspension in spinner flasks or Erlenmeyer flasks according to Example 2. The flow cytometry data shows high levels of NKX6.1, a transcription factor required for functional β cells, and high levels of endocrine pancreas markers such as synaptophysin (data not shown) and chromogranin with both suspension formats.

[027] Figure 2f shows qRT-PCR results for expression of selected genes associated with differentiation for the EDTA-treated cells of the human embryonic stem cell line H1 which were further differentiated from stage 1 to pancreatic endoderm cells by suspension in spinner flasks or Erlenmeyer flasks according to Example 2. The data is compared to expression in WA01 hES cells. The RT-PCR results show high levels of expression of pancreatic precursor genes.

[028] Figure 3a shows a micrograph of cells of the human embryonic stem cell line H1, which had been lifted from a static culture following treatment with Accutase®. As shown in Figure 3a, the cells were removed from the surface as small aggregates.

[029] Figure 3b shows phase contrast micrographs of cells of the human embryonic stem cell line H1, which had been lifted from a static culture following treatment with Accutase® and which were then expanded in suspension culture for three days. Visible in Figure 3b is the formation of a substantially uniform, spherical population of cell clusters.

[030] Figure 3c shows a micrograph of clusters of cells of the human embryonic stem cell line H1, which had been lifted from a static culture following treatment with Accutase®, which were then expanded in suspension culture for three days, and which were then serially passaged using Accutase® dissociation.

[031] Figure 4a shows micrographs of suspension cultured human embryonic stem cells of the cell line H1 using a directed differentiation protocol at different stages of differentiation. Visible in Figure 4a are micrographs of the cells at each stage of differentiation.

[032] Figure 4b shows the results of flow cytometry for markers of differentiation (CXCR4, CD56 and PDX1) for suspension cultured human embryonic stem cells of the cell line H1 using a directed differentiation protocol at different stages of differentiation (hours after beginning differentiation). At the end of the differentiation process on day 4 of stage 4, a high percentage of the cells were positive for PDX1 expression.

[033] Figure 4c shows the non-fasting blood glucose levels of SCID-Bg Mice transplanted with differentiated cells encapsulated in a TheraCyte™ device.

[034] Figure 5a shows flow cytometry data for the EDTA-treated cells of the human embryonic stem cell line H1 prior to transition to differentiation culture for markers

associated with pluripotency and differentiation. As shown in Figure 5a, high expression of the pluripotency markers CD9, SSEA4, TRA-1-60 and TRA-1-80 was observed.

[035] Figure 5b shows phase contrast images of the cells and flow cytometry data for CXCR4/CD184 and CD99 (markers of differentiation) and CD9 (a pluripotency marker) for three different feed settings during stage 1. The conditions tested were as follows: (A) media change 24 hours after initiation of differentiation, no media change at 48 hours; (B) media change 24 hours after initiation of differentiation and glucose bolus addition at 48 hours; and (C) no media change throughout stage 1 with glucose and GDF8 bolus added 24 hours after initiation of differentiation, then a glucose bolus added at 48 hours post initiation.

[036] Figure 5c shows phase contrast images of the differentiated cells exhibiting pancreatic endoderm morphology, which were differentiated using the following feed settings during the formation of definitive endoderm: (A) media change 24 hours after initiation of differentiation, no media change at 48 hours; (B) media change 24 hours after initiation of differentiation and glucose bolus addition at 48 hours; and (C) no media change throughout stage 1 with glucose and GDF8 bolus added 24 hours after initiation of differentiation, then a glucose bolus added at 48 hours post initiation.

[037] Figure 5d shows the results of flow cytometry for select markers of pancreatic gene expression (NKX6.1 and chromogranin) and select non-pancreatic genes (CDX2 and SOX2) for differentiated cell as the end of stage 4, which were differentiated using the following feed settings during formation of definitive endoderm: (A) media change 24 hours after initiation of differentiation, no media change at 48 hours; (B) media change 24 hours after initiation of differentiation and glucose bolus addition at 48 hours; and (C) no media change throughout stage 1 with glucose and GDF8 bolus added 24 hours after initiation of differentiation, then a glucose bolus added at 48 hours post initiation.

[038] Figure 5e shows qRT-PCR results for select pancreatic and non-pancreatic gene expression for differentiated cells as the end of stage 4, which were differentiated using the following feed settings during formation of definitive endoderm: (A) media change 24 hours after initiation of differentiation, no media change at 48 hours; (B) media change 24 hours after initiation of differentiation and glucose bolus addition at 48 hours; and (C) no media change throughout stage 1 with glucose and GDF8 bolus added 24 hours after initiation of differentiation, then a glucose bolus added at 48 hours post initiation. The data are shown as fold difference in expression versus undifferentiated H1 (WA01) hES cells (baseline expression of 1).

[039] Figure 5f shows the expression of C-peptide in SCID-Bg mice that were implanted with cells differentiated according to condition A (media change 24 hours after initiation of differentiation, no media change at 48 hours). Each SCID-Bg mouse was implanted with 5 million of the cells under the kidney capsule. As shown in Figure 5f, by 12 weeks post implantation, human c-peptide was detectable at levels above 1ng/mL, and at 16 weeks c-peptide levels were an average of 2.5ng/mL.

[040] Figure 5g shows the effect of glucose treatment for selected SCID-Bg mice pre- and post-administration (*e.g.* implantation) of cells differentiated according to condition A (media change 24 hours after initiation of differentiation, no media change at 48 hours). As shown in Figure 5g, glucose treatment induced a significant increase in circulating human c-peptide from an average of 0.93ng/mL in a fasted state to 2.39ng/mL in a fed state.

[041] Figure 5h shows the effect of streptozotocin (STZ) administration (*i.e.* STZ-induced diabetes) on SCID-Bg mice that had been administered cells differentiated according to condition A (media change 24 hours after initiation of differentiation, no media change at 48 hours). As evident from Figure 5h, animals with a graft of functional GSIS competent tissue (*i.e.* those that had been administered the cells) maintained normal blood glucose levels unlike the untreated controls which developed frank diabetes.

[042] Figure 6a shows micrographs of cells of the human embryonic stem cell line H1 grown on Cytodex® 3 microcarrier beads prior to differentiation.

[043] Figure 6b shows micrographs of cells of the human embryonic stem cell line H1 grown on Cytodex® 3 microcarrier beads at various stages of differentiation.

[044] Figure 6c shows the cell count (cells/cm^2) as a function of days of differentiation for cells of the human embryonic stem cell line H1 grown and differentiated on plates in media containing Activin A (AA) and WNT3A (WTN3A/AA plate), microcarriers in media containing Activin A and WNT3A (WTN3A/AA microcarriers), plates in media containing MCX and GDF8 (MCX/GDF8 plate) and microcarriers in media containing MCX and GDF8 (MCX/GDF8 microcarriers).

[045] Figure 6d shows the cell count (cells/ml) as a function of days of differentiation for cells of the human embryonic stem cell line H1 grown and differentiated on plates in media containing Activin A and WNT3A (WTN3A/AA plate), microcarriers in media containing Activin A and WNT3A (WTN3A/AA microcarrier), plates in media containing MCX and GDF8 (MCX/GDF8 plate) and microcarriers in media containing MCX and GDF8 (MCX/GDF8 microcarriers).

[046] Figure 6e shows flow cytometry results for the first stage of differentiation of cells grown on a microcarrier culture or planar culture in the presence of: (a) WNT3A and AA; or (2) MCX and GDF8 as a dot plot of cell expression of CXCR4/CD184 (Y-axis) and CD9 (X-axis).

[047] Figure 6f shows flow cytometry results for the first stage of differentiation of cells grown on a microcarrier culture or planar culture in the presence of: (a) WNT3A and AA; or (2) MCX and GDF8 as total expression of each of the markers (CXCR4 and CD9).

[048] Figure 6g shows qRT-PCR results for expression of selected genes associated with differentiation for cells of the human embryonic stem cell line H1, which were differentiated by growth on planar culture or on microcarrier beads in suspension culture in the presence of: (a) WNT3A and AA; or (2) MCX and GDF8.

[049] Figure 7 shows the cell counts at various stages of differentiation in a Bioreactor from stage 1, day 1 to stage 4, day 3 for cells differentiated according to the protocol of Example 7. Cell counts are shown as million cells/ml as determined by an image-based cytometer (NucleoCounter®).

[050] Figure 8 shows the average daily bioreactor medium pH levels as a function of time (days of differentiation) during the differentiation protocol of Example 7. pH levels were determined by a NOVA BioProfile® FLEX (Nova Biomedical Corporation, Waltham, MA).

[051] Figure 9 shows the average daily bioreactor medium lactate levels as a function of time (days of differentiation) during the differentiation protocol of Example 7. Lactate levels were determined by a NOVA BioProfile® FLEX (Nova Biomedical Corporation, Waltham, MA).

[052] Figure 10 shows the average daily bioreactor medium glucose levels as a function of time (days of differentiation) during the differentiation protocol of Example 7. Glucose levels were determined by a NOVA BioProfile® FLEX (Nova Biomedical Corporation, Waltham, MA).

[053] Figure 11 shows the undifferentiated gene expression, as determined by qRT-PCR, for stage 0, day 1 (*i.e.* twenty-four hours after inoculation) cells differentiated according to the protocol of Example 7 for the pluripotency array, which contains select genes associated with pluripotency.

[054] Figure 12 shows the undifferentiated gene expression, as determined by qRT-PCR, for stage 0, day 1 (*i.e.* twenty-four hours after inoculation) cells for the definitive endoderm ("DE") array, which contains select genes associated with definitive endoderm (*see* Example 7).

[055] Figure 13 shows the undifferentiated gene expression, as determined by qRT-PCR, for stage 0, day 3 (*i.e.* seventy-two hours after inoculation) cells for the pluripotency array, which contains select genes associated with pluripotency (*see* Example 7).

[056] Figure 14 shows the undifferentiated gene expression, as determined by qRT-PCR, for stage 0, day 3 (*i.e.* seventy-two hours after inoculation) cells for the DE array, which contains select genes associated with DE (*see* Example 7).

[057] Figure 15 shows the results of fluorescence-activated cell sorting (FACS) for CD9, CD184/CXCR4, SSEA4, TRA-1-60 and TRA-1-81 for undifferentiated stage 0, day 3 (*i.e.* seventy-two hours after inoculation) cells (*see* Example 7). The results are also shown in Table 8.

[058] Figure 16 shows the undifferentiated gene expression, as determined by qRT-PCR, for select genes of stage 0, day 1 (*i.e.* twenty-four hours after inoculation) and stage 0, day 3 (*i.e.* seventy-two hours after inoculation) cells differentiated according to the protocol of Example 7. Specifically, Figure 16 shows a modest increase in gene expression for GATA4, GSC, MIXL1, and T and a ≥100x increase in GATA2 expression during the stage 0 process prior to directed differentiation.

[059] Figure 17 shows the undifferentiated gene expression, as determined by qRT-PCR, for the DE array, which contains select genes associated with DE, for stage 0, day 1 (*i.e.* twenty-four hours after inoculation) and stage 0, day 3 (*i.e.* seventy-two hours after inoculation) cells differentiated according to the protocol of Example 7. Specifically, Figure 17 shows a ≥100x increase in CER1, FGF17, and FGF4 expression during the stage 0 process prior to directed differentiation.

[060] Figures 18 and 19 show the gene expression for stage 1, day 1 cells differentiated according to the protocol of Example 7. Figure 18 shows the gene expression, as determined by qRT-PCR, for the pluripotency array, which contains select genes associated with pluripotency, for stage 1, day 1 cells. Figure 19 shows the gene expression, as determined by qRT-PCR, for the DE array, which contains select genes associated with DE, for stage 1, day 1 cells. Figures 18 and 19 illustrate significant alterations in gene expression patterns such as a ~700x increase in FOXA2 expression and a 1000x increase in CER1, EOMES, FGF17, FGF4, GATA4, GATA6, GSC, MIXL1, and T expression.

[061] Figures 20 and 21 show the gene expression for stage 1, day 3 cells differentiated according to the protocol of Example 7. Figure 20 shows the gene expression, as determined by qRT-PCR, for the pluripotency array, which contains select genes associated with pluripotency, for stage 1, day 3 cells. Figure 21 shows the gene expression, as determined by

qRT-PCR, for the DE array, which contains select genes associated with DE, for stage 1, day 3 cells.

[062] Figure 22 shows the results of FACS for CD9, CD184 (also known as CXCR4) and CD99 for stage 1, day 3 cells differentiated according to the protocol of Example 7. A near complete transition from a CD9 expressing/CXCR4 negative pluripotent cell population at the initiation of differentiation (Figure 15) to a homogeneous population of CXCR4 expressing cells (98.3% of cells CXCR4 positive, ± 1.9SD) at the end of stage 1 (Figure 22) was observed.

[063] Figure 23 shows the gene expression, as determined by qRT-PCR, for the DE array, which contains select genes associated with DE, for stage 1, day 3; stage 2, day 1; and stage 2, day 3 cells differentiated according to the protocol of Example 7. Figure 23 shows that HNF4α and GATA6 expression levels at stage 2 days 1 and 3 increased, while genes expressed at high levels on day 3 of stage 1 (CXCR4, EOMES, FGF17, FGF4, MNX1, PRDM1, SOX17, and VWF) showed reduced expression by the end of stage 2.

[064] Figure 24 shows the gene expression of the foregut genes AFP, PDX1, and PROX1, as determined by qRT-PCR, for stage 2, day 1 cells and stage 2, day 3 cells differentiated according to the protocol of Example 7. As shown in Figure 24, the expression of these genes increased.

[065] Figure 25 shows the results of FACS for PDX1, FOXA2, chromogranin, NKX2.2 and SOX2 for stage 3, day 3 cells grown in stage 3 medium (Table 7) differentiated according to the protocol of Example 7. As shown in Figure 25, the cells expressed markers consistent with an endodermal pancreatic lineage as measured by PDX1 and FOXA2 expression (90.9% ± 11.9SD PDX1 positive and 99.2% ± 0.6SD FOXA2 positive).

[066] Figure 26 shows the gene expression, as determined by qRT-PCR, for the stage 4 array, which contains select genes associated with stage 4, for stage 3, day 1 and stage 3, day 3 cells differentiated according to the protocol of Example 7. Figure 26 illustrates that these cells exhibit increased levels of a host of genes commonly expressed in the pancreas (ARX, GAST, GCG, INS, ISL1, NEUROD1, NGN3, NKX2.2, NKX6.1, PAX4, PAX6, PTF1A, and SST).

[067] Figure 27 shows the results of FACS for NKX6.1, chromagranin (CHGA), CDX2, SOX2, NKX2.2, PDX1, FOXA2 and NEUROD for stage 4, day 3 cells differentiated according to the protocol of Example 7. As shown in Figure 27, stage 4 day 3 the cells retained high levels of PDX1 and FOXA2 expression and further developed an expression

pattern consistent with a mix of pancreatic endocrine cells (28.1% ± 12.5SD chromogranin positive) and pancreatic progenitor cells (58.3% ± 9.7SD positive for NKX6.1).

[068] Figure 28 shows the gene expression, as determined by qRT-PCR, for the stage 4 array, which contains select genes associated with stage 4, for stage 3, day 3; stage 4, day 1 and stage 4, day 3 cells differentiated according to the protocol of Example 7. Figure 28 shows an increased expression level of genes commonly expressed in the pancreas (ARX, GAST, GCG, IAPP, INS, ISL1, MAFB, NEUROD1, NGN3, NKX2.2, NKX6.1, PAX4, PAX6, PTF1A, and SST).

[069] Figure 29 shows the average results of FACS for NKX6.1, chromagranin (CHGA), CDX2, SOX2, NKX2.2, PDX1, FOXA2 and NEUROD for stage 4, day 3 cells differentiated according to the protocol of Example 7. Specifically, Figure 29 shows the average FACS expression pattern of pancreatic precursors generated at a 3L scale from different seed material lots.

[070] Figure 30 shows the average results of FACS for NKX6.1, chromagranin (CHGA), CDX2, SOX2, NKX2.2, PDX1, FOXA2 and NEUROD for stage 4, day 3 cells differentiated according to the protocol of Example 7. Prior to differentiation in stage 4, day 3 cells, the cells were expanded to form ISM and then grown at stage 0 in either a custom in-house medium "IH3" or Essential8™, both of which were supplemented with 0.5% BSA. The cells grown in the IH3 medium are the "IH3-P grown cells" and the cells grown in Essential8™ are the "EZ8 grown cells." No significant difference in expression patterns was observed between the cells grown in the different media.

[071] Figure 31 shows the average results of FACS for NKX6.1, chromagranin (CHGA), CDX2, SOX2, NKX2.2, PDX1, FOXA2 and NEUROD for stage 4, day 3 cells, which were previously grown at different pH levels in stage 0 (*see* Example 7). No significant change in the stage 4, day 3 cell profile was observed.

[072] Figure 32 compares the results of FACS for NKX6.1, chromogranin (CHGA), CDX2, SOX2, NKX2.2, PDX1, FOXA2 and NEUROD for stage 4, day 3 cells, which were not treated with Anti-Foam C, and stage 4, day 3 cells, which were treated with Anti-Foam C emulsion (94 ppm) (*see* Example 7). Anti-Foam C emulsion (Sigma Cat#A8011) was not observed to affect the profile of stage 4 day 3 cells.

[073] Figures 33 to 35 show the gene expression, as determined by qRT-PCR, for select genes for cells differentiated according to the protocol of Example 8. Figure 33 shows the gene expression, as determined by qRT-PCR, for select genes of cells, twenty-four hours prior to the start of differentiation (*see* Example 8). As shown in Figure 33, cells from the

bioreactor retained expression for genes characteristic of pluripotency (POU5F1, NANOG, SOX2, and ZFP42) and showed minimal or no induction of genes characteristic of differentiation (AFP, and FOXA2: <50 fold increase; FOXD3, GATA2, GATA4, GSC, HAND2, MIXL1, and T: <10 fold increased expression). Figure 34 shows the gene expression, as determined by qRT-PCR, for select genes of cells twenty-four hours after the start of differentiation. Figure 35 shows the gene expression, as determined by qRT-PCR, for select genes of cells seventy-two hours after the start of differentiation.

[074] Figure 36(a) to 36(e) show the gene expression, as determined by qRT-PCR, for select genes for cells differentiated from stage 2 to stages 3 and 4 according to the protocol of Example 8. Specifically, these Figures show the gene expression of the cells at stage 2, day 1; stage 2, day 2; stage 2, day 3; stage 3, day 3; and, depending on the gene, stage 4, day 1. Figure 36(a) shows the gene expression for AFP, ATOH1, and CDX2. Figure 36(b) shows the gene expression for GAST, HAND1, HHEX, and HNF4a. Figure 36(c) shows the gene expression for NKX2.2, NKX6.1, OSR1, and PDX1. Figure 36(d) shows the gene expression for PROX1, PFT1a, SOX17, and SOX2. Figure 36(e) shows the gene expression for SOX9. The data are shown as difference in expression versus undifferentiated H1 (WA01) hES cells (baseline expression of 1).

[075] Figure 37 show the gene expression, as determined by qRT-PCR, for select genes for cells at stage 4, day 3 of differentiation according to the protocol in Example 8. As shown in Figure 37, at the end of differentiation at stage 3, day 3 the cells have differentiated into pancreatic progenitor cells characterized by high expression levels of PDX1 ($>1x10^6$ fold induction) and other pancreatic genes (>1000 fold induction of ARX, GCG, GAST, INS, ISL, NEUROD1, NGN3, NKX2.2, NKX6.1, PAX4, PTF1a, and SST) and near total loss of OCT4/POU5F1 expression as compared to undifferentiated H1 human embryonic stem cells.

[076] Figure 38 shows the daily cell counts during the differentiation protocol according to Example 8. Specifically, Figure 38 shows cell density as a function of the process day. Figure 38 shows the cell counts for differentiation protocols of two reactor runs (PRD1205 and PRD1207) carried out at pH 6.8 and 7.2. For comparison, the cell counts for cell drift are also shown.

[077] Figure 39(a) to Figure 39(d) illustrate the *in vivo* bioactivity of stage 4 day 3 cells, which were differentiated according to the protocol of Example 8 and were implanted into SCID-Bg mice. The cells were implanted subcutaneously via a TheraCyte™ device, under the kidney capsule or implanted after incubation in an ultra-low attachment dish. The mice were monitored for blood glucose and C-peptide levels every four weeks following graft

implantation. Figure 39(a) shows the C-peptide levels after implantation of 5 x 10^6 or 10 x 10^6 stage 4 day 3 cells in a TheraCyte™ device as a function of time. Figure 39(b) shows the non-fasting glucose levels in animals after implantation of 5 x 10^6 or 10 x 10^6 stage 4 day 3 cells in a TheraCyte™ device. The mice in Figure 39(b) were treated with STZ to ablate host β-cell function prior to implantation. Figure 39(c) shows the C-peptide level produced after implantation of previously-cyropreserved stage 4 day 3 cells in a TheraCyte™ device as a function of time (weeks post implantation). Figure 39(d) compares the C-peptide levels of mice treated by a kidney graft of never cryopreserved/fresh stage 4, day 3 cells or cryopreserved stage 4, day 3 cells implanted immediately after thaw (D0) or 1 day after thaw (D1).

[078] Figure 40A to Figure 40D show FACS plots for CXCR4, CD99, and CD9 of cells differentiated for three days according to the protocol of Example 9 which were treated at stage 1, day 1 with: MCX compound and GDF-8 (Figure 40A); MCX only (Figure 40B); WNT3A and Activin A (Figure 40C); and WNT3A only (Figure 40D). These figures indicate that in suspension culture, addition of 3μM MCX in the absence of a TGF-β family member on day one of differentiation generates definitive endoderm at levels comparable to that obtained when cells are treated with 3μM MCX plus 100ng/ml GDF-8 or 20ng/ml WNT-3a plus 100ng/ml Activin A on day one.

[079] Figures 41A to 41D show FACS plots for CXCR4, CD99, and CD9 of cells differentiated for three days according to the protocol of Example 10, which were treated with various amounts of MCX at stage 1, day 1. Specifically, the cells at stage 1, day 1were treated with: 4 μM of MCX (Figure 41A); 3 μM of MCX (Figure 41B); 2 μM of MCX (Figure 41C); and 1.5 μM of MCX (Figure 41D).

[080] Figure 42A and Figure 42B show FACS plots for CXCR4, CD99, and CD9 of cells differentiated for three days according to the protocol of Example 11. Specifically, these Figures show the role of media exchange frequency in suspension culture. Figure 42A shows FACS plots for CXCR4, CD99, and CD9 of cells differentiated for three days according to the protocol of Example 10 with full media exchange at stage 1. Figure 42B shows FACS plots for CXCR4, CD99, and CD9 of cells differentiated for three days according to the protocol of Example 10 without a media exchange on day 3. The data suggest that in the suspension culture system, cultures which receive a media exchange on day three (Figure 42A) of differentiation resulted in definitive endoderm with a comparable efficiency to cultures which did not receive a media exchange on day three (Figure 42B).

[081] Figure 43A and Figure 43B show FACS plots for CXCR4, CD99, and CD9 of cells differentiated for three days according to the protocol of Example 12. Specifically, these Figures show the role of GlutaMAX™ in suspension culture. The cells were cultured at stage 1 in a medium supplemented with 1X GlutaMAX™ (Figure 43A) or free of GlutaMAX™ or any glutamine (0 M GlutaMAX™) (Figure 43B). The data suggest that in the suspension culture system, addition of GlutaMAX™ does not appear to influence the efficiency with which definitive endoderm is generated

[082] Figures 44A to 44D show the effects of various amounts of sodium bicarbonate on cells differentiated according to the protocol of Example 13. Figure 44A and Figure 44B show FACS plots for CXCR4, CD99, and CD9 of cells differentiated for three days according to the protocol of Example 13 with either 3.64 g/l (Figure 44A) or 2.49 g/l (Figure 44B) added at stage 1. Figure 44C and Figure 44D show phase contrast micrographs of cells differentiated for three days according to the protocol of Example 13 with either 3.64 g/l (Figure 44C) or 2.49 g/l (Figure 44D) added at stage 1.

[083] Figure 45 shows daily cell counts for cell density as a function of differentiation for cells differentiated according to the protocol of Example 14. The cells counts were obtained using an image-based cytometer (NucleoCounter®).

[084] Figure 46 shows the average daily bioreactor medium pH levels as a function of time (days of differentiation) during the differentiation protocol of Example 14. pH levels were determined by a NOVA BioProfile® FLEX (Nova Biomedical Corporation, Waltham, MA).

[085] Figure 47 shows the average daily bioreactor medium glucose levels as a function of time (days of differentiation) during the differentiation protocol of Example 14. Glucose levels were determined by a NOVA BioProfile® FLEX (Nova Biomedical Corporation, Waltham, MA).

[086] Figure 48 shows the average daily bioreactor medium lactate levels as a function of time (days of differentiation) during the differentiation protocol of Example 14. Lactate levels were determined by a NOVA BioProfile® FLEX (Nova Biomedical Corporation, Waltham, MA).

[087] Figure 49 shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for the pluripotency array, which contains select genes associated with pluripotency, for stage 0, day 1 to 3 and stage 1, day 1 to day 3 cells differentiated according to the protocol of Example 14. Figure 50 shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for the DE array, which contains select genes associated with DE, for stage 0, day 1 to 3, stage 1, day 1

to day 3 and stage 2, day 1 to day 3 cells differentiated according to the protocol of Example 14.

[088] Figure 51 shows the results of FACS for markers associated with pluripotency (CD184/CXCR4, SSEA4, TRA-1-60 and TRA-1-81) for stage 0, cells prior to being differentiated according to the protocol of Example 14. Specifically, Figure 51 shows high expression of markers associated with pluripotency.

[089] Figure 52 shows FACS plots for the definitive endoderm markers CXCR4, CD99, and CD9 of cells differentiated to the end of stage 1 according to the protocol of Example 14.

[090] Figure 53 shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for GAPDH, AFP, HHEX, HNF4α, PDX1, and PROX1 for stage 2, day 1; stage 2, day 2 and stage 2, day 3 cells differentiated according to the protocol of Example 14. Figure 53 shows an increase in expression of foregut genes (AFP, HHEX, PDX1, and PROX1).

[091] Figure 54 shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for GAPDH, AFP, CDX2, GAST, HNF4A, NKX2-2, OSR1, PDX1 and PFT1A for stage 2, day 1 to day 3 and stage 3, day 1 to day 3 cells differentiated according to the protocol of Example 14. As shown in Figure 54, expression for PDX1 increased 60 fold from 12,000x over control at the end of stage 2 day 3 to 739,000x over control at the end of stage 3, day 3.

[092] Figure 55 shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for certain genes for stage 3, day 1 to 3 and stage 4, day 1 to day 3 cells differentiated according to the protocol of Example 14. Specifically, the top panel of Figure 55 shows the gene expression for GAPDH, AFP, ALB, ARX, CDX2, CHGA, GAST, GCG, IAAP, INS, ISL1, and MAFB. The bottom panel of Figure 55 shows the gene expression of MAFB, MUCS, NEUROD1, NEUROG3, NKX2-2, NKX6-1, PAX4, PDX1, POUSF1, PTF1A, SST and ZlC1.

[093] Figure 56 shows end stage micrographs for cells differentiated according to the protocol of Example 14. Visible in Figure 56 are representative micrographs (4X) of cell clusters at stage 0 and at the end of differentiation of stages 1 to 4.

[094] Figures 57 to 80 show the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for cells differentiated according to various embodiments of the protocol of Example 15 after 0 hours, 6 hours, 24 hours, 30 hours, 48 hours and 72 hours of differentiation for the following genes: AFP (Figure 57); CD99 (Figure 58); CD9 (Figure 59); CDH1 (Figure 60); CDH2 (Figure 61); CDX2 (Figure 62); CER1

(Figure 63); CXCR4 (Figure 64); FGF17 (Figure 65); FGF4 (Figure 66); FOXA (Figure 67); GADPH (Figure 68); GATA4 (Figure 69); GATA6 (Figure 70); GSC (Figure 71); KIT (Figure 72); MIXL1 (Figure 73); MNX1 (Figure 74); NANOG (Figure 75); OTX2 (Figure 76); POUF5F1 (Figure 77); SOX17 (Figure 78); SOX7 (Figure 79) and T (Figure 80).

[095] Figure 81 shows the percentage of cells in G0/G1 of Cell Cycle for cells after 6 hours, 24 hours, 30 hours, 48 hours, and 72 hours of differentiation according to various embodiments of the protocol of Example 15. Specifically, Figure 81 shows the results for clusters that were treated on the first day of differentiation with one of six conditions: (1) Neat, (2) 3μM MCX plus 100ng/ml GDF-8 (Catalog # 120-00, Peprotech), (3) 3μM MCX only, (4) 100ng/ml GDF-8 only, (5) 20ng/ml WNT-3A (Catalog # 1324-WN-002, R&D Systems, MN) plus 100ng/ml Activin A (Catalog # 338-AC, R&D Systems, MN), or (6) 20ng/ml WNT-3A only.

[096] Figure 82 shows the effects of EDU treatment on the cell clusters differentiated according to the protocol of Example 15. The left hand panel of shows percentage of cells in G2/M of Cell Cycle for cells after 0 hours, 6 hours, 24 hours, 30 hours, 48 hours, and 72 hours of differentiation according to various embodiments of the protocol of Example 15. Specifically, the left hand panel shows the results for clusters that were treated on the first day of differentiation with one of six conditions: (1) Neat, (2) 3μM MCX plus 100ng/ml GDF-8 (Catalog # 120-00, Peprotech), (3) 3μM MCX only, (4) 100ng/ml GDF-8 only, (5) 20ng/ml WNT-3A (Catalog # 1324-WN-002, R&D Systems, MN) plus 100ng/ml Activin A (Catalog # 338-AC, R&D Systems, MN), or (6) 20ng/ml WNT-3A only. In one set of data, these clusters were also treated with EDU. The right hand panel of Figure 82 shows the % Cells that are EDU positive 0 hours, 6 hours, 24 hours, 30 hours, 48 hours, and 72 hours of differentiation according to various embodiments of the protocol of Example 15.

[097] Figure 83 shows the general operational parameters used in the protocols of Example 15.

[098] Figure 84 shows the amount of EDU incorporation of cells after 6 hours, 24 hours, 30 hours, 48 hours, and 72 hours of differentiation according to various embodiments of the protocol of Example 15. Specifically, Figure 84 shows the results for EDU incubated cells clusters that were treated on the first day of differentiation with one of six conditions: (1) Neat, (2) 3μM MCX plus 100ng/ml GDF-8 (Catalog # 120-00, Peprotech), (3) 3μM MCX only, (4) 100ng/ml GDF-8 only, (5) 20ng/ml WNT-3A (Catalog # 1324-WN-002, R&D Systems, MN) plus 100ng/ml Activin A (Catalog # 338-AC, R&D Systems, MN), or (6) 20ng/ml WNT-3A only.

[099] Figure 85 shows the percentage of cells in G0/G1 of Cell Cycle for cells after 6 hours, 24 hours, 30 hours, 48 hours, and 72 hours of differentiation according to various embodiments of the protocol of Example 15. Specifically, Figure 85 shows the results for clusters that were treated on the first day of differentiation with one of six conditions: (1) Neat, (2) 3μM MCX plus 100ng/ml GDF-8 (Catalog # 120-00, Peprotech), (3) 3μM MCX only, (4) 100ng/ml GDF-8 only, (5) 20ng/ml WNT-3A (Catalog # 1324-WN-002, R&D Systems, MN) plus 100ng/ml Activin A (Catalog # 338-AC, R&D Systems, MN), or (6) 20ng/ml WNT-3A only.

[0100] Figure 86 shows the percentage of cells in S-phase of Cell Cycle for cells after 6 hours, 24 hours, 30 hours, 48 hours, and 72 hours of differentiation according to various embodiments of the protocol of Example 15. Specifically, Figure 86 shows the results for clusters that were treated on the first day of differentiation with one of six conditions: (1) Neat, (2) 3μM MCX plus 100ng/ml GDF-8 (Catalog # 120-00, Peprotech), (3) 3μM MCX only, (4) 100ng/ml GDF-8 only, (5) 20ng/ml WNT-3A (Catalog # 1324-WN-002, R&D Systems, MN) plus 100ng/ml Activin A (Catalog # 338-AC, R&D Systems, MN), or (6) 20ng/ml WNT-3A only.

[0101] Figure 87 shows the percentage of cells in S-phase of Cell Cycle for cells after hours, 6 hours, 24 hours, 30 hours, 48 hours, and 72 hours of differentiation according to various embodiments of the protocol of Example 15. Specifically, Figure 87 shows the results for clusters that were treated on the first day of differentiation with one of six conditions: (1) Neat, (2) 3μM MCX plus 100ng/ml GDF-8 (Catalog # 120-00, Peprotech), (3) 3μM MCX only, (4) 100ng/ml GDF-8 only, (5) 20ng/ml WNT-3A (Catalog # 1324-WN-002, R&D Systems, MN) plus 100ng/ml Activin A (Catalog # 338-AC, R&D Systems, MN), or (6) 20ng/ml WNT-3A only.

[0102] Figures 88A to 88E show the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for cells differentiated according to various embodiments of the protocol of Example 15 after 0 hours, 6 hours, 24 hours, 30 hours, 48 hours and 72 hours of differentiation. Figure 88A shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for CD99, CD9, CDH1, and CDH2. Figure 88A shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for CXD2, CER1, CXCR4, and FGF17. Figure 88C shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for FGF4, FOXA, GATA4, and GATA6. Figure 88D shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for

GSC, KIT, MIXL1 and MNX1. Figure 88E shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for NANOG, OTX2, POUF5F1, and SOX17. Figure 88F shows the gene expression, as determined by qRT-PCR as a fold expression versus undifferentiated cells, for SOX7 and T. The underlying data for Figures 88A to 88F is shown in Figure 58 to 67 and 69 to 80.

[0103] Figure 89 shows the gene expression pattern, as determined by qRT-PCR, of pluripotent cells cultured in ectodermal differentiation medium according to the protocol of Example 16. As shown in Figure 89, the cells differentiated towards the neural cell lineage. Specifically, the left panel of Figure 89 shows the gene expression pattern for an induced pluripotent stem cell line generated from umbilical tissue cells (UTC). The right panel of Figure 89 shows the gene expression pattern for the WB0106 sub-clone of the H1 hES cell line.

[0104] Figure 90 shows the gene expression pattern, as determined by qRT-PCR, of pluripotent cells cultured in mesodermal differentiation medium according to the protocol of Example 16. As shown in Figure 90, the cells differentiated towards cardiac cell lineage. Specifically, the left panel of Figure 90 shows the gene expression pattern for an induced pluripotent stem cell line generated from umbilical tissue cells (UTC). The right panel of Figure 90 shows the gene expression pattern for the WB0106 sub-clone of the H1 hES cell line.

[0105] Figure 91 shows the gene expression pattern, as determined by qRT-PCR, of pluripotent cells cultured in ectodermal differentiation medium according to the protocol of Example 16. As shown in Figure 91, the cells differentiated towards neural cell lineage. Specifically, the left panel of Figure 91 shows the gene expression pattern for an induced pluripotent stem cell line generated from umbilical tissue cells (UTC). The right panel of Figure 91 shows the gene expression pattern for the WB0106 sub-clone of the H1 hES cell line.

[0106] Figure 92 shows the protein expression pattern for PAX6, SOX2, and POU5F1/OCT4, as determined by FACS, of pluripotent cells cultured for three days in ectodermal differentiation medium according to the protocol of Example 16. Specially, the left panels of Figure 92 show the expression pattern for PAX6, SOX2, and POU5F1/OCT4 for an induced pluripotent stem cell line generated from umbilical tissue cells (UTC). The right panel of Figure 92 shows the protein expression pattern for PAX6, SOX2, and POU5F1/OCT4 for the WB0106 sub-clone of the H1 hES cell line.

[0107] Figure 93 shows the gene expression pattern, as determined by qRT-PCR, of pluripotent cells cultured in mesodermal differentiation medium according to the protocol of Example 16. As shown in Figure 93, the cells differentiated towards cardiac cell lineage. Specifically, the left panel of Figure 93 shows the gene expression pattern for an induced pluripotent stem cell line generated from umbilical tissue cells (UTC). The right panel of Figure 93 shows the gene expression pattern for the WB0106 sub-clone of the H1 hES cell line.

[0108] Figure 94 shows micrographs for cells differentiated in mesodermal differentiation medium according to the protocol of Example 16. As shown in Figure 94, the cells differentiated towards cardiac cell lineage. Specifically, the left hand panels of Figure 94 show micrographs of cells of the WB0106 sub-clone of the H1 hES cell line at day 3, day 5 and day 10 of differentiation. The right hand panel of Figure 94 shows a micrograph of induced pluripotent stem cell line generated from umbilical tissue cells (UTC iPSCs) after 10 days of differentiation.

[0109] Figure 95 shows micrographs for cells differentiated in ectodermal differentiation medium according to the protocol of Example 16. As shown in Figure 95, the cells differentiated towards the neural cell lineage. Specifically, the left hand panels of Figure 95 show micrographs of cells of the WB0106 sub-clone of the H1 hES cell line at day 3, day 5 and day 10 of differentiation. The right hand panel of Figure 95 shows a micrograph of induced pluripotent stem cell line generated from umbilical tissue cells (UTC iPCS) after 10 days of differentiation.

DETAILED DESCRIPTION

[0110] This application is directed to preparing embryonic stem cells and other pluripotent cells that maintain pluripotency in aggregated cell cluster for differentiation to endoderm progenitor cells, pancreatic endocrine cells, mesoderm cells or ectoderm cells. For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections that describe or illustrate certain features, embodiments or applications of the present invention.

DEFINITIONS

[0111] Stem cells are undifferentiated cells defined by their ability, at the single cell level, to both self-renew and differentiate. Stem cells may produce progeny cells, including self-

renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate *in vitro* into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm, and ectoderm). Stem cells also give rise to tissues of multiple germ layers following transplantation and contribute substantially to most, if not all, tissues following injection into blastocysts.

[0112] Stem cells are classified by their developmental potential. "Cell culture" or "culturing" refer generally to cells taken from a living organism and grown under controlled conditions ("in culture" or "cultured"). A *primary cell culture* is a culture of cells, tissues, or organs taken directly from an organism before the first subculture. Cells are *expanded* in culture when they are placed in a growth medium under conditions that facilitate one or both of cell growth and division, resulting in a larger population of the cells. When cells are expanded in culture, the rate of cell proliferation is sometimes measured by the amount of time needed for the cells to double in number (referred to as *doubling time).*

[0113] "Expanding", as used herein is the process of increasing the number of pluripotent stem cells by culturing, such as by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 75%, 90%, 100%, 200%, 500%, 1000% or more, and levels within these percentages. It is appreciated that the number of pluripotent stem cells which can be obtained from a single pluripotent stem cell depends on the proliferation capacity of the pluripotent stem cell. The proliferation capacity of the pluripotent stem cell can be calculated by the doubling time of the cell, *i.e.*, the time needed for a cell to undergo a mitotic division in the culture, and the period that the pluripotent stem cell can be maintained in the undifferentiated state, which is equivalent to the number of passages multiplied by the days between each passage.

[0114] Differentiation is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell such as, a nerve cell or a muscle cell. A differentiated cell or a differentiation-induced cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term "committed", when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. "De-differentiation" refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the lineage of a cell defines the heredity of the cell, *i.e.*, which cells it came from and to what cells it can give rise. The

lineage of a cell places the cell within a hereditary scheme of development and differentiation. A lineage-specific marker refers to a characteristic specifically associated with the phenotype of cells of a lineage of interest and can be used to assess the differentiation of an uncommitted cell to the lineage of interest.

[0115] "Markers", as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker as compared to an undifferentiated cell. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

[0116] As used herein, a cell is "positive for" a specific marker or "positive" when the specific marker is sufficiently detected in the cell. Similarly, the cell is "negative for" a specific marker, or "negative" when the specific marker is not sufficiently detected in the cell. In particular, positive by FACS is usually greater than 2%, whereas the negative threshold by FACS is usually less than 1%. Positive by PCR is usually less than 34 cycles (Cts); whereas negative by PCR is usually more than 34.5 cycles.

[0117] As used herein, "cell density" and "seeding density" are used interchangeably herein and refer to the number of cells seeded per unit area of a solid or semisolid planar or curved substrate.

[0118] As used herein, "suspension culture" refers to a culture of cells, single cells or clusters, suspended in medium rather than adhering to a surface.

[0119] As used herein, "serum free" refers to being devoid of human or animal serum. Accordingly, a serum free culture medium does not comprise serum or portions of serum.

[0120] In attempts to replicate the differentiation of pluripotent stem cells into functional pancreatic endocrine cells in cell culture, the differentiation process is often viewed as progressing through a number of consecutive stages. As used herein, the various stages are defined by the culturing times, and reagents set forth in the Examples included herein.

[0121] "Definitive endoderm", as used herein, refers to cells which bear the characteristics of cells arising from the epiblast during gastrulation and which form the gastrointestinal tract and its derivatives. Definitive endoderm cells express at least one of the following markers: FOXA2 (also known as hepatocyte nuclear factor 3-β (HNF3β)), GATA4, GATA6, MNX1, SOX17, CXCR4, Cerberus, OTX2, brachyury, goosecoid, C-Kit, CD99, and MIXL1. Markers characteristic of the definitive endoderm cells include CXCR4, FOXA2 and SOX17.

Thus, definitive endoderm cells may be characterized by their expression of CXCR4, FOXA2, and SOX17. In addition, depending on the length of time cells are allowed to remain in stage 1, an increase in HNF4α may be observed.

[0122] "Pancreatic endocrine cells," as used herein, refer to cells capable of expressing at least one of the following hormones: insulin, glucagon, somatostatin, ghrelin, and pancreatic polypeptide. In addition to these hormones, markers characteristic of pancreatic endocrine cells include one or more of NGN3, NeuroD1, ISL1, PDX1, NKX6.1, PAX4, ARX, NKX2.2, and PAX6. Pancreatic endocrine cells expressing markers characteristic of β cells can be characterized by their expression of insulin and at least one of the following transcription factors: PDX1, NKX2.2, NKX6.1, NeuroD1, ISL1, HNF3β, MAFA, PAX4, and PAX6.

[0123] Used interchangeably herein are "d1", "d 1", and "day 1"; "d2", "d 2", and "day 2"; "d3", "d 3", and "day 3", and so on. These number letter combinations refer to a specific day of incubation in the different stages during the stepwise differentiation protocol of the instant application.

[0124] "Glucose" and "D-Glucose" are used interchangeably herein and refer to dextrose, a sugar commonly found in nature.

[0125] Used interchangeably herein are "NeuroD" and "NeuroD1" which identify a protein expressed in pancreatic endocrine progenitor cells and the gene encoding it.

[0126] "LDN" and "LDN-193189" refer ((6-(4-(2-(piperidin-1-yl)ethoxy)phenyl)-3-(pyridin-4-yl)pyrazolo[1,5-a]pyrimidine, hydrochloride; DM-3189)), a BMP receptor inhibitor available under the trademark STEMOLECULE™ from Stemgent, Inc., Cambridge, MA, USA.

ISOLATION, EXPANSION AND CULTURE OF PLURIPOTENT STEM CELLS

[0127] Pluripotent stem cells may express one or more of the designated TRA-1-60 and TRA-1-81 antibodies (Thomson *et al.* 1998, *Science* 282:1145-1147). Differentiation of pluripotent stem cells *in vitro* results in the loss of TRA-1-60, and TRA-1-81 expression. Undifferentiated pluripotent stem cells typically have alkaline phosphatase activity, which can be detected by fixing the cells with 4% paraformaldehyde, and then developing with Vector® Red as a substrate, as described by the manufacturer (Vector Laboratories, Inc., Burlingame, CA). Undifferentiated pluripotent stem cells also typically express OCT4 and TERT, as detected by RT-PCR.

[0128] Another desirable phenotype of propagated pluripotent stem cells is a potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm tissues. Pluripotency of stem cells can be confirmed, for example, by injecting cells into severe combined immune-deficiency ("SCID") mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining histologically for evidence of cell types from these three germ layers. Alternatively, pluripotency may be determined by the creation of embryoid bodies and assessing the embryoid bodies for the presence of markers associated with the three germinal layers.

[0129] Propagated pluripotent stem cell lines may be karyotyped using a standard G-banding technique and compared to published karyotypes of the corresponding primate species. It is desirable to obtain cells that have a "normal karyotype," which means that the cells are euploid, wherein all human chromosomes are present and not noticeably altered. Pluripotent cells may be readily expanded in culture using various feeder layers or by using matrix protein coated vessels. Alternatively, chemically defined surfaces in combination with defined media such as mTeSR®1 media (StemCell Technologies, Vancouver, BC, Canada) may be used for routine expansion of the cells.

[0130] Culturing in a suspension culture according to the method of some embodiments of the invention is effected by seeding the pluripotent stem cells in a culture vessel at a cell density that promotes cell survival and proliferation, but limits differentiation. Typically, a seeding density that maintains undifferentiation of cells is used. It will be appreciated that although single-cell suspensions of stem cells may be seeded, small clusters of cells may be advantageous.

[0131] To provide the pluripotent stem cells with a sufficient and constant supply of nutrients and growth factors while in the suspension culture, the culture medium can be replaced or replenished on a daily basis or at a pre-determined schedule such as every 1-5 days. Large clusters of pluripotent stem cells may cause cell differentiation, thus, measures may be taken to avoid large pluripotent stem cell aggregates. According to some embodiments of the invention, the formed pluripotent stem cell clusters are dissociated, for example, every 2-7 days and the single cells or small clumps of cells are either split into additional culture vessels (*i.e.*, passaged) or retained in the same culture vessel and processed with replacement or additional culture medium.

[0132] Large pluripotent stem cell clumps, including a pellet of pluripotent stem cells resulting from centrifugation, can be subjected to one or both of enzymatic digestion and mechanical dissociation. Enzymatic digestion of pluripotent stem cell clumps can be

performed by subjecting the clump to an enzyme, such as type IV Collagenase, Dispase® or Accutase®. Mechanical dissociation of large pluripotent stem cell clumps can be performed using a device designed to break the clumps to a predetermined size. Additionally, or alternatively, mechanical dissociation can be manually performed using a needle or pipette.

[0133] The culture vessel used for culturing the pluripotent stem cells in suspension according to the method of some embodiments of the invention can be any tissue culture vessel (*e.g.*, with a purity grade suitable for culturing pluripotent stem cells) having an internal surface designed such that pluripotent stem cells cultured therein are unable to adhere or attach to such a surface (*e.g.*, non-tissue culture treated vessel, to prevent attachment or adherence to the surface). Preferably to obtain a scalable culture, culturing according to some embodiments of the invention is effected using a controlled culturing system (preferably a computer-controlled culturing system) in which culture parameters such as temperature, agitation, pH, and oxygen are automatically monitored and controlled using a suitable device. Once the desired culture parameters are determined, the system may be set for automatic adjustment of culture parameters as needed to enhance pluripotent stem cell expansion and differentiation.

[0134] The pluripotent stem cells may be cultured under dynamic conditions (*i.e.*, under conditions in which the pluripotent stem cells are subject to constant movement while in the suspension culture, *e.g.* a stirred suspension culture system) or under non-dynamic conditions (*i.e.*, a static culture) while preserving their, proliferative, pluripotent capacity and karyotype stability over multiple passages.

[0135] For non-dynamic culturing of pluripotent stem cells, the pluripotent stem cells can be cultured in petri dishes, T-flasks, HyperFlasks® (Corning Incorporated, Corning, NY), CellStacks® (Corning Incorporated, Corning, NY) or Cell Factories (NUNC™ Cell Factory™ Systems (Thermo Fisher Scientific, Inc., Pittsburgh, PA)) coated or uncoated. For dynamic culturing of pluripotent stem cells, the pluripotent stem cells can be cultured in a suitable vessel, such as spinner flasks or Erlenmeyer flasks, stainless steel, glass or single use plastic shaker or stirred tank vessels. The culture vessel can be connected to a control unit and thus present a controlled culturing system. The culture vessel (*e.g.*, spinner flask or Erlenmeyer flask) may be agitated continuously or intermittently. Preferably the cultured vessel is agitated sufficiently to maintain the pluripotent stem cells in suspension.

[0136] The pluripotent stem cells may be cultured in any medium that provides sufficient nutrients and environmental stimuli to promote growth and expansion. Suitable media include E8™, IH3 and mTeSR®1 or mTeSR®2. The media may be changed periodically to

refresh the nutrient supply and remove cellular by-products. According to some embodiments of the invention, the culture medium is changed daily.

SOURCES OF PLURIPOTENT STEM CELL

[0137] Any pluripotent stem cell may be used in the methods of the invention. Exemplary types of pluripotent stem cells that may be used include established lines of pluripotent cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily, before approximately 10 to 12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells (hESCs) or human embryonic germ cells, such as, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell Research Institute, Madison, WI, USA). Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells.

[0138] Also suitable are inducible pluripotent cells (IPS) or reprogrammed pluripotent cells that can be derived from adult somatic cells using forced expression of a number of pluripotent related transcription factors, such as OCT4, NANOG, Sox2, KLF4, and ZFP42 (*Annu Rev Genomics Hum Genet* 2011, 12:165-185). The human embryonic stem cells used in the methods of the invention may also be prepared as described by Thomson *et al.* (U.S. Patent No. 5,843,780; Science, 1998, 282:1145-1147; *Curr Top Dev Biol* 1998, 38:133-165; *Proc Natl Acad Sci* U.S.A. 1995, 92:7844-7848). Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, Ga.), or cells derived from adult human somatic cells, such as, for example, cells disclosed in Takahashi *et al.*, Cell 131: 1-12 (2007). Pluripotent stem cells suitable for use in the present invention may be derived according to the methods described in Li *et al.* (*Cell Stem Cell* 4: 16-19, 2009); Maherali *et al.* (*Cell Stem Cell* 1: 55-70, 2007); Stadtfeld *et al.* (*Cell Stem Cell* 2: 230-240); Nakagawa *et al.* (*Nature Biotechnology* 26: 101-106, 2008); Takahashi *et al.* (*Cell* 131: 861-872, 2007); and U.S. Patent App. Pub. No. 2011-0104805. Other sources of pluripotent stem cells include induced pluripotent cells (IPS, *Cell*, 126(4): 663-676). Other sources of cells suitable for use in the methods of invention include human umbilical cord tissue-derived cells, human amniotic fluid-derived cells, human placental-derived cells, and human parthenotes. In one embodiment, the umbilical cord tissue-derived cells may be obtained using the methods of U.S. Patent No. 7,510,873, the disclosure of which is incorporated by reference in its entirety as it pertains to the isolation and characterization of the cells. In another embodiment, the

placental tissue-derived cells may be obtained using the methods of U.S. App. Pub. No. 2005/0058631, the disclosure of which is incorporated by reference in its entirety as it pertains to the isolation and characterization of the cells. In another embodiment, the amniotic fluid-derived cells may be obtained using the methods of U.S. App. Pub. No. 2007/0122903, the disclosure of which is incorporated by reference in its entirety as it pertains to the isolation and characterization of the cells.

[0139] Characteristics of pluripotent stem cells are well known to those skilled in the art, and additional characteristics of pluripotent stem cells continue to be identified. Pluripotent stem cell markers include, for example, the expression of one or more (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or all) of the following: ABCG2, cripto, FOXD3, CONNEXIN43, CONNEXIN45, OCT4, SOX2, NANOG, hTERT, UTF1, ZFP42, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81. In one embodiment, the pluripotent stem cells suitable for use in the methods of the invention express one or more (*e.g.* 1, 2, 3 or all) of CD9, SSEA4, TRA-1-60, and TRA-1-81, and lack expression of a marker for differentiation CXCR4 (also known as CD184) as detected by flow cytometry. In another embodiment, the pluripotent stem cells suitable for use in the methods of the invention express one or more (*e.g.* 1, 2 or all) of CD9, NANOG and POU5F1/OCT4 as detected by RT-PCR.

[0140] Exemplary pluripotent stem cells include the human embryonic stem cell line H9 (NIH code: WA09), the human embryonic stem cell line H1 (NIH code: WA01), the human embryonic stem cell line H7 (NIH code: WA07), and the human embryonic stem cell line SA002 (Cellartis, Sweden). In one embodiment, the pluripotent stem cells are human embryonic stem cells, for example, H1 hES cells. In alternate embodiments, pluripotent stem cells of non-embryonic origin are used.

Differentiation of Cells Expressing Markers Characteristic of the Pancreatic Endoderm Lineage from Pluripotent Stem Cells

Expansion of Pluripotent Stem Cells

[0141] The present invention, in some of the embodiments as described below, relates to isolating and culturing stem cells, in particular culturing stem cell clusters, which retain pluripotency in a dynamic suspension culture system. Pluripotent cell clusters may be differentiated to produce functional β cells.

[0142] The pluripotent stem cells used in the methods of the present invention are

preferably expanded in dynamic suspension culture prior to differentiation toward a desired end point. Advantageously, it has been found that the pluripotent stem cells can be cultured and expanded as clusters of cells in suspension in a suitable medium without loss of pluripotency. Such culturing may occur in a dynamic suspension culture system wherein the cells or cell clusters are kept moving sufficiently to prevent loss of pluripotency. Useful dynamic suspension culture systems include systems equipped with means to agitate the culture contents, such as via stirring, shaking, recirculation or the bubbling of gasses through the media. Such agitation may be intermittent or continuous, as long as sufficient motion of the cell clusters is maintained to facilitate expansion and prevent premature differentiation. Preferably, the agitation comprises continuous stirring such as via an impeller rotating at a particular rate. The impeller may have a rounded or flat bottom. The stir rate of the impeller should be such that the clusters are maintained in suspension and settling is minimized. Further, the angle of the impeller blade may be adjusted to aid in the upward movement of the cells and clusters to avoid settling. In addition, the impeller type, angle and rotation rate may all be coordinated such that the cells and clusters are in what appears as a uniform colloidal suspension.

[0143] Suspension culturing and expansion of pluripotent stem cell clusters may be accomplished by transfer of static cultured stem cells to an appropriate dynamic culture system such as a disposable plastic, reusable plastic, stainless steel or glass vessel, *e.g.* a spinner flask or an Erlenmeyer flask. For example, stem cells cultured in an adherent static environment, i.e., plate or dish surface, may first be removed from the surface by treatment with a chelating agent or enzyme. Suitable enzymes include, but are not limited to, type I Collagenase, Dispase® (Sigma Aldrich LLC, St. Louis, MO) or a commercially available formulation sold under the trade name Accutase® (Sigma Aldrich LLC, St. Louis, MO). Accutase® is a cell detachment solution comprising collagenolytic and proteolytic enzymes (isolated from crustaceans) and does not contain mammalian or bacterial derived products. Therefore, in one embodiment, the enzyme is a collagenolytic enzyme or a proteolytic enzyme or a cell detachment solution comprising collagenolytic and proteolytic enzymes. Suitable chelating agents include, but are not limited to, ethylenediaminetetraacetic acid (EDTA). In some embodiments, the pluripotent stem cell cultures are incubated with the enzyme or chelating agent, preferably until colony edges began to curl and lift, but prior to full detachment of colonies from the culture surface. In one embodiment, the cell cultures are incubated at room temperature. In one embodiment, the cells are incubated at a temperature of more than 20°C, more than 25°C, more than 30°C or more than 35°C, for example, at a

temperature of between about 20°C and about 40°C, between about 25°C and about 40°C, between about 30°C and about 40°C, for example, about 37°C. In one embodiment, the cells are incubated for at least about 1, at least about 5, at least about 10, at least about 15, at least about 20 minutes, for example between about 1 and about 30 minutes, between about 5 and about 30 minutes, between about 10 and about 25 minutes, between about 15 and about 25 minutes, for example, about 20 minutes. In one embodiment, the method involves the step of removing the enzyme or chelating agent from the cell culture after treatment. In one embodiment, the cell culture is washed once or twice or more, after removal of the enzyme or chelating agent. In one embodiment the cell culture is washed with an appropriate culture medium, such as mTeSR®1 (Stem Cell Technologies, Vancouver, BC, Canada). In one embodiment, a Rho-kinase inhibitor (for example, Y-27632, Axxora Catalog#ALX-270-333, San Diego, CA). The Rho-kinase inhibitor may be at a concentration of about 1 to about 100 μM, about 1 to 90 μM, about 1 to about 80 μM, about 1 to about 70 μM, about 1 to about 60 μM, about 1 to about 50 μM, about 1 to about 40 μM, about 1 to about 30 μM, about 1 to about 20 μM, about 1 to about 15 μM, about 1 to about 10 μM, or about 10 μM. In one embodiment, the Rho-kinase inhibitor is added at least 1 μM, at least 5 μM or at least 10 μM. The cells may be lifted from the surface of the static culture system with a scraper or rubber policeman. Media and cells may be transferred to a dynamic culture system using a glass pipette or other suitable means. In a preferred embodiment, the media in the dynamic culture system is changed daily.

[0144] The invention provides, in one embodiment, methods of culturing and expanding pluripotent stem cells in a three-dimensional suspension culture. In particular, the methods provide for the culturing and expanding pluripotent stem cells by forming aggregated cell clusters of these pluripotent stem cells. The cell clusters may form as a result of treating pluripotent stem cell cultures with an enzyme (*e.g.* a neutral protease, for example Dispase®) or a chelating agent prior to culturing the cells. The cells may preferably be cultured in a stirred or shaken suspension culture system. In one embodiment, the invention further provides for formation of cells expressing markers characteristic of the pancreatic endoderm lineage from such clusters of pluripotent stem cells.

[0145] Preferably, the cell clusters are aggregated pluripotent stem cells. The aggregated stem cells express one or more markers of pluripotency, for example, one or more (*e.g.* 1, 2, 3 or all) of the markers CD9, SSEA4, TRA-1-60, and TRA-1-81, and lack expression of one or more markers for differentiation, for example, lack expression of CXCR4. In one embodiment, the aggregated stem cells express the markers for pluripotency CD9, SSEA4,

TRA-1-60, and TRA-1-81, and lack expression of a marker for differentiation CXCR4.

[0146] One embodiment is a method of culturing pluripotent stem cells as cell clusters in suspension culture. The cell clusters are aggregated pluripotent stem cells, cultured in a dynamic stirred or shaken suspension culture system. The cell clusters may be transferred from a planar adherent culture using an enzyme, such as a neutral protease, for example Dispase, as a cell lifting agent to a stirred or shaken suspension culture system. Exemplary suitable enzymes include, but are not limited to, type IV Collagenase, Dispase® or Accutase®. The cells maintain pluripotency in a stirred or shaken suspension culture system, in particular a stirred suspension culture system.

[0147] Another embodiment of the invention is a method of culturing pluripotent stem cells as cell clusters in suspension culture, wherein the cell clusters are aggregated pluripotent stem cells transferred from a planar adherent culture using a chelating agent, for example EDTA, and cultured in a stirred or shaken suspension culture system. The cell clusters maintain pluripotency in a stirred or shaken suspension culture system, in particular a stirred (dynamically agitated) suspension culture system.

[0148] Another embodiment of the invention is a method of culturing pluripotent stem cells as cell clusters in suspension culture, wherein the cell clusters are aggregated pluripotent stem cells transferred from a planar adherent culture using the enzyme Accutase®, and cultured in a stirred or shaken suspension culture system. The cell clusters maintain pluripotency in the dynamically agitated suspension culture system.

[0149] The cell clusters of the invention may be differentiated into mesoderm cells, such as cardiac cells, ectoderm cells, such as neural cells, single hormone positive cells or pancreatic endoderm cells. The method may further include differentiation, for example differentiation of the pancreatic endoderm cells into pancreatic precursor cells and pancreatic hormone expressing cells. In another embodiment, pancreatic precursor cells are characterized by expression of β cell transcription factors PDX1 and NKX6.1.

[0150] In one embodiment, the step of differentiation is carried out after at least 12 hours, at least 24 hours, at least 36 hours, at least 48 hours, at least 72 hours, at least 96 hours, at least 120 hours, at least 144 hours, at least 168 hours, at least 196 hours or more, preferably about 48 hours to about 72 hours in the suspension culture system. Differentiation may be carried out using a stage-wise progression of media components, such as that described in the examples (*e.g. see* Table A and Tables 1a and 1c).

[0151] In a preferred embodiment, a three-dimensional cell cluster is produced by growing pluripotent stem cells in a planar adherent culture; expanding the pluripotent stem cells to

aggregated cell clusters; and transferring the clusters of pluripotent stem cells from the planar adherent culture to a dynamic suspension culture using an enzyme or chelating agent. A further preferred embodiment is a method of expanding and differentiating pluripotent stem cells in a dynamically agitated suspension culture system by growing pluripotent stem cells in a planar adherent culture; expanding the pluripotent stem cells to aggregated cell clusters; and transferring the clusters of pluripotent stem cells from the planar adherent culture to a dynamic suspension culture using an enzyme or chelating agent; and differentiating the pluripotent cell clusters in a dynamic agitated suspension culture system to generate a pancreatic precursor cell population.

[0152] Another embodiment is a transplantable stem cell derived cell product comprising differentiated stem cells prepared from suspension of expanded pluripotent stem cell clusters that are differentiated to pancreatic precursor cells. More particularly, a transplantable stem cell derived product is produced by growing pluripotent stem cells in a planar adherent culture; expanding the pluripotent stem cells to aggregated cell clusters; and transferring the clusters of pluripotent stem cells from the planar adherent culture to a dynamic suspension culture using an enzyme or chelating agent; and differentiating the pluripotent cell clusters in a dynamically agitated suspension culture system. The transplantable stem cell derived cell product is preferably used to treat diabetes.

[0153] In another embodiment, the method includes transplantation into a diabetic animal for further *in vivo* maturation to functional pancreatic endocrine cells.

[0154] Another embodiment is a method of expanding and differentiating pluripotent stem cells in a suspension culture system comprising growing pluripotent stem cells in a planar adherent culture; removing the pluripotent stem cells from the planar adherent culture using an enzyme; adhering the pluripotent stem cells to microcarriers in static culture; expanding the pluripotent cells in a dynamically agitated suspension culture system; and differentiating the pluripotent cells in a dynamically agitated suspension culture system to generate a pancreatic precursor cell population.

[0155] The microcarriers may be of any form known in the art for adhering cells, in particular the microcarriers may be beads. The microcarrier can be comprised of natural or synthetically-derived materials. Examples include collagen-based microcarriers, dextran-based microcarriers, or cellulose-based microcarriers. For example, microcarrier beads may be modified polystyrene beads with cationic trimethyl ammonium attached to the surface to provide a positively charged surface to the microcarrier. The bead diameter may range from about 90 to about 200 μm, alternately from about 100 to about 190 μm, alternatively from

about 110 to about 180 μm, alternatively from about 125 to 175 μm in diameter. Microcarrier beads may also be a thin layer of denatured collagen chemically coupled to a matrix of cross-linked dextran. Microcarrier beads may be glass, ceramics, polymers (such as polystyrene), or metals. Further, microcarriers may be uncoated, or coated, such as with silicon or a protein such as collagen. In a further aspect the microcarrier can be comprised of, or coated with, compounds that enhance binding of the cell to the microcarrier and enhance release of the cell from the microcarrier including, but not limited to, sodium hyaluronate, poly(monostearoylglyceride co-succinic acid), poly-D,L-lactide-co-glycolide, fibronectin, laminin, elastin, lysine, n-isopropyl acrylamide, vitronectin, and collagen. Examples further include microcarriers that possess a microcurrent, such as microcarriers with a particulate galvanic couple of zinc and copper that produces low levels of biologically relevant electricity; or microcarriers that are paramagnetic, such as paramagnetic calcium-alginate microcarriers.

[0156] In some embodiments, the population of pancreatic endoderm cells is obtained by a stepwise differentiation of pluripotent cell clusters. In some embodiments, the pluripotent cells are human embryonic pluripotent stem cells. In one aspect of the present invention, a cell expressing markers characteristic of the definitive endoderm lineage is a primitive streak precursor cell. In an alternate aspect, a cell expressing markers characteristic of the definitive endoderm lineage is a mesendoderm cell.

[0157] In some embodiments, the present invention relates to a stepwise method of differentiating pluripotent cells comprising culturing stage 3-5 cells in a dynamic suspension culture. In some embodiments, the pancreatic endoderm population generated is transplanted into diabetic animals for further *in vivo* maturation to functional pancreatic endocrine cells. The invention also provides for systems or kits for use in the methods of the invention.

[0158] The invention also provides a cell or population of cells obtainable by a method of the invention. The invention also provides a cell or population of cells obtained by a method of the invention.

[0159] The invention provides methods of treatment. In particular, the invention provides methods for treating a patient suffering from, or at risk of developing, diabetes.

[0160] The invention also provides a cell or population of cells obtainable or obtained by a method of the invention for use in a method of treatment. In particular, the invention provides a cell or population of cells obtainable or obtained by a method of the invention for use in a method of treating a patient suffering from, or at risk of developing, diabetes. The diabetes may be Type 1 or Type 2 diabetes.

[0161] In one embodiment, the method of treatment comprises implanting cells obtained or obtainable by a method of the invention into a patient.

[0162] In one embodiment, the method of treatment comprises differentiating pluripotent stem cells in vitro into stage 1, stage 2, stage 3, stage 4, or stage 5 cells, for example as described herein, and implanting the differentiated cells into a patient.

[0163] In one embodiment, the method further comprises the step of culturing pluripotent stem cells, for example as described herein, prior to the step of differentiating the pluripotent stem cells.

[0164] In one embodiment, the method further comprises the step of differentiating the cells in vivo, after the step of implantation.

[0165] In one embodiment, the patient is a mammal, preferably a human.

[0166] In one embodiment, the cells may be implanted as dispersed cells or formed into clusters that may be infused into the hepatic portal vein. Alternatively, cells may be provided in biocompatible degradable polymeric supports, porous non-degradable devices or encapsulated to protect from host immune response. Cells may be implanted into an appropriate site in a recipient. The implantation sites include, for example, the liver, natural pancreas, renal subcapsular space, omentum, peritoneum, subserosal space, intestine, stomach, or a subcutaneous pocket.

[0167] To enhance further differentiation, survival or activity of the implanted cells in vivo, additional factors, such as growth factors, antioxidants or anti-inflammatory agents, can be administered before, simultaneously with, or after the administration of the cells. These factors can be secreted by endogenous cells and exposed to the administered cells in situ. Implanted cells can be induced to differentiate by any combination of endogenous growth factors known in the art and exogenously administered growth factors known in the art.

[0168] The amount of cells used in implantation depends on a number of various factors including the patient's condition and response to the therapy, and can be determined by one skilled in the art.

[0169] In one embodiment, the method of treatment further comprises incorporating the cells into a three-dimensional support prior to implantation. The cells can be maintained in vitro on this support prior to implantation into the patient. Alternatively, the support containing the cells can be directly implanted in the patient without additional *in vitro* culturing. The support can optionally be incorporated with at least one pharmaceutical agent that facilitates the survival and function of the transplanted cells.

[0170] In certain embodiments of the invention, one or more of the following may be used in the methods of the invention.

Table A

Component/Condition	Stage	Suitable Amounts
Activin A (AA)	1,3	Stage 1: about 100 mg/ml Stage 3: about 5 ng/ml, from about 3 ng/ml to about 6 ng/ml
AlbuMAX®	3-5	About 0.1%
ALK5 inhibitor	4, 5	About 1 μM, about 500 to about 1000 nM, about 600 to about 1000 nM, about 700 to about 1000 nM, about 800 to about 1000 nM, about 100 nM, about 500 nM or about 1 μM, from about 0.6 to about 1 μM
BSA	1-5	About 2%, 0.1% to about 2%
Cypi (Cyp26 inhibitor)	4, 5	About 100 nM, from about 80 nM to about 120 nM, from about 50 nM to about 150 nM
FGF7 ("F7")	2, 3	About 50 ng/mL, from about 30 ng/ml to about 60 ng/ml, from about 25 ng/ml to about 55 ng/ml
GDF8	1	About 100 ng/mL, from about 80 ng/ml to about 150 ng/ml, from about 75 ng/ml to about 125 ng/ml, from about 75 ng/ml to about 150 ng/ml
Glucose	1-5	Stages 1 to 4: About 8 mM, from about 1 m M to about 8 mM, from about 3 mM to about 5 mM or Stages 3 and 4 About 25 mM, from about 10 to about 25 mM or Stage 5 Less than about 11 mM, from about 1 mM to about 10 mM or Stage 5 More than about 25 mM, from about 25 mM to about 50 mM

Component/Condition	Stage	Suitable Amounts
ITS-X	1-5	About 1:50,000, about 1:200, about 1:1000, about 1:10,000
LDN	3	About 100 nM, from about 80 nM to about 120 nM, from about 50 nM to about 150 nM
L-Glutamine	1-5	About 2 mM, from about 1 mM to about 3 mM, from about 2 mM to about 6 mM, from about 1 mM to about 6 mM
MCX	1	About 3 μM, about 2 μM, about 1 μM to about 5 μM, about 2 μM to about 4 μM, about 1 μM to about 3 μM, about 2 μM to about 3 μM
Retinoic Acid	3	About 2 μM, from about 1 μM to about 3 μM, form about 1.5 μM to about 2.5 μM
SANT	3, 4	About 0.25 μM, from about 0.1 μM to about 0.3 μM, from about 0.2 to about 0.3 μM. from about 0.1 μM to about 0.25 μM
SCIO (an Alk5 inhibitor)	4	About 100 nM, about 2 μM
TppB or TPB	4	About 500 nM, about 100 nM, from about 50 nM to about 550 nM, from about 50 nM to about 150 nM, from about 200 nM to about 500 nM, from about 300 nM to about 550 nM, about 50nM, from about 25nM to about 75nM
Wnt3A	1	About 20 ng/ml, from about 10 ng/ml to about 25 ng/ml, from about 18 ng/ml to about 30 ng/ml, from about 18 ng/ml to about 22 ng/ml
Y-27632	0	About 10 μM, from about 5 μM to about 15 μM, from about 5 μM to about 10 μM

[0171] Publications cited throughout this document are hereby incorporated by reference in their entirety. The present invention is further illustrated, but not limited, by the following examples.

EXAMPLES

[0172] The present invention is further illustrated by the following non-limiting examples.

Example 1

Suspension and Clustering of Human Embryonic Stem Cells of the Cell Line H1 with Dispase/Neutral Protease

[0173] Cells of the human embryonic stem cell line H1, (WA01 cells, WiCell, Madison WI) at passage 41 were washed once with PBS (Catalog# 14190, Invitrogen) and treated with a 1mg/mL solution of Dispase® (Neutral Protease, Sigma Aldrich Co LLC, Catalog# D4818, St. Louis, MO) in DMEM/F12 (Invitrogen Catalog#11330, Grand Island, NY). Cells were incubated at 37°C for 15-25 minutes until colony edges began to curl and lift, but prior to full detachment of colonies from the culture surface. Dispase® was then removed and the culture dish was washed twice with mTeSR®1 (Stem Cell Technologies, Vancouver, BC, Canada) media containing 10 μM Y-27632 (Axxora Catalog#ALX-270-333, San Diego, CA). The mTeSR®1 media containing 10 μM Y-27632 was then added to the culture dish at 5mL/60cm^2 and the cells were lifted from the surface with a scrapper or rubber policeman. Media and cells were then transferred to a 50mL conical tube using a glass pipette and clusters were centrifuged at 90g (rcf) for 3 minutes.

[0174] After centrifugation, media was aspirated and cells were gently re-suspended and briefly triturated in 12mL mTeSR®1 media containing 10 μM Y-27632 per 225-240 cm^2 of total planar culture (equivalent to one T225 flask or four 10cm dishes, approximately 90 million cells). The cell suspension was then transferred (1mL/well) to Ultra Low Binding Culture 6 well dishes (Corning Incorporated, Catalog#3471, Corning, NY) containing 2mL/well of fresh mTeSR®1 media with 10 μM Y-27632. Cells lifted in this manner resembled fragments of monolayer, with the average diameter of lifted fragments around 20-30 microns (Figure 1a) each consisting of clumps of cells. These monolayer fragments were incubated in suspension for 2 hours, (incubation time can range from 0.5-4 hours) at which point aggregates of fragments were observed. The aggregates were then triturated briefly with a glass 10ml pipette, and incubated overnight (the aggregates can proceed directly into suspension) in the low binding plate (aggregates can also be incubated in non-treated cell culture plastic and standard tissue culture treated plastic).

[0175] After overnight incubation (18-24 hours), cells and media were transferred directly to a 125mL spinner flask (Corning Incorporated, Catalog# 4500-125, Corning NY) containing 25 mL mTeSR®1 media stirred at 50 rpm (can range from 30-80+ rpm) to make a final volume of approximately 75mL. Media was changed daily for 4 days. Pluripotency

was determined after 4 days in culture and flow cytometry results showed high expression for the markers of pluripotency (CD9, SSEA4, TRA-1-60, and TRA-1-81) with almost no expression of a marker for differentiation (CXCR4). *See* Figure 1b. These data demonstrate that H1 hES cells can be successfully transferred as cell clusters to suspension culture from a planar adherent culture format with Dispase® as a cell lifting agent and maintain pluripotency in a stirred (dynamic) suspension culture system. This example can also be carried out in shaken rather than stirred suspension systems with plates and Erlenmeyer flasks with comparable results.

[0176] After 4 days in suspension culture (differentiation can also begin 24-120 hours after formation of aggregates, preferably culture for 2-3 days before beginning differentiation), the pluripotent cell aggregates were differentiated with a stage-wise progression of media components to induce the cells to form a pancreatic fate. The spinner agitation was turned up for differentiation of the aggregates to a speed of 65 rpm. The media and components are shown in Table 1a.

[0177] At the end of stage 1 samples were run for flow cytometry and PCR. Suspension differentiated cultures formed a uniform and homogeneous population of cells in loose aggregates at the end of stage1 (Figure 1c), with expression of a marker for pluripotency (CD9) nearly eliminated, while the markers for definitive endoderm differentiation were quite high, 97.2% positive for CXCR4 (CD184) and 97.3% positive for CD99 (Figure 1d). These results correlated with qRT-PCR results which showed a dramatic decrease in the expression of pluripotency genes (CD9, NANOG, and POU5F1/OCT4) and a large increase in genes associated with definitive endoderm (CXCR4, CERBERUS, GSC, FOXA2, GATA4, GATA6, MNX1, and SOX17) versus undifferentiated WA01 hES cells (Figure 1e).

[0178] The definitive endoderm clusters were then further differentiated toward a primitive foregut by removing the TGF-β family member, GDF8, and adding FGF7 to the media. After three days culture with FGF7, the clusters were differentiated to a pancreatic PDX1 expressing fate by addition of all-trans-retinoic acid to either a media containing high glucose (25mM) and low concentration of lipid rich bovine serum albumin (AlbuMAX® (Life Technologies Corporation, Carlsbad, CA)) or a media containing a relatively low glucose concentration (8mM) and 2% fatty acid free bovine serum albumin. The detailed addition of components to these media is listed in Table 1a. At the end of the differentiation the samples were analyzed for expression of markers of pancreatic precursor cells. It was observed that the clusters differentiated with either condition - low glucose + 2% FAF-BSA (A) or high glucose + 0.1% AlbuMAX® (B) - as measured by flow cytometry expressed high levels of

NKX6.1, a transcription factor required for functional β cells, and high levels of endocrine pancreas markers such as synaptophysin and chromogranin (Table 1b). These results were consistent with RT-PCR results which showed high levels of multiple pancreatic precursor genes expressed in samples from both condition A and B (data not shown).

[0179] Typical morphologies of cell clusters as they progressed through differentiation from definitive endoderm (DE) (Figure 1c) to primitive foregut and onto pancreatic endoderm (Figure 1f) demonstrated visible morphological changes to cells and cell clusters. Typically, pluripotent clusters appear dense and dark by phase contrast microscopy, then become looser in appearance as cells progress to primitive foregut in stage 2. This morphology reverses following all-trans-retinoic acid treatment and the clusters again become more dense and uniform with a smooth cluster border.

[0180] Cells differentiated according to condition B through stage 4 were held for an additional 5 days in stage 5 media containing an ALK5 inhibitor (*see* Table 1c). This additional maturation in culture resulted in a significant increase in endocrine marker expression: INS, GCG, SST, PPY, and PCSK1. The cell clusters were then implanted into the kidney capsule of SCID-Bg mice according to IACUC approved study protocol, and the mice were followed for 20 weeks with fasted/fed c-peptide measured every 2 to 4 weeks. After 4 weeks post implantation, following a 20 hour fast and then glucose stimulation, c-peptide was not detectable. By 6 weeks, 2 of 5 mice positive showed some (0.087 & 0.137 ng/mL) human c-peptide, and by 10 weeks, 5 of 5 mice were positive (0.085 - 0.291 ng/mL) for c-peptide. At 16 weeks, following 20 hour fast and glucose stimulation, all 4 mice (4/4) were positive (0.377 – 3.627 ng/mL) for c-peptide expression.

[0181] These results indicate that a pluripotent cell aggregate can be formed and then differentiated in suspension culture to generate a pancreatic precursor cell population characterized by expression of β cell transcription factors like PDX1 and NKX6.1. Furthermore, differentiated cell clusters that were implanted and allowed to mature *in vivo* expressed insulin in response to glucose challenge at physiologically appropriate levels.

Table 1 a: Differentiation Protocol

	Stage 1	Stage 2	Stage 3	Stage 4
Basal Media (final glucose concentration)	MCDB131 8mM glucose		MCDB131 8mM (A) or 25mM glucose (B)	
Protein Supplement	2% Fatty Acid Free Bovine Serum Albumin (FAF-BSA) and 2mM L-Glutamine		2% Fatty Acid Free Bovine Serum Albumin (FAF-BSA) and 2mM L-Glutamine (A) or 0.1% Albumax (Bovine Serum Albumin) and 2mM L-Glutamine (B)	
Growth factors AND/OR Small molecules	MCX (3μM) For 0-24 hours GDF8 (100ng/mL) for 0-96 hours ITS-X (1:50,000)	FGF7 (50ng/ml) ITS-X (1:50,000)	FGF7 (50ng/ml) ITS-X (1:200) RA (2μM) SANT (0.25μM) AA (5ng/mL) LDN (100nM)	ITS-X (1:200) SANT (0.25μM) Cypi (100nM) TppB (500nM) LDN (100nM)
Total Days	**4**	**3**	**4**	**5**
Media Exchanges	Every 24 hours	Every 24 hours	Every 24 hours	Every 24 hours

[0182] As used in this example and throughout the specification, TppB (also known as TpB) is a PKC activator having the chemical name (2S,5S)-(E,E)-8-(5-(4-(trifluoromethyl)phenyl)-2,4-pentadienoylamino)benzolactam and CAS number 497259-23-1.

Table 1b: Flow Cytometry Results for Selected Markers of Differentiation

Glucose (mmol/L)	BSA	% NKX6.1	% SOX9	% Insulin	% Synaptophysin	% Chromogranin
Low (A)	2% FAF BSA	55	7	5.5	34.5	26.5
High (B)	0.1% Albumax	48	7	0.5	26.9	30

Table 1c: Differentiation Protocol

	Stage 5
Basal Media (final glucose concentration)	MCDB131 (25mM glucose)
Protein Supplement	0.1% AlbuMAX® (Bovine Serum Albumin) and 2mM L-Glutamine
Growth factors **AND/OR** **Small molecules**	ITS-X (1:200) Cypi (100nM) LDN (100nM) ALKVi (10mM)
Total Days	**5**
Media Exchanges	Every 24 hours

Example 2

Suspension and Clustering of Human Embryonic Stem Cells of the Cell Line H1 with EDTA

[0183] Cells of the human embryonic stem cell line H1, (WA01 cells, WiCell, Madison WI) at passage 41 were washed once with PBS (Catalog# 14190, Invitrogen) and treated with EDTA, a non-enzymatic cell lifting/passaging agent (Lonza, Catalog# 17-7-11E,). Cells were incubated at room temperature for 8 minutes. EDTA was then removed and after 1 or 2 more minutes (9-10 minutes total EDTA exposure) the plate was rinsed with mTeSR®1 media containing 10 μM Y-27632 (Axxora Catalog#ALX-270-333, San Diego, CA) and dislodged cells were collected in a 50 ml conical tube using a glass pipet. One additional rinse of the plate with mTeSR®1 media containing 10 μM Y-27632 was performed and pooled with dislodged cells. Note that some cells remained on the plate after 9-10 minutes of exposure to EDTA at room temperature, and lifted cells were not completely disaggregated to a single cell suspension. Instead, the cells were removed from the surface as small aggregates. Media and cells were then transferred to a 50 ml conical tube using a glass pipet and a cell count was performed (NucleoCounter®-ChemoMetec A/S, Cat#YC-T100, Denmark). Additional mTeSR®1 media containing 10 μM Y-27632 was added as needed to make a concentration of cells at 1.0 to 1.5 million cells/ml.

[0184] Cells were not centrifuged, as the clusters were loosely aggregated and would disassociate to single cells if centrifuged to a pellet and re-suspended by pipette. Instead, media and cells in the tube were gently swirled until a uniform suspension was formed. If desired, one can also lengthen the period of EDTA treatment and take cells to near a single cell suspension. The cell suspension was then transferred to two non-tissue culture treated 6 well dishes (Becton Dickinson, Catalog# Falcon 351146, Franklin Lakes, NJ) in a 37°C humidified 5% CO_2 incubator at 3 ml/well with a glass pipette. Cells were incubated in suspension for 2 hours at which point aggregates were observed. The aggregates were then triturated by gentle pipetting with a glass pipette to disrupt large aggregates and create a homogeneous, uniform cluster suspension, then incubated undisturbed overnight.

[0185] Then 18-24 hours later, cells and media were spun down in 50mL conical tubes at 90g (rcf) for 3 minutes. The spent media supernatant was discarded, the cell aggregates were suspended in fresh mTeSR®1 and the suspension was transferred to a spinner flask (Corning Incorporated, Catalog#4500-125, Corning NY) stirred at 55 rpm in a 37°C humidified 5% CO_2 incubator. Media was changed daily for 2 days. Pluripotency was determined after 2 days in stirred suspension culture before transition to differentiation culture. The flow cytometry results for CD9, SSEA4, TRA-1-60, TRA-1-81, and CXCR4 expression are shown in scatter plot format in Figure 2a. These data show high expression for the markers of pluripotency (CD9, SSEA4, TRA-1-60, TRA-1-81) and low or no expression of a marker for differentiation (CXCR4). These results indicate that H1 hES cells can be transferred to suspension culture from a planar adherent culture format using a non-enzymatic lifting method and maintain pluripotency in a dynamic agitated suspension culture system.

[0186] After 2 days in suspension culture, the pluripotent cell aggregates were differentiated with a stage-wise progression of media components to induce the cells to form a pancreatic fate. The spinner agitation was maintained at a speed of 55 rpm. The media and components are shown in Table 2a.

[0187] At the end of stage 1 samples were run for flow cytometry and PCR. Suspension differentiated cultures formed a uniform and homogeneous population of cells in loose aggregates at the end of stage1 (Figure 2b), with expression of a marker for pluripotency (CD9) nearly eliminated, while CXCR4 (CD184), a marker for definitive endoderm differentiation, was quite high, 95.9% ± 1.8sd (Figure 2c) across three spinner flasks. These results correlated with qRT-PCR results which showed a dramatic decrease in the expression of pluripotency genes (CD9, NANOG, and POU5F1/OCT4) and a large increase in genes

associated with definitive endoderm (CXCR4, CERBERUS, GSC, FOXA2, GATA4, GATA6, MNX1, and SOX17) versus undifferentiated WA01 hES cells (Figure 2d).

[0188] The definitive endoderm clusters from spinner flasks were then pooled and distributed to either another spinner flask or an Erlenmeyer flask (shaken agitation system) and directed for further differentiation toward a primitive foregut by removing GDF8, and adding FGF7 to the media. After three days culture with FGF7, the clusters were differentiated to a pancreatic PDX1 expressing fate by addition of all-trans-retinoic acid to a media containing a relatively low glucose concentration (8mM) and 2% fatty acid free bovine serum albumin. The detailed addition of components to these media is listed in Table 2a. At the end of the differentiation the samples were analyzed for expression of markers of pancreatic precursor cells. Using flow cytometry, high levels of NKX6.1, a transcription factor required for functional β cells, and high levels of endocrine pancreas markers such as synaptophysin and chromogranin (Table 2b and Figure 2e) were observed with both suspension formats. These results were consistent with RT-PCR results which showed very similar high levels of multiple pancreatic precursor genes expressed in samples generated in spinner flask format or Erlenmeyer flask format (Figure 2f).

[0189] These results demonstrate that a pluripotent cell aggregate can be formed and then differentiated in suspension culture in multiple suspension culture formats, including a stirred system or a shaken suspension system, to generate a pancreatic precursor cell population characterized by expression of β cell transcription factors like PDX1 and NKX6.1.

Table 2a: Media Components and Differentiation Protocol

	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4
Basal Media	mTeSR®1	MCDB131 (8mM Glucose) 3.64g/L $NaCO_3$	MCDB131 (8mM Glucose) 2.41g/L $NaCO_3$		
Supplement	mTeSR®1	2% FAF-BSA 1:50,000 ITS-X 1x GlutaMax		2% FAF-BSA 1:200 ITS-X 1x GlutaMax	
Growth factors		GDF8 *(d2 only)* 100ng/ml	FGF7 50 ng/ml	FGF7 50 ng/ml	
Small molecules	Y-27632 (*day 1 only*) [10 µM]	MCX *(0-24 hours)* [2 µM]		RA [2 µM] SANT [0.25 µM] TPPB [100 nM] *LDN (Day 1 only)* [100 nM]	SANT [0.25 µM] Cypi [100 nM] ALK5 inh [1 µM] TPPB [100 nM]
Days	**3**	**3**	**3**	**3**	**3**
NOTES:	**1 d NTCT 2 days SF**	**Media change Day 1 and 2, No change d3**	**Media change Day 1 and 3, No change d2**	**Media change Day 1 and 2, No change d3**	**Media change d1 And d2, No change d3**

Table 2b: Flow Cytometry Results for Selected Markers of Differentiation

Sample	%NKX6.1	%CDX2	%SOX2	%NKX2.2	%Synaptophysin	%Chromogranin
Spinner Flask (avg)	68.1	4.0	31.3	36.2	27.0	25.3
Erlenmeyer Flask	65.8	7.9	28.1	30.0	30.7	17.0

Example 3

Suspension Clustering and Serial Suspension Passage of Human Embryonic Stem Cells of the Cell Line H1

[0190] Cells of the human embryonic stem cell line H1, (WA01 cells, WiCell, Madison WI) at passage 40 grown on tissue culture treated polystyrene coated with Matrigel® (Corning Incorporated, Corning NY) were washed twice with PBS (Catalog# 14190, Invitrogen) and treated with a half strength solution of Accutase® (one part PBS to one part Accutase®, Sigma-Aldrich, Catalog# A-6964, St. Louis, MO). Cells were incubated at room temperature for 3 ½ minutes. (Accutase® is a cell detachment solution comprised of collagenolytic and proteolytic enzymes (isolated from crustaceans) and does not contain mammalian or bacterial

derived products.) Accutase® was then removed and after 3 more minutes (6 1/2 minutes total Accutase® exposure), the plate was rinsed with mTeSR®1 media containing 10 μM Y-27632 and dislodged cells were collected in a 50 ml conical tube using a glass pipet. One additional rinse of the plate with mTeSR®1 media containing 10 μM Y-27632 was performed and pooled with dislodged cells. Some cells remained on the plate after the exposure to Accutase® and lifted cells were not completely disaggregated to a single cell suspension. Rather the cells were removed from the surface as small aggregates (Figure 3a). Media and cells were then transferred to a 50 ml conical tube using a glass pipette and a cell count was performed. Additional mTeSR®1 media containing 10 μM Y-27632 was added as needed to make a concentration of cells at 1.0 to 1.5 million cells/ml.

[0191] Cells were not centrifuged, as the clusters were loosely aggregated and would disassociate to single cells if centrifuged to a pellet and resuspended by pipette. Instead, media and cells in the tube were gently swirled until a uniform suspension was formed. The cell suspension was then transferred to two ultra-low binding culture 6 well dishes in a 37°C humidified 5% CO_2 incubator at 3ml/well with a glass pipette. Cells were incubated in suspension for 90 minutes at which point aggregates were observed. The aggregates were then triturated briefly, and transferred directly to a 125ml spinner flask containing 25 ml mTeSR®1 media stirred at 55 rpm (total final volume was approximately 75mL). Media was changed daily for 3 days, and pluripotency was determined on the 3rd day in culture. Phase contrast microscope images of the clusters show a uniform, spherical population of clusters that formed after 90 minutes in static suspension culture and expanded over three days in culture (Figure 3b). At the end of three days in suspension culture, the cells were assayed for pluripotency by flow cytometry results for the markers CD9, SSEA4, TRA-1-60, TRA-1-81, and CXCR4. The cells maintained high expression of markers for pluripotency (CD9, SSEA4, TRA-1-60, TRA-1-81) and almost no expression for CXCR4, a marker of differentiation (Table 3). These data show that H1 hES cells can be transferred to suspension culture from a planar adherent culture format using an enzymatic lifting method, such as Accutase®, and will maintain pluripotency in a dynamic agitated suspension culture system.

[0192] The pluripotent clusters were then serially passaged using Accutase® dissociation for an additional 20 passages. At each passage, 50 million cells were gravity settled for 2 minutes in a 50 ml conical tube, washed twice with PBS and treated with a half strength solution of Accutase® in a 37°C water bath with gentle swirling of the tube at two and four minutes after addition of Accutase®. After six minutes incubation Accutase® was aspirated from the tube without disturbing the cell pellet. The cells were then incubated 3 more

minutes (9 minutes total Accutase® exposure). The tube was then rinsed with mTeSR®1 media containing 10 μM Y-27632, triturated twice using a glass pipet, and the suspended cells passed through a 70 micron cell strainer (BD Falcon, Cat#352350, Franklin Lakes, NJ). Two additional rinses of the tube with mTeSR®1 media containing 10 μM Y-27632 were performed and passed through the cell strainer.

[0193] Media and cells in the tube were gently swirled until a uniform suspension was formed. The cell suspension was then transferred to ultra-low binding culture 6 well dishes in a 37°C humidified 5% CO_2 incubator at 3ml/well with a glass pipette and incubated in suspension for 2 hours (tested 0-28 hrs) at which point aggregates were transferred to a glass spinner flask with a final volume of 80 ml of media. Alternatively, the cell suspension could be directly placed into a spinner flask agitated at 55 rpm or an Erlenmeyer flask shaken at 40 rpm, and clusters formed in the stirred suspension (Figure 3c) in a final volume of 80 ml of media.

[0194] Using this serial passage method, the cells were passaged 20 times, with an approximate split ratio of 1:3 at each passage. Pluripotency was measured at each passage by flow cytometry and karyotype was determined using a florescent *in-situ* hybridization (FISH) assay for chromosomes 12 and 17; two chromosomes identified as potentially unstable in hES cells. The flow cytometry results for CD9, SSEA4, TRA-1-60, TRA-1-81, and CXCR4 expression are shown in scatter plot format and show high expression for the markers of pluripotency and low or no expression of a marker for differentiation (CXCR4), while FISH assays for chromosomes 12 and 17 showed normal copy number. These data indicate that H1 hES cells can be maintained in suspension culture with routine serial passage using Accutase®, a non-mammalian, enzymatic cell dissociation method, and will maintain pluripotency and stable karyotype in a dynamic agitated suspension culture system, generating $1x10^9$ cells per original input cell over the course of 20 passages. EDTA can also be used for this serial suspension for 6 passages.

Table 3: Flow Cytometry for Pluripotency of the Cells as a Function of Time based on Results for the Markers CD9, SSEA4, TRA-1-60, TRA-1-81, and CD184 (CXCR4)

Passage (culture day)	CD9	SSEA4	TRA-1-60	TRA-1-81	CD184
1 (3)	92.0%	100.0%	57.4%	58.6%	0.2%
2 (4)	73.3%	99.9%	63.5%	54.3%	0.1%
3 (3)	87.5%	99.7%	65.8%	63.6%	0.1%
4 (4)	86.7%	99.8%	60.9%	68.2%	0.1%
5 (3)	79.3%	99.7%	67.6%	69.9%	0.3%
6 (3)	79.3%	99.7%	67.6%	69.9%	0.3%
7 (3)	93.7%	100.0%	60.1%	58.8%	0.2%
8 (3)	83.0%	99.0%	73.0%	68.0%	0.5%
9 (4)	94.6%	100.0%	65.5%	64.2%	0.1%
10 (4)	96.3%	100.0%	77.3%	75.0%	0.2%
11 (4)	97.3%	100.0%	69.1%	61.3%	0.2%
12 (4)	91.6%	100.0%	56.9%	62.7%	0.6%
13 (4)	97.3%	99.9%	62.9%	63.2%	0.2%
14 (4)	97.1%	100.0%	71.1%	82.4%	1.0%
15 (4)	96.1%	99.6%*	79.0%	74.2%	0.2%
16 (4)	87.7%	99.9%	77.1%	72.5%	0.3%
17 (4)	98.6%	99.7%	69.9%	57.7%	0.3%
18 (4)	97.7%	100.0%	68.6%	56.6%	0.2%
19 (4)	97.1%	100.0%	79.4%	70.4%	0.1%
20 (4)	96.9%	100.0%	57.4%	55.7%	0.4%

Example 4

Directed Differentiation of Suspension Cultured Human Embryonic Stem Cells of the Cell Line H1

[0195] Cells of the human embryonic stem cell line H1, (WA01 cells, WiCell, Madison WI) at passage 40 were lifted from a planar adherent culture using Accutase® and transferred to suspension culture format. The cells were maintained in a dynamic agitated suspension culture system for 30 passages using the method described in Example 3.

[0196] Pluripotency was confirmed through the first 20 passages as shown in Table 3, with stable high levels of pluripotency markers maintained throughout the culture, as measured by flow cytometry. To confirm pluripotency and demonstrate their ability to provide a cell source for treatment of diabetes, cells were differentiated to a pancreatic precursor in a dynamic agitated suspension culture system through a step-wise progression of different media containing morphogens or growth factors intended to recapitulate normal pancreatic

development. This process gives rise to a pancreatic precursor cell population characterized by a high PDX1 and NKX6.1 co-expression. When these cells were transplanted, they matured further to functional glucose stimulated insulin secreting tissue able to secrete insulin in response to glucose and maintain normal blood glucose in a streptozotocin induced model of diabetes. *See* Figure 4c and Table 4c.

[0197] In order to generate these pancreatic precursor cells, H1 human embryonic stem cells that had been expanded and maintained in a dynamic agitated suspension culture system for 16 passages were differentiated using the method described in Example 3. In summary, the cells were expanded for 30 passages, tested for pluripotency for the first 20 of these passages; the cells were differentiated on the 16^{th} passage. Pluripotent cells in cluster format were transferred from mTeSR®1 media to FBC solution (Table 4a) at 4°C for 3 hours. Cell clusters were then moved to a 3 liter glass suspension bioreactor regulated by a Sartorius Stedim Biostat B-DCU (Goettingen, Germany) control unit and suspended in differentiation media at 0.55 million cells/mL according to Table 4b. The cells were maintained 14 days in the closed sterile suspension bioreactor regulated for temperature, pH, and dissolved oxygen (DO) (FermProbe® pH electrode 225mm, Model # F-635, and dissolved oxygen OxyProbe® 12mm Sensor, model number D-145 from Broadley-James Corporation, Irvine CA).

[0198] Throughout the run, media bicarbonate levels were maintained at 3.64g/L with pH maintained at pH 7.4 by regulation of CO_2 flow in a total media volume of $\leq$1.6 liters. The bioreactor head space was continuously perfused with CO_2, air, and O_2, under control of the Sartorious control system with a 20% dissolved oxygen set-point for stage 1 and a 30% dissolved oxygen set-point for stage 2 onward with a constant gas flow of 150cc/minute. Oxygen flow was regulated in response to dissolved oxygen content and CO_2 flow was regulated in response to pH. Temperature was maintained at 37°C throughout the run by an electric heated jacket. At the initiation of the run and for each media exchange (93% of media removed per exchange) the impeller (3" stainless steel pitch blade impeller operated at 70 rpm) was stopped and media was removed or added by peristaltic pump through a dip tube in the bioreactor connected to C-Flex® tubing using a Terumo™ tube welder to maintain a closed system. Images of cells/clusters were taken at the end of each stage of differentiation, and flow cytometry samples were collected and assayed for CXCR4 expression at stage 1 day 3 and 3 days later at the end of stage 2 (Figure 4a). A near total population transition from a CXCR4 negative pluripotent cell population at the initiation of differentiation (Table 3, passage 16) to a population of CXCR4 expressing (98.5% of cells CXCR4 positive, Figure 4b) cells was observed. These cells then transitioned to a nearly CXCR4 negative state 3

days later at the end of stage 2 (7.0% of cells CXCR4 positive), and by the end of stage 3 the cells had almost completely transitioned to a CD56 positive state. At the end of the differentiation process on day 4 of stage 4, the cells were 88.5% positive for PDX1 expression (Figure 4b) and showed an expression pattern consistent with a mix of pancreatic endocrine cells (33.5% chromogranin positive) and pancreatic progenitor cells (65.7% positive for NKX6.1). This stage specific marker expression pattern indicated an efficient stage-wise differentiation from a pluripotent population to pancreatic cells. At the end of the differentiation process 2.77 million cells/ mL were generated (4.1 billion cells in 1.5 Liter), indicating a total mass expansion of 5 differentiated cells per each input hES cell.

[0199] At the end of the run, 500mL were removed for centrifugation and washing and were tested in an animal model of engraftment, maturation, and function. The remaining 1000mL of cell suspension was processed in a kSep®400 system (KBI Biosystems, Durham NC) to wash, filter, and concentrate the cell product in a fully closed loop system. The cell product was concentrated from a starting volume of 1 liter to 50mL of concentrated cells at a final concentration of 41 million cells/mL. These concentrated cells were then dispensed into 24 vials with 1.2 ml fill volume using an automated vial fill machine (Fill-It, TAP, Hertfordshire UK) and frozen by placing into a liquid nitrogen freezer.

[0200] The 500mL differentiated cells that were washed and concentrated by standard centrifugation were transplanted at a dose of 5 million cells per SCID-Bg mouse placed either directly under the kidney capsule, or placed inside an immune-protective macro encapsulation device (TheraCyte™, Irvine CA) that was implanted subcutaneously (6 animals per condition). By 12 weeks post implantation, the implanted cells expressed significant levels of circulating human C-peptide (>0.1ng/mL) as detected by ELISA (human c-peptide custom ELISA Mercodia cat# 10-1141-01) in response to fasting and then feeding and by 16-20 weeks animals had over 1ng/mL of circulating c-peptide (Table 4c).

[0201] At 27 weeks (190 days) post implantation, two animals with device encapsulated immune-protected grafts were each treated with a single high dose of streptozoticin (STZ) to selectively kill all endogenous mouse β islet cells and induce diabetes (250mg/Kg). For the next two weeks after an STZ treatment sufficient to induce frank diabetes in a control animal the engrafted animals' blood glucose levels remained within normal range (<150mg/dL). At 29 weeks post implantation and two weeks after STZ administration the two animals were then tested for glucose stimulated insulin secretion (GSIS) and showed a marked increase in circulating human c-peptide in response to glucose administration. Furthermore, when each

of the grafts were removed at day 209 (29.5 weeks) post implantation, the animals' blood glucose levels increased dramatically to >500mg/dL.

[0202] These results demonstrate that a human embryonic stem cell derived cell product to treat diabetes can be prepared from suspension of expanded and differentiated stem cells. The product can be generated in a scalable, stirred, closed loop bioreactor system and the cell product can be processed with a closed loop wash and concentration as required for commercial cGMP manufacturing. This human embryonic stem cell derived cell product can treat diabetes in a widely used animal model of diabetes as shown by GSIS competence, ability to regulate blood glucose, and the return to a diabetic state upon removal of the cell therapy.

Table 4a Composition of FBC solution

Component	Amount (mg/L)	Function	Grade
Dextrose, Anhydrous	901	Sugar	USP[a]
Potassium Chloride	559	Salt	USP
Sodium Bicarbonate	2000	Buffer	USP
Sucrose	6846	Sugar	USP
Mannitol	3644	Sugar Alcohol	USP
Calcium Chloride Dihydrate ($CaCl_2.2H_2O$)	70	Salt	USP
Magnesium Chloride ($MgCl_2.6H_2O$)	1017	Salt	USP
Potassium Bicarbonate ($KHCO_3$)	500	Buffer	USP
Potassium Monophosphate (KH_2PO_4)	1361	Buffer	NF[b]/FCC[c]
Lactobionic Acid	35830	Cell Stabilizer	NA[d]
L-Glutathione	922	Anti-oxidant	NA
HCl	To adjust pH	Acid	ACS[e]
Sodium Hydroxide	To adjust pH	Base	NF/FCC
Water for Injection (WFI)	To prepare the solutions	To prepare the solutions	USP

[a] USP = United States Pharmacopeia

[b] NF = National Formulary

[c] FCC = Food Chemicals Codex

[d] NA = Not applicable

[e] ACS = American Chemical Society

Table 4b: Media Components and Differentiation Protocol

	Stage 1	Stage 2	Stage 3	Stage 4
Basal Media **(final glucose conc.)**	MCDB131 3.64 g/l $NaCO_3$ (8 mM glucose)			
Protein /Amino Acid Supplement	2% Fatty Acid Free Bovine Serum Albumin (FAF-BSA) and 2mM L-Glutamine			
Growth factors **AND/OR** **Small molecules**	MCX (3 µM) For 0-24 hours GDF8 (100 ng/mL) for 24-72 hours ITS-X (1:50,000)	FGF7 (50ng/ml) ITS-X (1:50,000)	FGF7 (50 ng/ml) ITS-X (1:200) RA (2µM) SANT (0.25 µM) AA (5 ng/mL) TppB (200 nM) LDN (100 nM) for 0-24 hours stage3	ITS-X (1:200) SANT (0.25 µM) Cypi (100 nM) SCIO (2µM) TppB (100 nM)
Total Days	**3**	**3**	**3**	**5**
Media Exchanges	Time 0 and 24 hours	Time 0 and 48 hours	Time 0 and 24 hours	Time 0 and 48 and 96 hours

(Nomenclature: Time 0= first feeding of the new stage; Time 24, 48 or 96 hours = time after new stage media)

Table 4c: C-peptide expression (ng/mL)

C-Peptide (ng/mL)	4wk	8wk	12wk	16wk	20wk	24wk	29wk
Kidney Capsule Implant (N=6)	0.00	0.03	0.19	0.95	2.56		
STDEV	0.00	0.03	0.17	0.71	1.33		
Theracyte Device Implant (N=6)	0.00	0.02	0.35	0.58	1.45	2.49	2.85
STDEV	0.01	0.01	0.54	0.51	1.02	0.75	0.21

Example 5

Directed Differentiation in Suspension Format of Adherent Cultured Human Embryonic Stem Cells of the Cell Line H1

[0203] Cells of the human embryonic stem cell line H1, (WA01 cells, WiCell, Madison WI) at passage 41 were lifted from a planar adherent culture using EDTA and transferred to suspension culture format using the method described in Example 2.

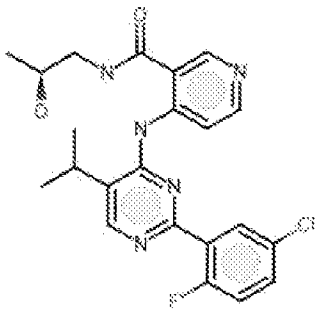
[0204] Pluripotency of the cellular aggregates was measured by flow cytometry as shown in Figure 5a and high expression of the pluripotency markers CD9, SSEA4, TRA-1-61, and TRA-1-80, indicating the cells were highly pluripotent, was observed. These pluripotent cells were then differentiated to a pancreatic precursor in a dynamically agitated suspension culture system through a step-wise progression of different media containing small molecules and growth factors intended to recapitulate morphogen drivers of normal pancreatic development. This process produces a pancreatic precursor cell population characterized by co-expression of the pancreatic cell transcription factors, PDX1 and NKX6.1. When these cells are transplanted they mature further to functional glucose stimulated insulin secreting tissue which can correct high blood glucose in a streptozotocin induced model of diabetes.

[0205] In order to generate the pancreatic precursor cell population, pluripotent cells in cluster format maintained in mTeSR®1 media were transferred to a 0.2 liter glass stirred suspension bioreactor (Dasgip, Catalog#DS0200 TBSC, Shrewsbury, MA) with controller regulated temperature, pH, and dissolved oxygen. Pluripotent cell clusters were cultured in the bioreactor for two days. At that time (stage 1, day 0) the media was exchanged and differentiation was initiated as the cell aggregates were suspended at approximately 0.7 million cells/mL in differentiation media according to Table 5a. The cells were then maintained in this closed sterile suspension bioreactor for 14 days. Throughout differentiation, media bicarbonate levels were maintained at 3.64g/L with pH maintained at 7.4 by regulation of CO_2 flow in a total volume of 0.3 liter. The bioreactor head space was sparged with CO_2 and air under control of the Dasgip control system with a 30% dissolved oxygen set-point under a constant gas flow of 5 liters/hour. Air flow was regulated in response to dissolved oxygen content and CO_2 flow was regulated in response to pH.

Table 5a: Media Components and Differentiation Protocol

	Stage 1	Stage 2	Stage 3	Stage 4
Basal Media **(final glucose concentration)**	MCDB131 3.64 g/l $NaCO_3$ (8 mM glucose)	MCDB131 3.64 g/l $NaCO_3$ (8 mM glucose)	MCDB131 3.64 g/l $NaCO_3$ (8 mM glucose)	MCDB131 3.64 g/l $NaCO_3$ (8 mM glucose)
Protein Supplement	2% Fatty Acid Free Bovine Serum Albumin (FAF-BSA) and 2mM L-Glutamine			
Growth factors **AND/OR** **Small molecules**	MCX (3 µM) As specified GDF8 (100ng/mL) As specified ITS-X (1:50,000)	FGF7 (50 ng/ml) ITS-X (1:50,000)	FGF7 (50 ng/ml) ITS-X (1:200) RA (2 µM) SANT (0.25 µM) AA (5 ng/mL) TppB (200 nM) LDN (100 nM) for 0-24 hours stage3	ITS-X (1:200) SANT (0.25 µM) Cypi (100 nM) SCIO (2µM) TppB (100 nM)
Total Days	**3**	**3**	**3**	**5**
Media Exchanges	As specified	Time 0 and 48 hours	Time 0 and 24 hours	Time 0 and 48 and 96 hours

[0206] As used in this example and throughout the specification, SCIO is an Alk5 inhibitor having the chemical name 4-{[2-(5-Chloro-2-fluorophenyl)-5-(1-methylethyl)pyrimidin-4-yl]amino}-N-(2-hydroxypropyl)pyridine-3-carboxamide and CAS number 674794-97-9. The chemical structure of SCIO is shown below:



[0207] Temperature was maintained at 37°C throughout the run. At the initiation of the run and for each media exchange (95% of media removed per exchange) the impeller was

stopped and media was removed and then added by peristaltic pump through a bioreactor dip tube connected to C-Flex® tubing using a Terumo™ tube welder to maintain a closed system.

[0208] Several different feed settings were tested during stage 1: (a) media change 24 hours after initiation of differentiation, no media change at 48 hours; (b) media change 24 hours after initiation of differentiation and glucose bolus addition at 48 hours; and (c) no media change throughout stage 1 with glucose and GDF8 bolus added 24 hours after initiation of differentiation, then a glucose bolus added at 48 hours post initiation.

[0209] Cell counts at the initiation, middle, and end of the process were taken for each reactor as listed in Table 5b. At the end of stage 1, cells were sampled for protein expression patterns by flow cytometry. Cells differentiated in condition A- media change 24 hours after initiation of differentiation to definitive endoderm, then no media change for next 48 hours – showed the best results as measured by induction of markers of differentiation (CD99 and CXCR4) and reduction in pluripotency marker expression (CD9) (Figure 5b). The higher expression of CXCR4 and CD99 in combination with lower expression of CD9 at the end of definitive endoderm formation correlated with the higher expression of pancreatic genes and lower expression of genes indicative of alternate organ fates later in differentiation (Figures 5d and 5e). Specifically, one or both of not changing media throughout the first stage of differentiation or adding glucose to the media in stage 1 in a bulk feeding format resulted in lower CXCR4 levels at the end of stage 1 which correlated with very different aggregate morphologies at the end of the four stage differentiation (Figure 5c). Specifically, conditions B and C had lower pancreatic gene expression (NKX6.1 and CHGA) and higher expression of non-pancreatic genes (CDX2 and SOX2) at the end of stage 4 as measured by flow cytometry (Figure 5d and Table 5b). These findings were borne out by qRT-PCR (Figure 5e), as condition A showed significantly higher expression of pancreatic genes than condition C, with condition B intermediate to A and C. Furthermore, Condition C expressed significantly higher levels of genes indicative of an alternative non-pancreatic fate, *e.g.* CDX2, AFP, and Albumin (Figure 5e). These data indicate that a homogeneous, high CXCR4 expressing definitive endoderm (DE) generated without a media change for the last 48 hours of DE formation is able to convert later to a pure pancreatic endoderm population.

[0210] At the end of the four stage differentiation, the cells differentiated according to condition A were removed from the bioreactor, washed with MCDB131 media containing 0.1% FAF-BSA and implanted in SCID-Bg mice. Each mouse was transplanted with 5 million cells directly under the kidney capsule. Every 4 weeks after implantation blood

draws were performed and blood glucose and c-peptide were measured. By 12 weeks post implantation, human c-peptide was detectable by ELISA at levels above 1ng/mL, and at 16 weeks c-peptide levels were an average of 2.5ng/mL (Figure 5f). At 20 weeks post-implantation c-peptide was measured in the animals in a fasted and then fed state. Glucose treatment induced a significant increase in circulating human c-peptide from 0.93ng/mL in a fasted state to 2.39ng/mL in a fed state (Figure 5g) indicating that the transplanted cells had matured to functional GSIS competent tissue. Furthermore, when the animals were given a streptozotocin (STZ) administration (mouse β cells are more sensitive to and preferentially destroyed by STZ compared to human β cells) to induce a diabetic state, the animals with a graft of functional GSIS competent tissue maintained normal blood glucose levels unlike the untreated controls which developed frank diabetes (Figure 5h). These results demonstrate that animals with a hES differentiated cell graft were protected from STZ induced diabetes by a functional pancreatic tissue graft.

Table 5b: Cell Counts and Flow Cytometry Data

Pluripotency (Condition)	**Viable Cell density (Million cells/mL)**	**CD9**	**CD184**	**SSEA4**	**TRA-1-60**	**TRA-1-81**	
(A)	0.723	93.8	0.2	100	74.3	67.3	
(B)	0.677	92.3	0.2	100	71.7	71	
(C)	0.738	89.9	0.1	100	75.3	72.1	
DE (Condition)	**Viable Cell density (Million cells/mL)**	**CD9**	**CD184**	**CD99**			
(A)	0.965	1.7	99.6	84.3			
(B)	1.22	4.8	93.1	81.2			
(C)	1.2	8.3	68	34.1			
PE (Condition)	**Viable Cell density (Million cells/mL)**	**NKX6.1**	**Synaptophysin**	**CDX2**	**SOX2**	**NKX2.2**	**CHGA**
(A)	0.795	47.5	48.4	2.9	23.8	61.7	55.7
(B)	0.98	44.4	38.5	10.3	21.4	45.4	41.5
(C)	1.33	15.4	5.8	37	18.4	9.6	6.7

Example 6

Directed Differentiation in Suspension Format of Microcarrier Adherent Cultured Human Embryonic Stem Cells of the Cell Line H1

[0211] Cytodex® 3 Microcarrier beads (C3) (Sigma-Aldrich Co LLC, St. Louis, MO, Catalog # C3275) were prepared for culture by soaking 400mg of the beads in 20ml volume silicon coated glass scintillation vials containing 15ml Dulbecco's PBS (DPBS), for 4-24 hours. Cytodex® 3 consists of a thin layer of denatured collagen chemically coupled to a matrix of cross-linked dextran. The denatured collagen layer on Cytodex® 3 is susceptible to digestion by a variety of proteases, including trypsin and collagenase, and provides the ability to remove cells from the microcarriers while maintaining maximum cell viability, function, and integrity.

[0212] After soaking, the beads were autoclaved and rinsed with sterile DPBS and re-suspended in mouse embryonic fibroblast conditioned media (MEF-CM) supplemented with 10 μM Y-27632. The beads were then transferred to 125ml Corning® glass spinner flasks (Corning Incorporated, Corning, NY) at a density of 100mg beads/flask. The spinner containing beads and MEF-CM with Y-27632 was equilibrated in a humidified 5% CO_2 incubator at 37°C for at least 60min.

[0213] Cells of the human embryonic stem cell line H1, (WA01 cells, WiCell, Madison WI) at passage 44 were lifted from a planar adherent culture using TrypLE™ (Life Technologies Corporation, Grand Island, NY) (8 minute incubation at 37°C to form a single cell suspension). The cells were then washed and suspended in MEF-CM with Y-27632 and 11 million hES cells were allowed to adhere to the beads for 6 hours in a static (still) incubation period. MEF-CM with Y-27632 was then added to a spinner flask to make a final media volume of 75mL, and the cells and beads were agitated in the glass spinner flask at an impeller speed of 50 rpm. The cells were grown in this manner for 5 days with a daily 50mL media exchange of MEF-CM. After 5 days in culture, the flasks contained $53x10^6$ cells (± $12x10^6$ SD). As a control, one million H1 hES cells were also seeded to 6 well tissue culture polystyrene dishes coated with a 1:30 dilution of Matrigel™ and maintained with a daily media change of MEF-CM.

[0214] After 5 days in pluripotent culture, these cells were then differentiated to a pancreatic precursor in a dynamic agitated suspension culture system through a step-wise progression of different media containing one or both of small molecules and growth factors

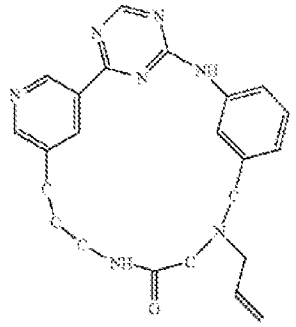
intended to recapitulate normal pancreatic development morphogens. Two media formulations were tested- as a method to recapitulate normal pancreatic development; one which used Activin A and Wnt3A to form DE, and another that used the MCX compound with GDF8 to form DE (Tables 6a and 6b, respectively). Media was changed daily, and samples were characterized by RT-PCR and flow cytometry to determine the cell properties. Phase contrast images of the cells on micro-carriers were taken and a time course of the cell morphology as pluripotent culture before differentiation of the cells was initiated is shown in Figure 6a. A time course showing the culture differentiating is shown in Figure 6b. A cell count was also taken at various time points through the experiment, and the results are presented as a function of surface area (cells/cm^2 in Figure 6c) or media volume (cells/mL in Figure 6d) for the media formulations in either a planar culture or a suspended microcarrier culture.

[0215] The cells were characterized at various points throughout the process by both flow cytometry and RT-PCR. Flow cytometry results for the first stage of differentiation, the formation of definitive endoderm, are shown as a dot plot of cell expression of CXCR4 (Y-axis) and CD9 (X-axis) in Figure 6e and the results are also expressed as total expression of each marker in Figure 6f. The results indicate that in all conditions a substantial majority of the cells form definitive endoderm, as defined by gain of CXCR4 expression and loss of the pluripotency surface marker, CD9. Furthermore, the more efficient formation of definitive endoderm occurs in rank order of treatment from MCX / GDF8 MicroCarriers > MCX / GDF8 Planar > WNT3A / AA MicroCarriers > WNT3A / AA Planar. There does appear to be a media specific effect on the cells, as cells treated with MCX / GDF8 show lower expression of CERBERUS (Cer 1), GOOSECOID, and FGF17 (Figure 6g) However, all treatment conditions show similar expression levels of definitive endoderm genes; CD99, CXCR4, FOXA2, KIT, and SOX17 (Figure 6g and Table 6c). These processes generate a pancreatic precursor cell population characterized by co-expression of the pancreatic cell transcription factors, PDX1 and NKX6.1. When these cells are transplanted they mature further to functional glucose stimulated insulin secreting tissue which can correct high blood glucose in a streptozotocin induced model of diabetes.

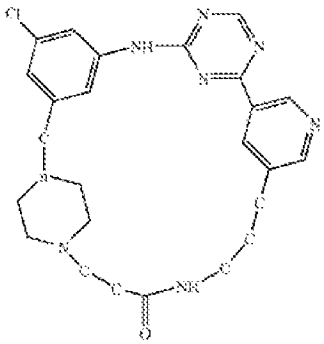
[0216] As used in Table 6a below, B27 is Gibco® B-27® Supplement (Life Technologies Corporation, Carlsbad, CA).

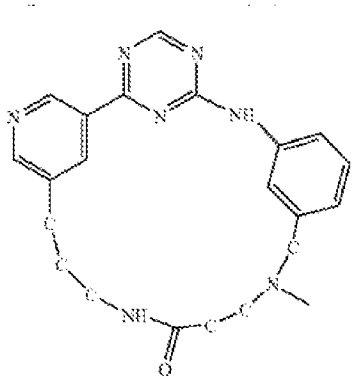
[0217] As used in this example, the MCX compound is 14-Prop-2-en-1-yl-3,5,7,14,17,23,27-heptaazatetracyclo[19.3.1.1~2,6- ~.1~8,12.~]heptacosa-

1(25),2(27),3,5,8(26),9,11,21,23-non- aen-16-one, which has the following formula (Formula 1):



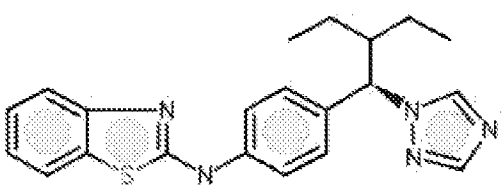
[0218] Other cyclic aniline-pyridinotriazines may also be used instead of the above-described MCX compound. Such compounds include but are not limited to 14-Methyl-3,5,7,14,18,24,28-heptaazatetracyclo[20.3.1.1~2,6~.- 1~8,12 ~]octacosa-1(26),2(28),3,5,8(27),9,11,22,24-nonaen-17-on- e and 5-Chloro-1,8,10,12,16,22,26,32-octaazapentacyclo[24.2.2.1~3,7~-1~9,13~.1 ~14,18~]tritriaconta-3(33),4,6,9(32),10-,12,14(31),15,17-nonaen-23-one. These compounds are shown below (Formula 2 and Formula 3):





[0219] Exemplary suitable compounds are disclosed in U.S. Patent App. Pub. No. 2010/0015711, the disclosure of which is incorporated in its entirety as it pertains to the MCX compounds, related cyclic aniline-pyridinotriazines, and their synthesis.

[0220] The Cyp26 inhibitor used at Stage 4 in this example was N-{4-[2-Ethyl-1-(1H-1,2,4-triazol-1-yl)butyl]phenyl}-1,3-benzothiazol-2-amine, which has a CAS number of 201410-53-9 and the following structure:



This Cyp26 inhibitor is also known as "Cypi." The structure and synthesis of this Cyp26 inhibitor are disclosed in U.S. Patent No. 7,378,433, the disclosure of which is incorporated in its entirety as it pertains to Cyp26 inhibitors and their synthesis.

Table 6a: Media Formulations and Differentiation Protocol

	Stage 1		Stage 2	Stage 3	Stage 4	
Basal Media	RPMI 11mM Glucose		DMEM/F12 17.5mM Glucose	DMEM 25mM Glucose		
Supplement	+0.2% FBS	+0.5% FBS	+2% FBS	+1% B27		
Growth Factors And/Or Small Molecules	AA (100ng/ml) Wnt3a (20ng/ml)	AA (100ng/ml)	FGF7 (50ng/ml)	Noggin (100ng/ml) RA (2μM) SANT1 (250nM)	Noggin (100ng/ml) ALK5i (1μM) TPB (50nM)	Noggin (100ng/ml) ALK5i (1μM)
Days	1d	2d	3d	4d	4d	2d

Table 6b: Media Formulations and Differentiation Protocol

	Stage 1	Stage 2	Stage 3	Stage 4
Basal media	MCDB131 8 mM Glucose	MCDB131 10.5 mM Glucose	MCDB131 25 mM Glucose	MCDB131 25 mM Glucose
Supplement	2 % FAF-BSA	2 % FAF-BSA	0.1% AlbuMAX®	0.1% AlbuMAX®
Growth factors Small molecule agonist/ antagonist	GDF8 100 ng/ml MCX (day 1only) 3 μM 1:50000 ITS-X	FGF7 50 ng/ml 1:50000 ITS-X	FGF7 (50 ng/ml) AA (5 ng/ml) RA (2 μM) SANT (250 μM) LDN 193189 1:200 ITS:X	PKC activator (500 nM) SANT (250 nM) LDN 193189 (200 nM) Cyp26 inhibitor (100 nM) 1:200 ITS:X
Days	4	3	4	6

Table 6c

Description	H1 hES Calibrator	WNT3A / AA PLANAR	WNT3A / AA MicroCarrier	MCX / GDF8 PLANAR	MCX / GDF8 MicroCarrier
GAPDH Control	1	1	1	1	1
AFP	1	0.6	0.0	4.7	0.0
CD9	1	1.0	0.9	0.3	0.5
CD99	1	10.5	10.9	18.5	7.1
CDH1	1	1.2	0.6	0.5	0.6
CDH2	1	24.8	28.4	47.8	27.8
CDX2	1	23.2	0.0	74.9	27.8
CER1	1	346.2	649.7	8.1	5.6
CXCR4	1	280.3	190.1	153.9	154.7
FGF17	1	1406.4	3174.5	92.0	112.9
FGF4	1	0.8	0.5	0.0	1.1
FOXA2	1	432.5	424.3	588.5	321.2
GATA4	1	252.4	165.3	1100.1	444.9
GATA6	1	607.1	939.9	709.4	312.0
GSC	1	49.0	81.6	0.3	0.6
KIT	1	16.3	17.9	12.3	8.0
MIXL1	1	33.2	95.6	16.0	19.1
MNX1	1	146.3	111.4	595.8	392.6
NANOG	1	0.4	0.5	0.0	0.2
OTX2	1	22.9	26.4	9.1	8.3
OCT4	1	1.5	1.1	0.0	0.5
SOX17	1	751.1	1198.2	1235.0	796.3
SOX7	1	0.6	1.7	5.5	0.7
T	1	64.1	7.1	22.3	212.9

Example 7

[0221] A sub-clone of the H1 (WA01) hES cell line - WB0106 was used for this example. WB0106 was derived at the WiCell Research Institute (Madison, WI) from H1 line seed material termed DDL-13. The WB0106 sub-clone of the H1 line was derived from a DDL-13 vial thawed at passage 23 into mTeSR®1 medium on a Matrigel™ substrate, and was subsequently passaged using EDTA. WB0106 was frozen at passage 28 and was selected for these studies on the basis of a normal karyotype (FISH and G-band), ability to differentiate to pancreatic progenitor cells, and competency to form clusters and expand in suspension culture.

[0222] A WB0106 WCB vial was then thawed into medium on a substrate of Matrigel™ in a T225 flask (Corning Incorporated, Corning, NY) and at the first passage the cells were

expanded into multiple T225 flasks. At the second passage the cells from multiple T225 flasks were combined and used to seed a single 2-Layer Cell Stack™ (CS2). Once the CS2 was 70% confluent, C-Flex® tubing assembly caps with adjacent pump tubing were attached to the media ports to close the system. After the system was closed with C-Flex® tubing bags or bottle were welded on via Terumo welder and liquid volumes (medium, $PBS^{-/-}$, Accutase®, or suspended cells) were transferred using a peristaltic pump.

[0223] To lift the cells from the CS2, cells were washed once with $PBS^{-/-}$, then treated with a half strength solution of Accutase® diluted with $PBS^{-/-}$ and incubated for 4 to 5 minutes. The Accutase® was then removed, and 3 minutes after application of the enzyme solution, the CS2 was tapped to encourage cell lifting. A bottle of medium supplemented with 0.5% BSA and containing 10 micromolar of the Rho Kinase inhibitor, Y-27632, was pumped into the CS2 to rinse and inactivate the residual Accutase® and the rinse was then collected. A second rinse volume was added, collected, and pooled with the first rinse. Then 2.0 -2.5 x 10^8 cells in 200mL were transferred into a 1 layer CellSTACK® and incubated at 37° for 2 hours in a humidified 5% CO_2 incubator. Using a closed loop of C-Flex® tubing with pump tubing attached between the two CellSTACK® media ports the cell suspension was triturated for 5 minutes at 75 rpm by peristaltic pump to homogenize the aggregates. The closed loop tubing was replaced with sterile 0.2 micron filters to allow gas exchange and the CellSTACK® was incubated overnight at 37° in a humidified 5% CO_2 incubator. After overnight incubation (12-22 hours, 18 hours optimal) the cells in the CellSTACK® formed rounded spherical aggregates (clusters) of pluripotent cells.

[0224] The medium supplemented with 0.5% BSA containing the suspended cell clusters were transferred from the CellSTACK® to a 1 liter disposable spinner flask (Corning; Corning, NY) along with 0.4 liter of fresh medium supplemented with 0.5% BSA and maintained at 55-65 rpm. Twenty four hours after transfer, the 1 liter disposable spinner flask was removed from the humidified 5% CO_2 incubator and the clusters allowed to settle for 5-10 minutes. The medium was then aspirated until 200mL remained in the vessel and 400mL of additional fresh culture medium was then added to the spinner flask. This process was repeated at the end of day 2 (48 hours after transfer).

[0225] At the end of day 3 (72 hours after transfer to the spinner flask from the CS2), the cell clusters were disassociated with Accutase® treatment for passaging and further expansion. The passage process was initiated by removing the 1 liter disposable spinner flask from the humidified 5% CO_2 incubator. The flask was placed on a spinner plate inside of a biosafety cabinet to maintain a homogeneous suspension of cells. The cell suspension was removed

from the spinner flask by 100mL pipette and distributed evenly between four 175mL conical polycarbonate tubes (ThermoFisher-Nalgene; Buffalo, NY) and centrifuged for 5 minutes at 80-200 rcf. The spent medium was aspirated without disturbing the cell pellets. Then 25mL of DPBS without calcium or magnesium ($DPBS^{-/-}$) was added to each tube, and the cells were combined into one conical tube and centrifuged for 5 minutes at 80-200 rcf. The $DPBS^{-/-}$ was aspirated from the conical tube and 30mL of a 50% Accutase®/50% $DPBS^{-/-}$ solution was added to the tube. The cell clusters were pipetted up and down 1-3 times, and then intermittently swirled for 4 minutes, then centrifuged for 5 minutes at 80-200 rcf. The Accutase® was then aspirated as completely as possible without disturbing the cell pellet and the conical tube was continuously and gently tapped for 3-5 minutes until the cell suspension appeared a uniform milky white. 10mL of medium supplemented with 0.5% BSA containing 10micromolar Rho Kinase inhibitor, Y-27632, was added to the cell suspension and triturated 2-4 times to inactivate the residual Accutase®. 90mL of medium supplemented with 0.5% BSA containing 10 micromolar Rho Kinase inhibitor, Y-27632, was added to the cells and the suspension passed through a 40 micron cell strainer (BD Falcon; Franklin Lakes, NJ).

[0226] The cell density in the 100mL volume of the filtered cell suspension was determined with a NC-100 NucleoCounter® (ChemoMetec A/S, Allerod, Denmark) and additional medium was added to give a final cell concentration of 1 x 10^6 cells/mL in medium supplemented with 0.5% BSA containing 10micromolar Rho Kinase inhibitor, Y-27632. Then 225mL (225 million cells) of the cell suspension was transferred to a 1 liter disposable spinner flask and incubated for 1 hour without agitation in a humidified 5% CO_2 incubator. The flask was then removed from the incubator and agitated at 100 rpm on a spinner plate in a biosafety cabinet for 1-3 minutes. While the cell suspension was mixing, an additional 225mL of medium supplemented with 0.5% BSA containing 10micromolar Rho Kinase inhibitor, Y-27632, was added to the cell suspension. The spinner flask was then returned to the humidified 5% CO_2 incubator for 30 minutes. The flask was then removed from the incubator and agitated at 100 rpm on a spinner plate in a biosafety cabinet for 1-3 minutes. While the cell suspension was mixing, an additional 150mL of medium supplemented with 0.5% BSA containing 10micromolar of the Rho Kinase inhibitor, Y-27632, was added to the cell suspension to make a final volume of 600mL and the flask returned to stirred suspension in the incubator. At both 24 and 48 hours after Accutase® dissociation cell clusters were allowed to settle to the bottom of the flask for 5-10 minutes. Being sure to minimize any cluster loss, 400mL of spent medium was removed from the flask by aspiration and was

replaced with fresh medium. Using this process, H1 cells were converted from adherent culture on a substrate to suspension culture as cell clusters.

[0227] 72 hours after initial Accutase® treatment the process of cell cluster dissociation and spinner flask seeding (passaging) was repeated to maintain the cells in suspension for multiple passages (tested range: 1-10 passages). The above process was followed with the exception that after the first 24 hours no medium was removed, and 200mL of fresh medium was added. At 48 hours after Accutase® dissociation clusters were allowed to settle to the bottom of the flask for 5-10 minutes, 600mL was aspirated, and 400mL of fresh medium was added to the flask.

[0228] These suspension-passaged and cultured cells could then be cryopreserved and stored for future use. In order to prepare the suspension expanded cell for cryopreservation the cell clusters were dissociated with Accutase® as described above for suspension passaging, except cells were not passed through a 40 micron cell strainer. The cell count for the 100mL cell suspension generated from each 1 liter disposable flask was determined. The cell suspensions were then combined and centrifuged for 5 minutes at 80-200 rcf. The medium from the centrifuge tube was then removed as completely as possible without disturbing the cell pellet. Cold (<4°C) CryoStor®10 (Stem Cell Technologies, Inc., Vancouver, BC, Canada) was then added in a drop-wise manner to achieve a final concentration of 150 million cells per mL and the cell solution was held in an ice bath during transfer to a 1.8mL Corning® cryo vial (Corning Incorporated, Corning, NY) or 15mL Miltenyi cryo bag (Miltenyi Biotec Inc. Auburn, CA).

[0229] The suspension expanded cells were then frozen in a vial at high density in a controlled rate freezer as follows. The chamber was pre-cooled to 4°C and the temperature was held until sample vial temperature reached 6°C. The chamber temperature was then lowered 2°C/min until the sample reached -7°C. Once the sample vial reached -7°C, the chamber was cooled 20°C/min until the chamber reached -45°C. The chamber temperature was then allowed to briefly rise at 10°C/min until the chamber temperature reached -25°C, and the chamber was then further cooled at 0.8°C/min until the sample vial reached -45°C. The chamber temperature was then cooled at 35°C/min until the chamber reached -160°C. The chamber temperature was then held at -160°C for at least 10 minutes, after which the vials were transferred to gas phase liquid nitrogen storage.

[0230] In order to inoculate a stirred tank bioreactor the high density cryo-preserved cells were removed from the liquid nitrogen storage, thawed and used to seed a closed 3 liter glass bioreactor (DASGIP; Julich, Germany). Four or five vials were removed from gas phase

liquid nitrogen storage and placed directly in a 37°C water bath for 105 seconds. The thawed vial contents were then transferred via 2ml glass pipette to a 50ml conical tube. Then 9ml of medium (IH3 or Essential8™ medium ("E8™")) containing 0.5%BSA and supplemented with 10micromolar Rho Kinase inhibitor, Y-27632 was added to the tube in a drop wise manner. The cells were then centrifuged at 80-200rcf for 5 minutes. The supernatant from the tube was aspirated, 10ml fresh medium (IH3 or E8™) containing 0.5 % BSA and supplemented with 10micromolar Rho Kinase inhibitor, Y-27632 was added and the volume containing the cells was pipetted into a media transfer bottle (Cap2V8®, SaniSure, Moorpark, CA). The bottle contents were then pumped directly into the bioreactor via a sterile C-flex tubing weld by peristaltic pump. In preparation for pluripotent stem cell inoculation the bioreactor was prepared with 1.5L of medium (IH3 or E8™ supplemented with 0.5% BSA and containing 10micromolar Rho Kinase inhibitor, Y-27632), pre-warmed to 37°C, stirred at 70 rpm, regulated to 6.8-7.1 pH by CO_2, with a dissolved oxygen set-point of 30% (CO_2, air, O_2, and N_2 regulated). Immediately post-inoculation the bioreactor was sampled for cell count, and medium volume was adjusted as needed to give a final cell concentration of 0.225 x 10^6 cells/mL.

[0231] The cells inoculated into the stirred tank bioreactor formed cell clusters in the continuously stirred tank, and were maintained in pluripotency medium (IH3 or E8™, supplemented with 0.5% BSA) in the reactor for three days total. Medium was changed daily, with a partial media exchange performed 24 hours after inoculation as 1-1.3 liter of spent medium was removed and 1.5 liters of fresh medium added. Forty-eight hours after inoculation, 1.5-1.8 liters of spent medium was removed and 1.5 liters of fresh medium was added. At 72 hours after inoculation, pluripotent cell differentiation was initiated by removing >90% of the spent medium and adding differentiation medium (Table 7).

[0232] Once the staged differentiation process was initiated the cells were maintained for 12 or more days in the closed sterile suspension bioreactor regulated for temperature (37°C), pH (7.4 for differentiation), and dissolved oxygen (10% DO set-point for stage 1 and 30% DO set-point all other times, CO_2, O_2, N_2, and air regulated). Throughout the differentiation process, at each media exchange, the impeller was stopped 5-20 minutes prior to medium removal via dip-tube to allow clusters to settle. Medium in the bioreactor was removed or added to/from a closed bottle or bag by peristaltic pump through a dip tube connected to C-Flex® tubing using a Terumo™ tube welder to maintain a closed system. The impeller and heater were re-energized once sufficient medium was added to the vessel to fully submerge the impeller.

[0233] In order to monitor the bioreactor process, samples of medium containing cell clusters were drawn daily to determine cell number and viability (NucleoCounter®) as shown in Figure 7. A general expansion of cells was observed during the process, as the inoculum of 0.225 x 10^6 viable cells/mL expanded to generate an average of 0.92 x 10^6 viable cells/ mL at stage 4 day 3. By maintaining the cells at an acidic set-point (pH 7.0-6.8) during bioreactor inoculation and pluripotent cell clustering and culture, the average cell output at stage 4 day 3 increased to an average of 1.3 x 10^6 cells/ mL (Figure 7).

[0234] In addition to daily counts, bioreactor medium samples were analyzed by NOVA BioProfile® FLEX (Nova Biomedical Corporation, Waltham, MA). It was observed that, per the reactor set-points, the pH of the medium in stage 0 was acidic relative to a homeostatic standard pH of 7.4 common to most culture media and the reactor medium pH declined through stage 0 as a result of cellular metabolism (Figure 8). These results correlated with a trend of increasing lactic acid concentrations and decreasing glucose levels through the end of the 6th day of differentiation (Figures 9 and 10). Together, these data indicated the cells in the reactor were most rapidly growing and glucose consumptive through stage 0 and the first two stages of differentiation (day 1-6). However, from stage 3 onward, cell metabolism (reduced lactate levels and increased glucose levels) in the reactor declined correlating with a peak in cell numbers at stage 3 followed by a decline in cell density over the course of stage 4.

[0235] In order to determine if stage specific changes in pH and metabolism matched stage changes in mRNA expression patterns. A test of bioreactor cell samples was carried out using four Applied Biosystems® Low Density Arrays (Life Technologies Corporation, Carlsbad, CA) designated Pluripotency, Definitive Endoderm (DE), Gut Tube (GT), or stage 4 (S4) the results were compared to a historical undifferentiated H1 (WB0106) hES cell sample as control to standardize expression across all runs and arrays.

[0236] Using these arrays gene expression was determined for each stage of differentiation. It was also observed that seed material cells thawed into the bioreactor showed an undifferentiated gene expression pattern at stage 0 day 1 and stage 0 day 3 (24 and 72 hours after bioreactor inoculation: Figures 11, 12, 13, and 14). These results correlated well with flow cytometry results which showed high expression levels of CD9, SSEA4, TRA-1-60, and TRA-1-81, and the absence of CXCR4/CD184 (Figure 15 and Table 8). Although flow cytometry and qRT-PCR assays for genes expression showed robust and stable expression patterns for genes of pluripotency (CD9, NANOG, POU5F1, SOX2, TDGF, and ZFP42) consistent with a stable pluripotent state that was also noted a modest but variable increase in

gene expression for GATA4, GSC, MIXL1, and T; and a ≥100x increase in CER1, FGF17, FGF4 and GATA2 expression in some samples during the stage 0 process prior to directed differentiation (Figures 16 and 17).

[0237] At the completion of stage 0 (72 hours after reactor inoculation), the cells were moved into differentiation medium (Table 7) containing MCX and GDF8. Twenty-four hours after this media change significant alterations in gene expression patterns were noted (Figures 18 and 19), such as a ~700x increase in FOXA2 expression and a 1000x increase in CER1, EOMES, FGF17, FGF4, GATA4, GATA6, GSC, MIXL1, and T expression. These increased expression levels indicated the cells were transitioning through a mesendodermal fate. It was also noted that CDX2 levels were elevated at stage 1 day 1 versus undifferentiated cells (470x increase in expression vs. control), however this was a transient increase in expression and CDX2 levels dropped 94% from stage 1, day 1 to stage 1 day 3 returning to levels comparable to those observed prior to induction of differentiation (Figures 14, 19, and 21).

[0238] At 72 hours after exposure to the DE differentiation medium, the cells expressed a profile consistent with specification to definitive endoderm, as CXCR4 levels peaked and FOXA2 and SOX17 were expressed at >1000x over historical control. Consistent with definitive endoderm, it was also noted that the genes CER1, EOMES, FGF17, FGF4, GATA4, GATA6, GSC, MIXL1, and T dropped from elevated levels observed at stage 1 day 1 (Figures 20 and 21).

[0239] The changes in gene expression observed by qRT-PCR correlated with results observed by flow cytometry. A near complete transition was also seen from a CD9 expressing/CXCR4 negative pluripotent cell population at the initiation of differentiation (Figure 15) to a homogeneous population of CXCR4 expressing cells (98.3% of cells CXCR4 positive, ± 1.9SD) at the end of stage 1 (Figure 22).

[0240] Following the completion of definitive endoderm formation (stage 1) the medium was changed to one containing FGF7, a morphogen used to induce primitive foregut formation (stage 2). Consistent with formation of primitive foregut, HNF4α and GATA6 expression levels at stage 2 days 1 and 3 were increased, while genes expressed at high levels on day 3 of stage 1 (CXCR4, EOMES, FGF17, FGF4, MNX1, PRDM1, SOX17, and VWF) showed reduced expression by the end of stage 2 (Figure 23). The expression of foregut genes (AFP, PDX1, and PROX1) was increased (Figure 24).

[0241] After the cells had been cultured in stage 2 medium for 72 hours, the culture was switched to a stage 3 medium (Table 7). Once in this medium the cells expressed markers consistent with an endodermal pancreatic lineage as measured by PDX1 and FOXA2

expression (90.9% ± 11.9SD PDX1 positive and 99.2% ± 0.6SD FOXA2 positive) shown in Figure 25. These results were confirmed by data from samples analyzed by qRT-PCR for gene expression. Gene expression for PDX1 increased 5 fold in 24 hours from the end of stage 2 day3 (38,000x vs. H1) to the end of stage 3 day 1 (200,000x vs. H1) and doubled again 48 hours later on stage 3 day 3 (435,000x vs. H1). These data show the cells were specifying to a pancreatic fate (Figure 26). This observation was further supported by the increased levels of a host of genes commonly expressed in pancreas (ARX, GAST, GCG, INS, ISL1, NEUROD1, NGN3, NKX2.2, NKX6.1, PAX4, PAX6, PTF1A, and SST) as shown in Figure 26. In addition, very low or no OCT4/POU5F1 expression (2-10% of control or 32-37 sample Cts by qRT-PCR) and high expression levels for other markers of endodermal lineages AFP, ALB, and CDX2- was also seen, further indicating the specification and transition of the cell population in the bioreactor from a relatively plastic gut tube fate to a pancreatic fate.

[0242] At the end of the differentiation process on stage 4 day 3, the cells retained high levels of PDX1 and FOXA2 expression and further developed an expression pattern consistent with a mix of pancreatic endocrine cells (28.1% ± 12.5SD chromogranin positive) and pancreatic progenitor cells (58.3% ± 9.7SD positive for NKX6.1) as shown in Figure 27. This stage specific marker expression pattern indicated an efficient stage-wise differentiation from a pluripotent population to pancreatic precursor cells. The results observed with flow cytometry, were further confirmed with data from qRT-PCR. A host of genes commonly expressed in pancreas (ARX, GAST, GCG, IAPP, INS, ISL1, MAFB, NEUROD1, NGN3, NKX2.2, NKX6.1, PAX4, PAX6, PTF1A, and SST) all showed increased expression levels. (Figure 28).

[0243] The expression pattern observed in Figure 27 held consistent across multiple runs as multiple process variables, such as different seed materials, stage 0 medium, pH of stage 0 medium and the use of anti-foam, were tested. Multiple sources of seed material were tested and each efficiently generated a pancreatic endodermal fate with >90% FOXA2, >75% PDX1, and >50% NKX6.1 (Figure 29). Furthermore, it was noted that was no significant difference in expression patterns of bioreactor product when the cells were grown at stage 0 in a custom in-house medium called "IH3" supplemented with 0.5% BSA or a commercially available medium: Essential8™, supplemented with 0.5% BSA (Figure 30). When the role of pH in stage 0 culture was examined, it was noted that cells grown in stage 0 at a relatively low pH (6.8) had increased expansion in the bioreactor relative to the average run (Figure 7), but no significant change in the stage 4 day 3 cell profile (Figure 31). Additionally, the use

of Anti-Foam C emulsion (Sigma Cat#A8011) at 94 parts per million was seen to reduce bubbles produced by sparging but did not appear to affect the profile of cells from the end of stage 0 through stage 4 day 3 cell (Table 9 and Figure 32).

[0244] At the end of each bioreactor differentiation the product cells were cryopreserved. The cells were washed in MCDB131 with 3.63 g/L sodium bicarbonate or MCDB131 with 3.63 g/L sodium bicarbonate, glucose (8mM final), and 1x Glutamax, and then transferred to cold (<4°C) cryopreservation media comprised of 57.5% MCDB131 with 2.43g/L sodium bicarbonate, 30% Xeno-free KSR, 10% DMSO, and 2.5% HEPES (final concentration 25mM). The cells were then frozen in a controlled rate freezer (CRF) using a cooling profile that maintained the cell clusters in cryopreservation media at ambient temperature for a maximum of 15 minutes, reduced to a temperature of 4°C for 45min, and further reduced by 2.00 °C/min to -7.0°C (sample). The sample was then quickly cooled, reducing the temperature of the chamber at a rate of 25.0 °C /min to -45.0°C. A compensation increase was then provided by increasing the chamber temp 10.0 °C /min to -25.0°C (chamber). The sample was then cooled at 0.2 °C /min until the temperature reached -40.0°C. The chamber was then cooled to -160°C at a rate of 35.0°C /min and held at that temperature for 15 minutes. The samples were moved to a gas phase liquid nitrogen storage container at the termination of the CRF run.

[0245] The cells could be thawed by removal from vapor phase liquid nitrogen storage and transferring the vial to a 37°C water bath. The vial was gently swirled in the water bath for less than 2 minutes until a small ice crystal remained in the vial. The vial contents were then transferred to a 50ml conical and diluted drop-wise over two minutes using MCDB131 media with 2.43g/L sodium bicarbonate and 2% BSA to a final volume of 20ml total. The total cell number was then determined by NucleoCounter® and the cell suspension transferred to an ultra-low attachment culture dish for 1 hour. The cells were then isolated from the media in a 50ml conical, the supernatant removed and cells re-suspended in stage 4 media for analysis or in vivo study.

[0246] Alternatively after thawing, vialed cells were transferred to an empty 125mL glass Corning® spinner flask (Corning, Incorporated, Corning, NY) and 10mL MCDB131 medium containing 2.43g/L sodium bicarbonate and 2% BSA was added to the flask in a drop-wise manner. The final volume was then adjusted to 80mL of the same medium. The total cell number was determined by NucleoCounter® and the cell suspension stirred at 40-65 rpm overnight (12-28 hours). The cells were then characterized or used for *in vivo* study.

[0247] The composition of IH3 media is shown below as well as in U.S. Pub. App. No. 2013/0236973, the disclosure of which is incorporated in its entirety as it pertains to suitable cell culture media. The amount of BSA in IH3 media may vary.

Composition of IH3 Media	
Basal Media	**Added components**
DM-F12	1 X ITS-X, 0.5% reagent-grade FAF-BSA 1 ng/ml TGF-β1 100 ng/ml bFGF 20 ng/ml IGF-1 0.25 mM ascorbic acid

Table 7

Starting Day/Date:	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**
Basal Media	MCDB131 Cust ($3.64g/L\ NaCO_3$)	MCDB131 Cust ($3.64g/L\ NaCO_3$)	MCDB131 Cust ($3.64g/L\ NaCO_3$)	MCDB131 Cust ($3.64g/L\ NaCO_3$)
Supplement	2% FAF-BSA 2.5mM glucose 1:50,000 ITS-X Glutamax 1:100	2% FAF-BSA 2.5mM glucose 1:50,000 ITS-X Glutamax 1:100	2% FAF-BSA 2.5mM glucose 1:200 ITS-X Glutamax 1:100	2% FAF-BSA 2.5mM glucose 1:200 ITS-X Glutamax 1:100
Growth factors	***Day 1 and 2 only:*** ***GDF8*** 100 ng/mL	**FGF7** 50 ng/mL	**FGF7** 50ng/mL	None
Small molecules	***Day 1 only:*** **MCX** [2μM]		RA [2 μM] SANT [0.25 μM] TPPB [100 nM] ***Day 1 only*** ***LDN*** *[100 nM]*	SANT [0.25 μM] TPPB [100 nM]
Days	**3**	**3**	**3**	**3**
NOTES: All Days refer to 0H	**Media change Days 1 and 2, No change Day 3**	**Media change Days 1 and 3, No change Day 2**	**Media change Days 1 and 2, No change Day 3**	**Media change Day 1 and end of Day 3 if S4 is extended**

Table 8

BX replicate	Seed Material	CD9	CD184	SSEA4	TRA-1-60	TRA-1-81
1	KC	83.3	0.1	99.9	94.5	85.8
2	HW	95.5	0.2	100	91	84
3	ISM (Pink)	95.8	0.1	100	76.1	36.5
4	ISM (Pink)	93.2	0	99.9	78.6	64.5
5	ISM 1	97.8	0.2	99	74.8	66.4
6	ISM 2	98.6	0.2	100	92.2	86
7	ISM 1	98.1	0.1	99.9	88.8	80.3
8	ISM 1	99.1	0.1	99.9	93.8	83.3
9	ISM 2	97.2	0.1	99.9	88.3	81
10	ISM5	98	0.1	99.3	93.1	85.7
11	ISM6	72.6	0.2	99.9	94.7	88.9
12	ISM6	85.9	0.7	99.4	71.9	54.1
		CD9	**CD184**	**SSEA4**	**TRA-1-60**	**TRA-1-81**
	Average	93.6	0.1	99.8	87.8	76.6
	St. Deviation	8.3	0.1	0.3	7.6	15.5

Table 9

Stage-Day-Time	Viable Cell density (M cells/mL)	CD9	CD184	SSEA4	TRA-1-60	TRA-1-81			
S0D3-24H	0.626	95.8	0.1	99.8	87.9	74			
	Viable Cell density (M cells/mL)	**CD9**	**CD184**	**CD99**					
S1D3-24H	0.9	50.7	98.9	99					
	Viable Cell density (M cells/mL)	**NKX6.1**	**CHROMG.**	**NKX2.2**	**PDX1**	**FOXA2**			
S4D1-24H	0.943	69.3	14.2	23.6	98.8	99.7			
	Viable Cell density (M cells/mL)	**NKX6.1**	**CHROMG.**	**CDX2**	**SOX2**	**NKX2.2**	**PDX1**	**FOXA2**	**NEUROD**
S4D3-24H	1.002	66.2	35.6	0.3	15.8	38.1	99	99	45.6

Materials:

- human embryonic stem (hES) cell line H1, (WA01 cells, WiCell, Madison WI)
- PBS (Catalog# 14190, Invitrogen)
- Y-27632 (Axxora Catalog#ALX-270-333, San Diego, CA)
- EDTA, (Lonza, Catalog# 17-7-11E)
- NucleoCounter®-(ChemoMetec A/S, Cat#YC-T100, Allerod, Denmark)
- Non-Tissue Culture Treated 6 well dishes (Becton Dickinson, Catalog# Falcon 351146, Franklin Lakes, NJ)
- Accutase®, (Sigma, Catalog# A-6964, St. Louis, MO)
- pH, and dissolved oxygen (DO)bioreactor probes (FermProbe® pH electrode 225mm, Model # F-635, and DO OxyProbe® 12mm Sensor, Model # D-145 from Broadley-James Corporation, Irvine CA)
- Immune-protective macro encapsulation device (TheraCyte™, Irvine CA)
- Mm HUMAN C-PEPTIDE ELISA (MERCODIA CAT# 10-1141-01)
- GlutaMAX™, MCDB131, and ITS-X Invitrogen
- FAF-BSA (Proliant)
- Retinoic Acid, Glucose 45% (2.5M), SANT (Shh inhibitor) (Sigma)
- GDF8 (Peprotech)
- MCX
- FGF7 (R & D Systems)
- LDN-193189 (BMP receptor antagonist) (Stemgent)
- TPPB (PKC activator) (ChemPartner)
- MCDB 131 Custom Media

Example 8

Maturation and Function of Cryo-Preserved Bioreactor Generated Pancreatic Progenitor Clusters

[0248] In order to generate sufficient cells for each bioreactor study one passage 31 master cell bank vial of H1 hES (WB0106) cells was thawed. The cells were expanded under adherent conditions in mTeSR®1 media for several passages on Matrigel™ using EDTA passaging until sufficient cells were generated to seed five Matrigel™ coated 2-Layer CellSTACKs® (CS2). Once the adherent cells growing in the CS2 were 70% confluent, C-

Flex® tubing assembly caps with adjacent pump tubing were attached to the media ports to close the system. After the system was closed bags or bottle were welded on with C-Flex® via Terumo welder and all liquid volumes (medium, $PBS^{-/-}$, Accutase®, or suspended cells) were transferred using a peristaltic pump.

[0249] To lift the cells from the CS2s, cells were washed once with Dulbecco's Phosphate Buffered Saline without calcium or magnesium ($PBS^{-/-}$), then treated with a half strength solution of Accutase® diluted with an equal part of $PBS^{-/-}$ and incubated for 4-5 minutes. The Accutase® solution was then removed, and 3 minutes after application of the enzyme solution, the CS2s were tapped to encourage cell lifting. A bottle of mTeSR®1 containing 10micromolar Rho Kinase inhibitor, Y-27632, was pumped into the CS2s to rinse and inactivate the residual Accutase® and the rinse was then collected. A second rinse volume was added, collected, and pooled with the first rinse. 1.6-2.0 x 10^9 cells were recovered from the CS2s in a final volume of 2 liters. 2.0 -2.5 x 10^8 cells per layer, were transferred into four CS2s or eight 1 layer Cell Stacks™ and incubated at 37°C for 2 hours in a humidified 5% CO_2 incubator in a volume of 200mL per layer.

[0250] Using a closed loop of C-Flex® tubing with adjacent pump tubing attached between CellSTACK® media ports the cell suspension was triturated for 5 minutes at 75 rpm by peristaltic pump to homogenize the aggregates. The CellSTACKs® were then incubated overnight at 37°C for 18 hours in a humidified 5% CO_2 incubator. The 2 liters of cells and media from the Cell Stacks were then pooled and transferred, 1 liter each, into two 3 liter DASGIP bioreactors along with 1.5 liter of fresh mTeSR® medium per bioreactor. The cells were maintained for two additional days with mTeSR® medium before initiating differentiation, with a full media exchange 24 hours after bioreactor inoculation. Differentiation was then initiated and directed as described in Table 10. The cells were maintained 14 or 15 days total (2 days mTeSR® + 12 or 13 days of staged differentiation) in the closed sterile suspension bioreactor regulated for temperature (37°C), pH (drift, or regulated by CO_2 to 6.8 or 7.2 for pluripotent cells and 7.4 for differentiation), and dissolved oxygen (30% DO set-point, CO_2 / air regulated). The impeller was stopped for 5-20 minutes prior to each media exchange to allow clusters to settle. Medium was removed or added by peristaltic pump through a dip tube connected to C-Flex® tubing (Cole-Parmer North America, Vernon Hills, IL) using a Terumo™ tube welder to maintain a closed system. The impeller and heat jacket were re-energized once sufficient medium was added to submerge the impeller.

[0251] Two production runs were initiated in 3 liter reactors using these methods. In the first reactor run two different pH set points were tested over the first two days of pluripotent culture medium. Reactor 1 was set to pH 7.2 with a fixed CO_2 gas infusion rate of 5%, so the pH would "drift" lower as the reactor environment acidified over time due to metabolic activity of the cells. Reactor 2 was set to a pH of 7.2 regulated by CO_2 gas levels. In the second reactor run the pH was set to 6.8 for reactor 1 and 7.2 for reactor 2, both regulated by CO_2 gas levels.

[0252] In order to monitor the bioreactor process cell clusters were taken at the end of each stage of differentiation and assayed by flow cytometry (Table 11; Table 12). A near complete transition was observed from a CD9 expressing/CXCR4 negative pluripotent cell population at the initiation of differentiation to a homogeneous population of CXCR4 expressing cells (96.9-98.1% of cells CXCR4 positive) at the completion of definitive endoderm formation.

[0253] The results observed by flow cytometry correlated with results from paired samples analyzed by rt-PCR. Samples were tested throughout the process for gene expression characteristic of staged differentiation from pluripotency to a pancreatic fate. Prior to the initiation of directed differentiation, mRNA was tested from bioreactor cell clusters on a low density array for a panel of genes associated with pluripotency or early differentiation fates.

[0254] It was observed that cells from the bioreactor retained expression for genes characteristic of pluripotency (POU5F1, NANOG, SOX2, and ZFP42) and showed minimal or no induction of genes characteristic of differentiation (AFP, and FOXA2: <50 fold increase; FOXD3, GATA2, GATA4, GSC, HAND2, MIXL1, and T: <10 fold increased expression) as compared to undifferentiated H1 controls (Figure 33). However once the cells were contacted with stage 1 day 1 differentiation media gene expression patterns changed dramatically as levels of CDX2, CER1, FGF17, FGF4, FOXA2, GATA4, GATA6, GSC, MIXL1, MNX1, and Brachyury (T) expression increased to 100 to 1000 fold greater than undifferentiated H1 hES cells (Figure 34). By the end of stage 1 day 3 (formation of definitive endoderm), CD9, CDX2, FGF4, MIXL1, NANOG, POU5F1, and Brachyury (T) had decreased expression relative to stage 1-day 1 while expression of characteristic definitive endoderm genes such as CD99, CER1, CXCR4, FGF17, GATA4, GATA6, KIT, OTX, or SOX17 peaked (Figure 35).

[0255] At the end of stage 1 the cell culture medium was changed from one containing GDF8 to a medium containing FGF7. Several different gene expression patterns were noted: an increase in expression of certain genes over the course of stage 2 (AFP, ATOH1, HHEX,

OSR1, PDX1, PROX1, SOX2, and SOX9), a decrease in expression (HAND1 and SOX17), stable high expression throughout (HNF4α), or low/no expression (CDX2, GAST, NKX2.2, NKX6.1, and PTF1a) (Figure 36a-e). These patterns indicated that the cells in the reactor were becoming foregut (AFP, ATOH1, HHEX, HNF4α, OSR1, PDX1, PROX1, SOX2, and SOX9) expression for markers of mesoderm (HAND1 and SOX17) decreased. However, by the end of stage 2, the cells had not yet specified to a more mature gut or pancreatic fates (CDX2, GAST, NKX2.2, NKX6.1, and PTF1a).

[0256] By the end of stage 3 the cells had specified to a pancreatic lineage as measured by PDX1 expression demonstrated by >100,000 fold increase in mRNA vs. undifferentiated control (Figure 36) and 76-98% of the cells expressing PDX1 by flow cytometry (Table 11). Also observed was induction of other genes of the pancreas (GAST, NKX2.2, NKX6.1, PROX1, PTF1a, and SOX9) and gut such as AFP and CDX2; indicating the cells had begun to specify to a more mature fate.

[0257] By the end of the differentiation process on day 3 or 4 of stage 4, the cells showed an expression pattern consistent with a mix of pancreatic endocrine cells (47-54% Chromogranin positive) and pancreatic progenitor cells (33-52% positive for NKX6.1) as shown in Tables 11 and 12. This stage specific marker expression pattern indicated an efficient stage-wise differentiation from a pluripotent population to pancreatic progenitor cells characterized by high expression levels of PDX1 ($>1x10^6$ fold induction) and other pancreatic genes (>1000 fold induction of ARX, GCG, GAST, INS, ISL, NEUROD1, NGN3, NKX2.2, NKX6.1, PAX4, PTF1a, and SST) and near total loss of OCT4/POU5F1 expression as compared to undifferentiated H1 human embryonic stem cells (Figure 37).

[0258] At the end of the differentiation process 0.08-0.45 x 10^6 cells/ mL were generated (Figure 38: daily cell counts). The cells generated in this process were then cryo-preserved or directly implanted into an animal subcutaneously via a TheraCyte™ device or placed under the kidney capsule. In order to cryopreserve the cells, they were transferred to cryopreservation media comprised of 57.5% MCDB131 with 2.43g/L sodium bicarbonate, 30% Xeno-free KSR, 10% DMSO, and 2.5% HEPES (final concentration 25mM). Once the cell clusters were suspended in cryopreservation media at ambient temperature the cryo-vials were moved to the controlled rate freezer (CRF) within 15 minutes. The chamber temperature was then reduced to 4°C for 45min, and further reduced by 2.00 °C/min to -7.0°C (sample). The sample was then quickly cooled, reducing the temperature of the chamber at a rate of 25.0 °C /min to -45.0°C. A compensation increase was then provided by increasing the chamber temp 10.0 °C /min to -25.0°C (chamber). The sample was then

cooled at 0.2 °C /min until the temperature reached -40.0°C. The chamber was then cooled to -160°C at a rate of 35.0°C /min and held at that temperature for 15 minutes. The samples were moved to a gas phase liquid nitrogen storage container at the termination of the CRF run.

[0259] After the cells had been stored in gas phase liquid nitrogen the cells were thawed by removal from storage and transferred to a 37°C water bath. The vial was gently swirled in the water bath for less than 2 minutes until a small ice crystal remained in the vial. The vial contents were then transferred to a 50ml conical and diluted drop-wise over two minutes using MCDB131 media with 2.43g/L sodium bicarbonate and 2% BSA to a final volume of 20ml total. The total cell number was then determined by NucleoCounter® and the cell suspension transferred to an ultra-low attachment culture dish for 1 hour. The cells were then isolated from the media in a 50ml conical, the supernatant removed and cells re-suspended in stage 4 media. The cells were then either implanted into an animal subcutaneously via TheraCyte™ device or under the kidney capsule or the cells were incubated in an ultra-low attachment culture dish overnight and then implanted into an animal.

[0260] The animals were monitored for blood glucose and C-peptide levels every four weeks following graft implantation. Animals treated with non-cryopreserved pancreatic precursor cells inside a TheraCyte™ device or by direct placement of the cells under the kidney capsule matured to express over 1ng/mL C-peptide by 16 weeks and reached 2 ng/mL C-peptide by 20 weeks post-implantation (Figure 39a and 39d). Furthermore, when treated with STZ to ablate host β-cell function, the engrafted animals maintained normo-glycemia until the grafts were removed, indicating that the grafts were competent to protect the animals from diabetes induced by a single high dose of STZ (Figure 39b).

[0261] This pattern was also observed in animals treated with cryopreserved cells. Animals treated by kidney capsule graft with cryopreserved pancreatic precursor cells that had been cultured for 1 hour after thaw (1207B) had an average of 0.56 ng/mL and 1.09 ng/mL of C-peptide at 16 and 20 weeks, respectively, while cells cultured overnight after thaw (1207C) had an average of 0.81 ng/mL and 1.35 ng/mL of C-peptide at 16 and 20 weeks, respectively (Figure 39d). Animals treated with cryopreserved pancreatic precursor cells inside a TheraCyte™ device had over 1ng/mL C-peptide by 16 weeks, and similar to the non-cryopreserved controls, were able to express therapeutic levels of C-peptide one week after STZ treatment (0.98ng/mL, Figure 39c). These results indicate that cryopreserved pancreatic precursor cells can function comparably to non-cryopreserved controls when tested in an animal model.

Table 10

Starting Day/Date:	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4
Basal Media	mTeSR®1	MCDB131 (3.64g/L $NaCO_3$)	MCDB131 (3.64g/L $NaCO_3$)	MCDB131 (3.64g/L $NaCO_3$)	MCDB131 (3.64g/L $NaCO_3$)
Supplement		2% FAF-BSA 2.5mM glucose 1:50,000 ITS-X Glutamax 1:100	2% FAF-BSA 2.5mM glucose 1:50,000 ITS-X Glutamax 1:100	2% FAF-BSA 2.5mM glucose 1:200 ITS-X Glutamax 1:100	2% FAF-BSA 2.5mM glucose 1:200 ITS-X Glutamax 1:100
Growth factors		***Day 2 only:*** ***GDF8*** 100 ng/mL	**FGF7** 50 ng/mL	**FGF7** 50ng/mL	None
Small molecules	Y-27632 (day 0 only) [1:1000; 10 µM]	***Day 1 only:*** **MCX** [3µM]		RA [2 µM] SANT [0.25µM] TPPB [100 nM] ***Day 1 only*** ***LDN*** *[100 nM]*	SANT [0.25µM] TPPB [100nM]
Days	**3**	**3**	**3**	**3**	**3**
NOTES:		**Media change Days 1 and 2, No change D3**	**Media change Days 1 and 3, No change Day 2**	**Media change Days 1 and 2, No change D3**	**Media change Day 1 only Glucose Bolus Day 3**

Note:

- Basal media in Table 10 above may optionally include 5 mM glucose at stages 1-5 when Glutamax is not used in supplement.
- Cypi ([100 nM]) may optionally be added at stage 4 in Table 10 shown above.

Table 11

	Process Day	Name	CD9	CD184	SSEA4	TRA-1-60	TRA-1-81	
Pluripotency	2	Bx1	78.9	0.1	100	54.5	51.1	
		Bx2	66.5	0.0	100	63.5	72.3	
		Name	CD9	CD184				
DE (S1D2)	4	BX1	9.9	87.9				
		BX2	19.7	83.1				
DE (S1D3)	5	BX1	17.4	98.1				
		BX2	25.4	96.9				
		Name	Nkx6.1	Nkx2.2	PDX1			
PE (S3D3)	11	BX1	4.4	25.2	98.6			
		BX2	4.8	28.9	76.2			
		Name	Nkx6.1	Synaptophysin	CDX2	Sox2	Nkx2.2	Chrom.
PPC (S4d3)	14	BX1	33.2	67.4	2.1	13.0	69.3	51.1
		BX2	35.1	56.9	1.9	11.5	64.4	51.2

Table 12

	Process Day	Name	CD9	CD184	SSEA4	TRA-1-60	TRA-1-81	
Pluripotency	2	BX1	99.8	0.3	100.0	88.6	85.8	
		BX2	99.8	0.3	100.0	86.8	85.9	
		Name	CD9	CD184	CD99			
DE (S1d3)	5	BX1	88.3	99.2	97.0			
		BX2	78.3	99.3	96.9			
		Name	Nkx6.1				Nkx2.2	Chrom.
PE (S3d3)	11	BX1	6.3				23.2	8.5
		BX2	1.2				24.6	11.5
		Name	Nkx6.1	Synaptophysin	CDX2	Sox2	Nkx2.2	Chrom.
PPC (S4d3)	14	BX1	49.0		7.3	13.1	56.1	49.2
		BX2	52.6		3.1	19.9	54.5	47.4
PPC (S4d4)	15	BX1	48.4	53.1	0.4	4.9	60.3	44.3
		BX2	45.7	66.5	0.2	4.5	63.7	54.3

Calculation of Shear Stress Experienced by Cell Aggregates in a Stirred Tank Bioreactor

[0262] The shear stress experienced by cell aggregates in a 2.7 liter DASGIP stirred suspension bioreactor mixed at an agitation rate of 70 rpm in a 3l DASGIP bioreactor was determined. In order to calculate the shear stress values, the following stated assumptions were made.

Assumptions:

1. Max shear stress imposed on cell aggregates is not a result of turbulent eddies
2. Max shear stress imposed on cell aggregates is not a result of aggregate-aggregate or aggregate-impeller collision
3. Baffles (*i.e.* diptubes and probes) imposed shear stress are not addressed in these calculations

[0263] For the purposes of the calculations herein, the nomenclature and physical parameters listed below were used.

Nomenclature:

Abbreviation		*units*
P	Fluid Density	kg/m^3
μ	Fluid viscosity	Pa s
ϑ	Kinematic Viscosity	m^2/s
τ_{max}	Maximum Shear Stress	dyn/cm2
N	Agitation	rev/sec
P	Power consumed	$kg\ m^2/s^3$
P_N	Power Number	dimensionless
Re	Reynold's Number	dimensionless
ε	Power Dissipated per unit mass	m^2/s^3
D_i	Impeller Diameter	M
D_t	Tank Diameter	M
W	Impeller Widtch	M
V_L	Liquid volume	m^3
K_1-K_4	Calculated values based on Nagata Empirical Correlations	

Parameters:

Bioreactor Parameters		
D_i	0.08	m
D_t	0.13	m
W	0.04	m
V_L	0.0024	m3

Medium Parameters		
Density (ρ)	1000	kg/m^3
Viscosity (μ)	8.50E-04	Pa s
kinematic viscosity (ϑ)	8.50E-07	m^2/s

[0264] The listed medium and bioreactor parameters were applied to the equations below.

Equations:

Reynolds numbers:

$$Re = \frac{\rho N D_i^2}{\mu}$$

Maximum Shear Stress on aggregate (Cherry and Kwon 1990)

$$\tau_{max} = 5.33\rho\sqrt{\varepsilon\vartheta}$$

Power Dissipated (ε) per unit mass

$$\varepsilon = \frac{P}{V_L\rho}$$

Power consumed (P)

$$P = P_N N^3 D_i^5 \rho$$

Power Number calculation was based on the empirical correlation derived by Nagata (1975) for an unbaffled stirred tank.

$$P_N = \frac{K_1}{Re} + K_2\left[\frac{10 + 1.2Re^{0.66}}{10 + 3.2Re^{0.66}}\right]^{K_4}$$

Where

$$K_1 = 14 + \frac{W}{D_t}\left[670(\frac{D_i}{D_t} - 0.6)^2 + 185\right]$$

$$K_2 = 10^{K_3}$$

$$K_3 = 1.3 - 4\left[\frac{W}{D_t} - 0.5\right]^2 - 1.14\frac{D_i}{D_t}$$

$$K_4 = 1.1 + 4\left(\frac{W}{D_t}\right) - 2.5\left[\frac{D_i}{D_t} - 0.5\right]^2 - 7\left[\frac{W}{D_t}\right]^4$$

[0265] A maximum shear of at least 2.5dyn/cm^2 imposed on cell aggregates at an agitation rate of 70 rpm in a 2.7L DASGIP bioreactor was calculated. The cells comprising the outermost layer of the clusters experience the highest levels of shear stress. These shear stress values are highly dependent on the assumptions stated.

Example 9

Differentiation of Human Embryonic Stem Cells from Cell Line WA01 into Definitive Endoderm: role of MCX/GDF8 in Suspension Culture

[0266] Clusters from pluripotent human embryonic stem cell line H1 (NIH code: WA01) were seeded at cell densities ranging from 0.25 x 10^6 to 2 x 10^6 cells/ml in Erlenmeyer/Shaker flasks, spinner flasks, or uncoated ultra low-binding or non-tissue culture treated 6-well plates in MCDB-131 medium containing 3.64g/ml sodium bicarbonate and 5.5mM glucose (Catalog # A13051 DJ, Invitrogen, CA), which was supplemented with 2% fatty acid free BSA (Catalog # 68700, Proliant, IA), 1X GlutaMAX™ (Catalog # 35050-079, Invitrogen, CA), an additional 2.5mM glucose (Catalog # G8769, Sigma) and ITS-X at 1:50,000 stock concentration (Catalog # 51500056, Invitrogen, CA). MCDB-131 medium supplemented in this manner will be referred to as "stage 1 basal medium" for the purposes of this application. Clusters in this medium were treated on the first day of differentiation with either 3μM MCX (a 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, U.S. Patent Application No. 12/494,789; incorporated herein by reference in its entirety) and 100ng/ml GDF-8 (Catalog # 120-00, Peprotech), or 3μM MCX only, or 20ng/ml WNT-3A (Catalog # 1324-WN-002, R&D Systems, MN) plus 100ng/ml Activin A (Catalog # 338-AC, R&D Systems, MN) or 20ng/ml WNT-3A only. On day two, cells were transferred to fresh stage 1 basal media supplemented with either 100ng/ml GDF8 or 100ng/ml Activin A. Samples were collected for flow cytometry, PCR and Western Blot analysis at various time points ranging from time zero (immediately before addition of basal media plus supplements) up to 72 hours after beginning differentiation.

[0267] The efficiency with which definitive endoderm was generated was determined after 3 days of differentiation under each condition by measuring the percentage of cells expressing the cells surface markers CXCR4, CD99 and CD9 using flow cytometry. The data (as shown in FACS plots in Figure 40a-d and summarized in Table 13) indicates that in suspension culture, addition of 3μM MCX in the absence of a TGF-β family member on day one of differentiation generates definitive endoderm at levels comparable to that obtained when cells are treated with 3μM MCX plus 100ng/ml GDF-8 or 20ng/ml WNT-3A plus 100ng/ml Activin A on day one.

Table 13

Treatment (Day 1 → Day 2 and 3)	CD9 (% by FACS)	CD99 (% by FACS)	CD184 (% of Parent)
MCX + GDF8 → GDF8	1.5	0.0	95.3/95.4
MCX only → GDF8	6.4	0.0	93.6/93.6
WNT3a + Activin A → Activin A	3.3	22.1	98.1/97.5
WNT3a only → Activin A	31.7	6.2	87.8/86.1

Example 10

Differentiation of Human Embryonic Stem Cells from Cell Line WA01 into Definitive Endoderm: Dose Response of MCX Compound Concentration in Suspension Culture

[0268] Clusters from pluripotent human embryonic stem cell line H1 (NIH code: WA01) were seeded at cell densities ranging from 0.25 x 10^6 to 2 x 10^6 cells/ml in Erlenmeyer/shaker flasks or spinner flasks in stage 1 basal media as described in Example 9. Clusters were treated with stage 1 basal medium containing 1.5, 2, 3, or 4μM MCX on day one of differentiation and with fresh stage 1 basal medium containing 100ng/ml GDF-8 on day 2. No media exchange was performed on day three. Samples were collected for flow cytometry and PCR analysis at the end of day three of differentiation.

[0269] The efficiency with which definitive endoderm was generated was then determined by measuring the percentage of cells expressing the cells surface markers CXCR4, CD99 and CD9 using flow cytometry. The data (as shown in FACS plots in Figure 41A-D and summarized in Table 14) indicate that in suspension cultures, addition of MCX at concentrations less than 2μM results in progressively fewer definitive endoderm positive cells (as evidenced by a lower percentage of CXCR4 positive and a higher percentage of CD9 positive cells). Further, at concentrations above 4μM, MCX exhibits a deleterious effect on the cells, which results in decreased cell viability. However, by increasing BSA concentrations, the effects of MCX can be modulated such that concentrations ≥ 4 micromolar may be used. Conversely, concentrations ≤ 1.5 micromolar may be used to generate definitive endoderm when used with lower BSA concentrations.

Table 14

Treatment	CD9 (% by FACS)	CD184 (% by FACS)
4μM MCX	1.0	95.2
3μM MCX	0.2	96.0
2μM MCX	0.2	96.5
1.5μM MCX	68.4	67.8

Example 11

Differentiation of Human Embryonic Stem Cells from Cell Line WA01 into Definitive Endoderm: role of Media Exchange Frequency in Suspension Culture

[0270] Clusters from pluripotent human embryonic stem cell line H1 (NIH code: WA01) were seeded at cell densities ranging from 0.25 x 10^6 to 2 x 10^6 cells/ml in Erlenmeyer/shaker flasks or spinner flasks in stage 1 basal media as described in Example 9. Clusters were treated with stage 1 basal medium containing 3μM MCX on day one of differentiation and with fresh stage 1 basal medium containing 100ng/ml GDF-8 on day 2. Control cultures received a media exchange on day three; to a separate vessel, no media exchange was performed on day three. Samples were collected for flow cytometry and PCR analysis at the end of day three of differentiation.

[0271] The efficiency with which definitive endoderm was generated was then determined under each condition by measuring the percentage of cells expressing the cells surface markers CXCR4, CD99 and CD9 using flow cytometry. The results are shown in FACS plots in Figure 42A&B and summarized in Table 15.

Table 15

Treatment	CD9 (% by FACS)	CD99 (% by FACS)	CD184 (% by FACS)
Full Media Exchange at stage 1	0.2	72.4	90.2/89.6
Skip Feed at stage 1 day 3	0.9	68.3	89.2/89.8

Example 12

Differentiation of Human Embryonic Stem Cells from Cell Line WA01 into Definitive Endoderm: Use of GlutaMAX™ in Suspension Culture

[0272] Clusters from pluripotent human embryonic stem cell line H1 (NIH code: WA01) were seeded at cell densities ranging from 0.25 x 10^6 to 2 x 10^6 cells/ml in Erlenmeyer/shaker flasks or spinner flasks.

[0273] The example was carried out to determine whether Glutamax ™ supplementation was required for generation of definitive endoderm by suspending clusters in stage 1 basal media (described in Example 9*)* plus or minus GlutaMAX™, which was supplemented with 3μM MCX on day one of differentiation and with fresh stage 1 basal medium containing 100ng/ml GDF-8 on day 2. No media exchange was performed on day three. Samples were collected for flow cytometry and PCR analysis at the end of day three of differentiation.

[0274] The efficiency with which definitive endoderm was generated was determined under each condition by measuring the percentage of cells expressing the cells surface markers CXCR4, CD99 and CD9 using flow cytometry. The data and results are shown in FACS plots in Figure 43A&B and summarized in Table 16.

Table 16

Treatment	CD9 (% by FACS)	CD99 (% by FACS)	CD184 (% by FACS)
X GlutaMAX™	0.2	93.7	96.8/96.7
0 GlutaMAX™	1.3	95.6	97.7/97.3

Example 13

Differentiation of Human Embryonic Stem Cells from Cell Line WA01 into Definitive Endoderm: Role of Sodium Bicarbonate Concentration in Suspension Culture

[0275] Clusters from pluripotent human embryonic stem cell line H1 (NIH code: WA01) were seeded at cell densities ranging from 0.25 x 10^6 to 2 x 10^6 cells/ml in Erlenmeyer/shaker flasks or spinner flasks in either stage 1 basal media as described in Example 9 (containing 3.64g/l sodium bicarbonate), or in a modified stage 1 basal media which contained 2.43g/l sodium bicarbonate. Clusters were treated with stage 1 basal medium containing MCX and GDF-8 as described in Example 12. Samples were collected for flow cytometry at the end of day three of differentiation. Phase contrast images were also captured on each day of differentiation.

[0276] The efficiency with which definitive endoderm was generated was then determined by measuring the percentage of cells expressing the cells surface markers CXCR4, CD99 and CD9 using flow cytometry. The data is shown in FACS plots in Figures 44 A&B and summarized in Table 17. In suspension cultures, sodium bicarbonate levels, as low as 2.43g/L, appear to generate definitive endoderm less efficiently (on average, 87.4% of cells express CXCR4) than when the cells were differentiated in medium containing 3.64g/L (on average, 97.35% of cells express CXCR4). In addition, it was observed that differences in bicarbonate levels correlated with differences in cluster morphologies at the end of stage 1, as observed by phase contrast microscopy (Figures 44 C&D). Also, cells differentiated under high bicarbonate levels were noted to form looser clusters than cells differentiated in 2.43 g/L of bicarbonate.

Table 17

Treatment	CD9 (% by FACS)	CD99 (% by FACS)	CD184 (% by FACS)
3.64g/L Sodium bicarbonate	5.5	92.7	97.7/97.0
2.43g/L Sodium bicarbonate	12.3	66.7	86.4/88.4

Example 14

Generating Pancreatic Progenitor Clusters From Human Induced Pluripotent Stem Cells In A Scalable Bioreactor Process

[0277] Cell therapies will require large numbers (>10^8) of cells per dose. This example demonstrates a process capable of differentiating induced pluripotent stem cell (iPS cell) masses at 3 to 5 orders of magnitude greater than possible with current cell therapy manufacturing practices.

[0278] In this example, an iPS cell line was used – UTC (derived from umbilical tissue cells previously described in US patent application 13/330,931 (U.S. Pub. App. 2013/0157365), the disclosure of which is incorporated by reference as it pertains to deriving iPS cell lines). The cells were derived on mouse embryonic feeder cells using plasmid transfection in a "foot-print" free manner and cryo-preserved at passage 15.

[0279] From these cryopreserved cells, a series of cell banks were generated by thawing a source material vial directly onto human recombinant laminin (hrLaminin, Catalog# LN-521 from Biolamina, Stockholm, Sweden) in Essential8™ medium (E8™) from Life Technologies Corporation (Grand Island, NY) to generate an in-house seed material. This thawed and expanded material was called a "Pre-Pre Master Cell Bank" (Pre-Pre MCB) which served as seed material for future banks. Using the pre-pre MCB 3 sequential cell banks were then generated - a Pre-MCB, a MCB, and a working cell bank (WCB). One WCB vial was then thawed, expanded on hrLaminin using EDTA passaging for three passages in E8™. The cells were first seeded from thaw into a T225 flask (Corning; Corning, NY) and then passaged into multiple T225 flasks. The multiple T225 flasks were then passaged and combined to seed a single 1-Layer Cell Stack™ (CS1). Once the cells in the CS1 were confluent, cells were washed once with $PBS^{-/-}$, treated with a half strength solution of Accutase® diluted with $PBS^{-/-}$ and incubated for 4 to 5 minutes. The Accutase® was then removed, and 3 minutes after application of the enzyme solution, the CS1 was tapped to encourage cell lifting. E8™ supplemented with 0.5% BSA and containing 10micromolar of the Rho Kinase inhibitor, Y-27632, was added to the CS1 to rinse and inactivate the residual Accutase®. The rinse was then collected and a second rinse volume was added, collected, and pooled with the first rinse.

[0280] The cells were transferred in medium supplemented with 0.5% BSA and containing 10micromolar of the Rho Kinase inhibitor, Y-27632, to a 1 liter disposable spinner flask (Corning; Corning, NY) at a concentration of 1 x 10^6 cells/mL in 225mL liter. The cells were

allowed to cluster in static suspension for 60 minutes in a humidified 5% CO_2 incubator, then agitated for 5 minutes at 55-65 rpm and 225mL additional medium supplemented with 0.5% BSA and containing 10micromolar of the Rho Kinase inhibitor, Y-27632 was added. The cells were allowed to settle in static culture for 30 additional minutes, and then 150mL additional medium supplemented with 0.5% BSA and containing 10micromolar of the Rho Kinase inhibitor, Y-27632, was added to the spinner flask. Thereafter the cells were continuously stirred at 50-70 rpm in a humidified 5% CO_2 incubator. Twenty-four hours later the spinner flask was removed from the incubator and the clusters allowed to settle for 5-10 minutes. The medium was then aspirated until 200mL remained in the vessel and 400mL of additional fresh culture medium was then added to the spinner flask. This process was repeated at the end of day 2 (48 hours after transfer).

[0281] Then 72 hours after initial Accutase® treatment the process of cell cluster dissociation and spinner flask seeding (passaging) was repeated to maintain the cells in suspension for multiple passages (tested range: 1-10 passages).

[0282] Using this process UTC iPS cells were converted from adherent culture on a substrate to suspension culture as cell clusters and then expanded in suspension. These suspension passaged and cultured cells were then cryopreserved and stored for later use. In order to prepare the suspension expanded cell clusters for cryopreservation the cell clusters were dissociated with Accutase® as described above, except cells were not passed through a 40 micron c

[0283] ell strainer. The cells from each 1 liter disposable flask were then counted, combined as needed and centrifuged for 5 minutes at 80-200 rcf. The supernatant was then removed as completely as possible without disturbing the cell pellet. Cold (<4°C) CryoStor®10 was then added in a drop-wise manner to achieve a final concentration of 150 million cells per mL and the cell solution was held in an ice bath during transfer to a 1.8mL corning cryo vial (Corning; Corning, NY) or 15mL Miltenyi cryo bag(Miltenyi Biotec Inc. Auburn, CA).

[0284] The suspension expanded cells were then frozen in a vial at high density in a controlled rate freezer as follows. The chamber was pre-cooled to 4°C and the temperature was held until sample vial temperature reached 6°C. The chamber temp was then ramped down at 2°C/min until the sample reached -7°C. Once the sample vial reached -7°C, the chamber was cooled 20°C/min until the chamber reached -45°C. The chamber temperature was then allowed to briefly rise at 10°C/min until the chamber temperature reached -25°C, and the chamber was then further cooled at 0.8°C/min until the sample vial reached -45°C. The chamber temperature was then cooled at 35°C/min until the chamber reached -160°C.

The chamber temperature was then held at -160°C for at least 10 minutes, after which the vials were transferred to gas phase liquid nitrogen storage.

[0285] In order to inoculate a stirred tank bioreactor the high density cryo-preserved cells were removed from the liquid nitrogen storage, thawed and used to seed a closed 0.2 liter glass bioreactor (DASGIP; Julich, Germany). Cryo-vials were removed from gas phase liquid nitrogen storage and placed directly in a 37°C water bath for 105 seconds. The thawed vial contents were then transferred via 2mL glass pipette to a 50mL conical tube. Then 9mL of E8™ containing 0.5%BSA supplemented with 10micromolar Rho Kinase inhibitor, Y-27632 was then added to the tube in a drop wise manner. The cells were then centrifuged at 80-200rcf for 5 minutes. Afterwards, the supernatant was aspirated from the tube and, 10ml of fresh E8 containing 0.5%BSA and supplemented with 10micromolar Rho Kinase inhibitor, Y-27632 was added. This volume containing the cells was pipetted into a media transfer bottle (Cap2V8®, SaniSure, Moorpark, CA) and the bottle contents were pumped directly into the bioreactor via a sterile C-flex tubing weld by peristaltic pump. In preparation for pluripotent stem cell inoculation the bioreactor was prepared with 0.15L of E8™ supplemented with 0.5% BSA and 10micromolar Rho Kinase inhibitor, Y-27632, pre-warmed to 37°C, stirred at 70 rpm, regulated to 6.8-7.1 pH by CO_2, with a dissolved oxygen set-point of 30% (CO_2, air, O_2, and N_2 regulated). Immediately post-inoculation the bioreactor was sampled for cell count, and medium volume was adjusted as needed to give a final cell concentration of 0.225 x 10^6 cells/mL.

[0286] The cells inoculated into the stirred tank bioreactor formed cell clusters in the continuously stirred tank. After inoculation, the cell clusters were maintained in E8™ medium, supplemented with 0.5% BSA, in the reactor for three days. The medium was changed daily; 24 hours after inoculation 90% of spent medium was removed and 0.15 liters of fresh medium added. Forty-eight hours after inoculation, 90% of spent medium was removed and 0.15 liters of fresh medium was added. At 72 hours after inoculation, pluripotent cell differentiation was initiated by removing >90% of the spent medium and adding differentiation medium (Table 18).

[0287] Once the staged differentiation process was initiated the cells were maintained for 12 or more days in the closed sterile suspension bioreactor regulated for temperature (37°C), pH (7.4 for differentiation), and dissolved oxygen (10% DO set-point for stage 1 and 30% DO set-point all other times, CO_2, O_2, N_2, and air regulated). Throughout the differentiation process, at each media exchange, the impeller was stopped 5-20 minutes prior to medium removal via dip-tube to allow clusters to settle. Medium in the bioreactor was removed or

added to/from a closed bottle or bag by peristaltic pump through a dip tube connected to C-Flex® tubing using a Terumo™ tube welder to maintain a closed system. The impeller and heater were re-energized once sufficient medium was added to the vessel to fully submerge the impeller.

[0288] In order to monitor the bioreactor process samples of medium containing cell clusters were drawn daily to determine cell number and viability (NucleoCounter®) as shown in Figure 45. A general expansion of cells was observed during the process, as the inoculum of 0.225 x 10^6 viable cells/mL expanded to generate 0.65 x 10^6 viable cells/ mL at stage 4 day 3 (Figure 45).

[0289] In addition to daily counts, bioreactor medium samples were analyzed by NOVA BioProfile® FLEX (Nova Biomedical Corporation, Waltham, MA). It was observed that, per the reactor set-point at stage 0 (pH 6.8), the pH of the medium in stage 0 was acidic (pH 6.8) through stage 0 (Figure 46). The acidic set-point at stage 0 appeared to reduce the metabolic activity of the cells, at a relatively low lactic acid and high glucose levels in stage 0 media were observed. Once the cells began differentiation through to the end of stage 3, the cells consumed almost all of the glucose (Figure 47) in the media and generated high levels of lactic acid (Figure 48). Additionally increases in cell density were observed over the course of stages 1 and 2 (Figure 45).

[0290] In order to determine if stage specific changes in pH and metabolism matched stage changes in mRNA expression patterns as measured by qRT-PCR the following was done. Four Applied Biosystems Low Density Arrays were used (Life™ ,Carlsbad, CA) designated Pluripotency, Definitive Endoderm (DE), Gut Tube (GT), or stage 4 (S4). Results are presented as fold differences versus undifferentiated UTCiPS cell sample as control to standardize expression across all runs and arrays.

[0291] Using these arrays, gene expression was determined at each stage of differentiation. It was then observed that seed material cells thawed into the bioreactor showed an undifferentiated gene expression pattern at stage 0 day 1, 2, and 3 (24, 48, and 72 hours after bioreactor inoculation: Figures 49 and 50). These results correlated well with flow cytometry results which showed high expression levels of CD9, SSEA4, TRA-1-60, and TRA-1-81, and the absence of CXCR4/CD184 (Figure 51). These flow cytometry and qRT-PCR data showed robust and stable expression patterns for genes of pluripotency (CD9, NANOG, POU5F1, SOX2, TDGF, and ZFP42) and no expression of genes that are characteristically expressed during differentiation (CD99, CDH2, CDX2, CER1, CXCR4, EOMES, FGF17, FGF4, FOXA2, GATA2, GATA4, GATA6, GSC,HAND2, HNF4α, KIT, MNX1, MIXL1,

PRDM1, PTHR1R, SOX17, SOX7, T, TMPRSS2, and VWF) consistent with a stable pluripotent state.

[0292] At the completion of stage 0 (72 hours after reactor inoculation), the cells were moved into differentiation medium (Table 18) containing MCX and GDF8. Twenty-four hours after this media change significant alterations in gene expression patterns (Figures 49 and 50 fold expression versus undifferentiated control) were noted, such as a >10x increase in FOXA2, HAND2, PRDM1, PTH1R and SOX17 expression, >100x increase in CER1, FGF4, GATA4, GATA6, GSC, and MNX1 and a >1000x increase in EOMES, FGF17, MIXL1, and T expression. These increased expression levels indicated the cells were transitioning through a mesendodermal fate. It was also noted that CDX2 levels were elevated at stage 1 day 1 versus undifferentiated cells (2700x increase in expression vs. control), however this was a transient increase in expression and CDX2 levels dropped 97% by stage 1 day 3 to levels comparable to those observed prior to induction of differentiation (Figures 49 and 50 fold expression versus undifferentiated control).

[0293] At 72 hours after exposure to the stage 1 differentiation medium, the cells expressed a profile consistent with specification to definitive endoderm, as CXCR4 levels peaked at ~400x over historical control, FOXA2 was expressed at 136x over control and SOX17 was expressed at 470,000x over historical control. Consistent with definitive endoderm, it was also noted that gene expression of CER1, EOMES, FGF4, GSC, MIXL1, and T at the end of stage 1 (day 3) had dropped from the elevated levels observed at stage 1 day 1 (Figures 49 and 50 fold expression versus undifferentiated control).

[0294] These changes in gene expression observed with qRT-PCR correlated with results observed by flow cytometry. A near complete transition was seen from a CD9 expressing/CXCR4 negative pluripotent cell population at the initiation of differentiation (Figure 51) to a homogeneous population of CXCR4 expressing cells (98.6% of cells CXCR4 positive) at the end of stage 1 (Figure 52).

[0295] Following the completion of definitive endoderm formation (stage 1) the medium was changed to one containing FGF7, a morphogen used to induce primitive foregut formation. Consistent with formation of primitive foregut, HNF4α and GATA6 expression levels at stage 2 days 1 and 3 increased, while genes expressed at high levels on stage 1 day 3 (CXCR4, EOMES, FGF17, FGF4, MNX1, PRDM1, SOX17, and VWF) showed reduced expression by the end of stage 2 (Figures 50 and 53 fold expression versus undifferentiated control). The expression of foregut genes (AFP, HHEX, PDX1, and PROX1) was increased (Figure 53 fold expression versus undifferentiated control).

[0296] After the cells had been cultured in stage 2 medium for 72 hours, the culture was switched to a stage 3 medium (Table 18). Once in this medium the cells expressed markers consistent with an endodermal pancreatic lineage as measured by qRT-PCR assay for gene expression. Gene expression for PDX1 increased 60 fold from 12,000x over control at the end of stage 2 day 3 to 739,000x over control at the end of stage 3 day 3. These data indicated the cells were specifying to a pancreatic fate (Figure 54). Supporting this observation were increased expression levels versus undifferentiated control for a host of genes commonly expressed in pancreas (ARX, GAST, GCG, INS, ISL1, NEUROD1, NGN3, NKX2.2, NKX6.1, PAX4, PAX6, PTF1A, and SST) as shown in Figures 54 and 55. Interestingly no OCT4/POU5F1 expression (37 sample Cts by qRT-PCR) and high expression levels for other markers of endodermal lineages AFP, ALB, and CDX2 were also observed. This indicates that the cell population in the bioreactor differentiated from a pluripotent cell population first to a relatively plastic gut tube fate and then further differentiated to a pancreatic fate (Figures 54 and 55).

[0297] At the end of the four stage differentiation process the cells retained high levels of PDX1 (95.6% positive by FACS, ~1,000,000 fold induction over control by qRT-PCR) and FOXA2 (99.5% positive by FACS) expression. The cells showed an expression pattern consistent with pancreatic progenitor cells (39.2% positive for NKX6.1 by FACS) and a population of pancreatic endocrine cells (9.4% positive for PAX6, 12.4% positive for Chromogranin, 15.2% positive for NKX2.2; all by FACS). This stage specific marker expression pattern indicated an efficient stage-wise differentiation from a pluripotent population to pancreatic precursor cells. These results observed with flow cytometry, were confirmed by qRT-PCR. It was also noted that a host of genes commonly expressed in pancreas (ARX, GAST, GCG, IAPP, INS, ISL1, MAFB, NEUROD1, NGN3, NKX2.2, NKX6.1, PAX4, PAX6, PTF1A, and SST) all had increased expression levels on stage 4 day 3. (Figure 55). For reference, a representative micrograph (4x) of cell clusters at the end of each stage is shown in Figure 56.

Table 18

Starting Day/Date:	Stage 1	Stage 2	Stage 3	Stage 4
Basal Media	MCDB131 Cust ($3.64g/L\ NaCO_3$)	MCDB131 Cust ($3.64g/L\ NaCO_3$)	MCDB131 Cust ($3.64g/L\ NaCO_3$)	MCDB131 Cust ($3.64g/L\ NaCO_3$)
Supplement	2% FAF-BSA 2.5mM glucose 1:50,000 ITS-X Glutamax 1:100	2% FAF-BSA 2.5mM glucose 1:50,000 ITS-X Glutamax 1:100	2% FAF-BSA 2.5mM glucose 1:200 ITS-X Glutamax 1:100	2% FAF-BSA 2.5mM glucose 1:200 ITS-X Glutamax 1:100
Growth factors	***Day 1 and 2 only:*** ***GDF8*** 100 ng/mL	**FGF7** 50 ng/mL	**FGF7** 50ng/mL	None
Small molecules	***Day 1 only:*** **MCX** [2μM]		RA [2 μM] SANT [0.25 μM] TPPB [100 nM] ***Day 1 only*** ***LDN*** *[100 nM]*	SANT [0.25 μM] TPPB [100 nM]
Days	**3**	**3**	**3**	**3**
NOTES: All Days refer to 0H	**Media change Days 1 and 2, No change Day 3**	**Media change Days 1 and 3, No change Day 2**	**Media change Days 1 and 2, No change Day 3**	**Media change Day 1 and end of Day 3 if S4 is extended**

Table 18a

BX replicate	Seed Material	CD9	CD184	SSEA4	TRA-1-60	TRA-1-81
1	KC	83.3	0.1	99.9	94.5	85.8
2	HW	95.5	0.2	100	91	84
3	ISM (Pink)	95.8	0.1	100	76.1	36.5
4	ISM (Pink)	93.2	0	99.9	78.6	64.5
5	ISM 1	97.8	0.2	99	74.8	66.4
6	ISM 2	98.6	0.2	100	92.2	86
7	ISM 1	98.1	0.1	99.9	88.8	80.3
8	ISM 1	99.1	0.1	99.9	93.8	83.3
9	ISM 2	97.2	0.1	99.9	88.3	81
10	ISM5	98	0.1	99.3	93.1	85.7
11	ISM6	72.6	0.2	99.9	94.7	88.9
12	ISM6	85.9	0.7	99.4	71.9	54.1
		CD9	**CD184**	**SSEA4**	**TRA-1-60**	**TRA-1-81**
	Average	93.6	0.1	99.8	87.8	76.6
	St. Deviation	8.3	0.1	0.3	7.6	15.5

Table 18b

Stage-Day-Time	Viable Cell density (M cells/mL)	CD9	CD184	SSEA4	TRA-1-60	TRA-1-81
S0D3-24H	0.626	95.8	0.1	99.8	87.9	74
	Viable Cell density (M cells/mL)	**CD9**	**CD184**	**CD99**		
S1D3-24H	0.9	50.7	98.9	99		
	Viable Cell density (M cells/mL)	**NKX6.1**	**CHROMG.**	**NKX2.2**	**PDX1**	**FOXA2**
S4D1-24H	0.943	69.3	14.2	23.6	98.8	99.7
	Viable Cell density (M cells/mL)	**NKX6.1**	**CHROMG.**	**CDX2**	**SOX2**	**NKX2.2**	**PD PDX1**	**FOX FOXA2**	**NE NEUF**
S4D3-24H	1.002	66.2	35.6	0.3	15.8	38.1	9 99	9 99	45 45.6

Materials:

- human embryonic stem (hES) cell line H1, (WA01 cells, WiCell, Madison WI)
- PBS (Catalog# 14190, Invitrogen)
- Y-27632 (Axxora Catalog#ALX-270-333, San Diego, CA)
- EDTA, (Lonza, Catalog# 17-7-11E)
- NucleoCounter®-(ChemoMetec A/S, Cat#YC-T100, Allerod, Denmark)
- Non-Tissue Culture Treated 6 well dishes (Becton Dickinson, Catalog# Falcon 351146, Franklin Lakes, NJ)
- Accutase®, (Sigma-Aldrich, Catalog# A-6964, St. Louis, MO)

- pH, and dissolved oxygen (DO)bioreactor probes (FermProbe® pH electrode 225mm, Model # F-635, and DO OxyProbe® 12mm Sensor, Model # D-145 from Broadley-James Corporation, Irvine CA)
- Immune-protective macro encapsulation device (TheraCyte™, Irvine CA)
- HUMAN C-PEPTIDE ELISA (MERCODIA CAT# 10-1141-01)
- GlutaMAX™, MCDB131, and ITS-X Invitrogen
- FAF-BSA (Proliant)
- Retinoic Acid, Glucose 45% (2.5M), SANT (Shh inhibitor) (Sigma)
- GDF8 (Peprotech)
- MCX
- FGF7 (R & D Systems)
- LDN-193189 (BMP receptor antagonist) (Stemgent)
- TPPB (PKC activator) (ChemPartner)

Example 15

Differentiation of Human Embryonic Stem Cells from Cell Line WA01 into Definitive Endoderm: role of MCX/GDF8 as a cell cycle regulator in Suspension Culture

[0298] Clusters from pluripotent human embryonic stem cell line H1 (NIH code: WA01) were seeded at 0.5 x 10^6 cells/ml in Erlenmeyer shaker flasks in MCDB-131 medium containing 3.64g/ml sodium bicarbonate and 5.5mM glucose (Catalog # A13051 DJ, Invitrogen, CA), which was supplemented with 2% fatty acid free BSA (Catalog # 68700, Proliant, IA), 1X GlutaMAX™ (Catalog # 35050-079, Invitrogen, CA), an additional 2.5mM glucose (Catalog # G8769, Sigma) and ITS-X at 1:50,000 stock concentration (Catalog # 51500056, Invitrogen, CA). MCDB-131 medium supplemented in this manner will be referred to as stage 1 basal medium or "Neat" medium for the purposes of this example. 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, U.S. Patent Application No. 12/494,789; incorporated herein by reference in its entirety will be referred to as "MCX".

[0299] Clusters were treated on the first day of differentiation with one of six conditions: (1) Neat, (2) 3μM MCX plus 100ng/ml GDF-8 (Catalog # 120-00, Peprotech), (3) 3μM MCX only, (4) 100ng/ml GDF-8 only, (5) 20ng/ml WNT-3A (Catalog # 1324-WN-002, R&D

Systems, MN) plus 100ng/ml Activin A (Catalog # 338-AC, R&D Systems, MN), or (6) 20ng/ml WNT-3A only.

[0300] Media in each of the conditions was changed at 24 and 48 hours after the initiation of differentiation. At these times, cells in conditions 1, 2, 3, and 4 were changed to fresh stage 1 basal media supplemented with 100ng/ml GDF8 while cells in conditions 5 and 6 were changed to fresh stage 1 basal media supplemented with100ng/ml Activin A.

[0301] One hour prior to initiation of differentiation, and 5, 23, 29, 47, or 71 hours after the initiation of differentiation (referred to as "Time 0"), suspension samples were transferred to a non-tissue culture treated six well dish and incubated with EdU (Click-iT® EdU Kit, Life Technologies Corporation, Carlsbad, CA) for one hour. The EdU incubated cells were then assessed by flow cytometry at times 0, 6, 24, 30, 48, or 72 hours after initiation of differentiation to measure the percentage of cells in G0/G1, S, or G2/M stages of the cell cycle (Figures 81-87).

[0302] Following this protocol, significant differences in the percentage of cells in G0/G1, S, or G2/M stages of the cell cycle were observed (Figures 82-87) and it was noted that MCX and MCX+GDF8 treated cells had a nearly 40% reduction in the incorporation of EdU compared to the other four treatment conditions (Figure 81). This reduction in EdU incorporation was matched by a 38% increase in G0/G1 cells from the MCX+GDF8 treated sample and a 54% increase in G0/G1 cells for the MCX only treated cells. These changes to EdU incorporation and the increased transition to G0/G1at 6 hours following initiation of differentiation were not observed in cells treated with GDF8, WNT3A, WNT-3A + Activin A, or neat medium. Rather, cells treated with GDF8, WNT-3A, WNT-3A + Activin A, or neat medium demonstrated a minimal reduction in the percentage of cells with EdU incorporation (mean, 48.1%, SD±1.2) and an average 13% decrease in the number of cells in G0/G1 six hours after the initiation of differentiation (Standard Deviation, ± 5%) as shown in Figures 81 and 82.

[0303] Similar differences were observed later in the process in the spread between G0/G1 values for cells treated with MCX or MCX+GDF8 compared to the other treatment conditions. At 30 hours after time 0, MCX or MCX+GDF8 treated cells had 43-45% fewer cells in G0/G1 as compared to cells treated with WNT-3A + Activin A, GDF8, WNT-3A, or neat medium. This gap between percentage of G0/G1 cells was retained at 48 hours after initiation of differentiation, as 71.9-75.5% of cells treated with MCX or MCX+GDF8 were in G0/G1 of the cell cycle, while 48.5% of GDF8, 55.8% of WNT3A, 57.7% of WNT-3A + Activin A, or 49% of neat medium treated cells were in G0/G1. In addition to the observed

differences in EDU incorporation and G0/G1 profiles, MCX or MCX+GDF8 treated cells had 15-33% more cells in the S phase of cell cycle at 30 and 48 hours after time 0 when compared with WNT3A + Activin A, GDF8, WNT-3A, or neat medium treated cells (Figures 84 and 85).

[0304] The data (gene expression for CD99, CD9, CDH1, CDH2, CDX2, CER1, CXCR4, FGF17, FGF4, FOXA2, GATA4, GATA6, GSC, KIT, MIXL1, MNX1, NANOG, OTX2, POU5F1, SOX17, SOX7, and T, shown in Figures 57-80 and 88a-f) indicated that in suspension culture, addition of MCX with or without the TGF-β family member, GDF8, for the first day of differentiation generated definitive endoderm comparable to that obtained when cells are treated with 20ng/ml WNT-3A plus 100ng/ml Activin A on day one, as measured by gene expression at the end of definitive endoderm formation. However, consistent with the differences in cell cycle observed through the process of forming definitive endoderm, intermediate differences in gene expression were seen. In samples treated with MCX or MCX+GDF8 the genes T (brachyury), GATA4, and CDX2 were induced at levels substantially higher than cells treated with WNT-3A+Activin A or the other three tested conditions in the first 24 hours of differentiation (Figures 88 b, c, and d). Conversely, the expression of genes for pluripotency (NANOG and POU5F1/OCT4) was dramatically reduced by 24 hours in samples treated with MCX or MCX+GDF8 when compared to the starting cell population or the other four conditions tested (Figure 88e). The magnitude of induction of expression for genes such as FGF4, FOXA2, and SOX17 was much lower in MCX or MCX+GDF8 samples when compared to the other four conditions tested at 24 hours after the initiation of differentiation, however by 48 hours all samples expressed FGF4, FOXA2, and SOX17 at comparable levels. (Figure 88c and e).

Example 16

Generating Ectodermal and Mesodermal Tissues Using a Scalable Suspension Differentiation Process

[0305] This example demonstrates a process capable of both expanding and differentiating pluripotent stem cells (PSC) to achieve a scalable manufacturing process for generation of ectodermal or mesodermal tissues.

[0306] Two cell lines were suspension expanded to provide seed material for these studies: a sub-clone of the H1 (WA01)hES cell line - WB0106 and an induced pluripotent stem cell (iPSC) line generated from umbilical tissue cells (UTC). As described in prior examples,

suspension expanded cells were frozen at high density in a controlled rate freezer, then thawed to inoculate a closed 3 liter glass bioreactor (DASGIP; Julich, Germany) or disposable 3 liter single use bioreactor (Mobius®, EMD Millipore Corporation, Billerica, MA) at a final cell concentration of 0.225 x 10^6 cells/mL. The cells inoculated into the stirred tank bioreactor formed cell clusters in the continuously stirred tank, and were maintained in pluripotency medium (E8™, supplemented with 0.5% BSA) in the reactor for three days total. At 72 hours after inoculation, pluripotent cell differentiation was initiated by transferring cell clusters to plastic disposable Erlenmeyer flasks (PETG 125mL flask, Cat#4112, Thermo Scientific Rochester NY) in their respective differentiation medium (Table 19) to form mesoderm/cardiac tissue (1) or ectoderm/neural tissue (2).

[0307] Once the staged differentiation process was initiated, the cells were maintained for ten (10) days at 100 rpm in a humidified, 5% CO_2 incubator on a shaker platform (MAXQ 416hp, Thermo Scientific, Rochester NY). At 1 day, 3 days, 5 days, and 7 days after the initiation of differentiation the medium in the flask was exchanged for fresh medium made as described in Table 19. qRT-PCR samples were taken prior to starting differentiation for reference and then 3, 5, 7, and 10 days after initiating differentiation.

[0308] In order to determine if ectodermal or mesodermal specific changes in mRNA expression patterns could be detected by qRT-PCR, three Applied Biosystems Low Density Arrays (Life™, Carlsbad, CA) designated Pluripotency, Definitive Endoderm (DE), and stage 6 (S6) were used and the results were compared to the appropriate undifferentiated pluripotent stem cell sample as control to standardize expression.

[0309] Using these arrays, the gene expression pattern of pluripotent cells cultured in ectodermal (Figure 89) or mesodermal (Figure 90) differentiation medium was determined. It was observed that cells differentiated in shaker flasks under either condition demonstrated reduced pluripotent gene expression for genes of pluripotency like NANOG, POU5F1/OCT4, TDGF1, and ZFP42 over extended culture from day 3 to day 10 as measured by Pluripotency Array. The expression of CXCR4 increased in samples from hES or iPS cells differentiated to either ectoderm or mesoderm. These results correlated with qRT-PCR data showing high expression of genes characteristic of differentiation. Cells treated with ectodermal differentiation medium expressed increased levels of ARX, NEUROD, NKX6.1, PAX6 (>100 fold), and ZIC1 (>1000 fold) by qRT-PCR from 3 to 10 days after initiation of differentiation (Figure 91). These data were confirmed by FACS array, which showed that three (3) days after beginning the initiation of differentiation to an ectodermal fate both iPSC and hES cells maintained high expression of SOX2 (a gene required for both pluripotency

and neural stem cells), but lost expression of POU5F1/OCT4 (a gene required for pluripotency) while gaining PAX6 expression (a gene of neural and endocrine differentiation) (Figure 92).

[0310] Similar kinetics of differentiation in cells treated with mesodermal differentiation medium were also observed. As pluripotent gene expression dropped over the course of the 10 day differentiation (Figure 90), an early induction was observed for genes characteristic of the early, transient mesendoderm fate (CER1, EOMES, CKIT, and VWF) at day 3 and these genes expression levels declined to near baseline by day 10 (Figure 93). It was also observed that expression of characteristic mesoderm genes at 3, 5, 7, and 10 days after initiation of differentiation showed early and increasing gene expression (CDH2, CDX2, GATA6, HNF4α, MNX1, PRDM1, and SOX17 in Figure 93). The same pattern of gene induction was observed in both iPS and hES cell samples indicating the differentiation process was directed and not spontaneous in nature.

[0311] These changes in gene expression observed by qRT-PCR correlated with results observed by phase contrast microscopy and immunstained cryo-sections of clusters. By day 10 in the mesodermal differentiated suspension culture, about 1 in 10 clusters began to spontaneously "beat" suggesting the cells had differentiated to myo-cardial tissue (Figure 94, left panel, day 10, white bars). Stained cross sections of some clusters showed a striated, end to end, β-tubulin staining pattern indicative of muscle formation (Figure 94, right panel).

[0312] A strikingly different morphological pattern was observed for clusters differentiated to an ectodermal fate (Figure 95, left panel) when compared to clusters differentiated to mesoderm (Figure 94). The clusters throughout ectodermal differentiation were larger and denser than cells differentiated to a mesodermal fate, and the ectodermal differentiated cells expressed less total β tubulin. Those cells which did express β tubulin showed a more dendritic pattern of staining (Figure 95, right panel, white arrows) characteristic of neurons.

[0313] These results, in combination with qRT-PCR and FACS data, indicate that cells banked and expanded in suspension can be differentiated in suspension culture to mesodermal or ectodermal fates in a directed and reproducible manner.

Table 19

Starting Day/Date:	**Neural Differentiation Days 0-4**	**Neural Differentiation Day 5-10**	**Cardiac Differentiation Days 0-6**	**Cardiac Differentiation Days 7-10**
Basal Media	MCDB131 (2.5g/L $NaCO_3$ final)	MCDB131 Cust (2.5g/L $NaCO_3$ final)	MCDB131 Cust (2.5g/L $NaCO_3$ final)	MCDB131 Cust (2.5g/L $NaCO_3$)
Supplement	2% FAF-BSA 2.5mM glucose Glutamax 1:100 1:100 ITS-X	2% FAF-BSA 2.5mM glucose Glutamax 1:100 1:100 ITS-X or 1X B-27	2% FAF-BSA 2.5mM glucose Glutamax 1:100	2% FAF-BSA 2.5mM glucose Glutamax 1:100 1X B-27
Small molecules	***LDN** [100 nM]* ***ALKVi*** [7.5 μM]	***none***	***First 24 hrs only:*** MCX [2 μM] ***Days 3 and 4 only:*** IWP-4 [8 μM]	
Days	**3**	**3**	**3**	**3**
NOTES: All Days refer to time after initiation	**Media change: Days 0, 1 and 3**	**Media change: Days 5 and 7**	**Media change: Days 0, 1, 3, and 5**	**Media change Day 7**

Table 20

Materials:
human umbilical cord tissue-derived cells (as disclosed in U.S. Patent No. 7,510,873)
Inducible pluripotent stem cells
parthenotes
human embryonic stem (hES) cell line H1, (WA01 cells, WiCell, Madison WI)
PBS (Catalog# 14190, Invitrogen)
Y-27632 (Axxora Catalog#ALX-270-333, San Diego, CA)
EDTA, (Lonza, Catalog# 17-7-11E)
NucleoCounter®-(ChemoMetec A/S, Cat#YC-T100, Allerod Denmark)
Non-Tissue Culture Treated 6 well dishes (Becton Dickinson, Catalog# Falcon 351146, Franklin Lakes, NJ)
Accutase®, (Sigma, Catalog# A-6964, St. Louis, MO)
pH, and dissolved oxygen (DO)bioreactor probes (FermProbe® pH electrode 225mm, Model # F-635, and DO OxyProbe® 12mm Sensor, Model # D-145 from Broadley-James Corporation, Irvine CA)
Immune-protective macro encapsulation device (TheraCyte™, Irvine CA)
HUMAN C-PEPTIDE ELISA (MERCODIA CAT# 10-1141-01)
GlutaMAX™, MCDB131, and ITS-X (Life Technologies Corporation, Grand Island NY)
FAF-BSA (Proliant)
Retinoic Acid, Glucose 45% (2.5M), SANT (Shh inhibitor) (Sigma)
GDF8 (Peprotech)
MCX
IWP-4 (WNT3 inhibitor) Stemgent
MCDB131 media
MCDB131 media (customized ("MCDB131 Cust"))-modified to raise the $NaCO_3$ level to 3.64 g/L.

[0314] While the invention has been described and illustrated herein by reference to various specific materials, procedures and examples, it is understood that the invention is not restricted to the particular combinations of material and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art. It is intended that the specification and examples be considered as exemplary, only, with the true scope and spirit of the invention being indicated by the following claims. All references, patents, and patent applications referred to in this application are herein incorporated by reference in their entirety.

CLAIMS

1. An *in vitro* method of producing three-dimensional cell clusters of pluripotent stem cells comprising the steps of:

a. treating pluripotent stem cells cultured in a planar adherent culture with a chelating agent or enzyme to release cell aggregates from the planar adherent culture;
b. transferring the cell aggregates from the planar adherent culture to a dynamic suspension culture in the presence of a Rho-kinase inhibitor;
c. diluting the culture using culture media and a Rho-kinase inhibitor to a concentration of cells from about 1 to about 1.5 million cells/ml; and
d. expanding the suspension of cell aggregates to generate cell clusters, wherein the cell clusters maintain pluripotency.

2. The method of claim 1, wherein the pluripotent stem cells are treated with an enzyme selected from neutral protease or Accutase.

3. The method of claim 2, wherein the enzyme is neutral protease.

4. The method of any one of claims 1 to 3, wherein the treating in step a. does not completely disaggregate the cells to a single cell suspension.

5. The method of any one of claims 1 to 4, wherein the pluripotent stem cells are selected from the group consisting of induced pluripotent stem cells, human umbilical cord tissue-derived cells, parthenotes, human embryonic stem cells (hES) and amniotic fluid derived-cells.

6. The method of claim 5, wherein the cells are H1 hES.

7. The method of any one of claims 1 to 6, wherein the cells in the cell cluster express CD9, SSEA4, TRA-1-60, and TRA-1-81, and lack expression of CXCR4.

8. The method of any one of claims 1 to 7, wherein the pluripotent stem cells are treated with a chelating agent.

9. The method of claim 8, wherein the chelating agent is ethylenediaminetetraacetic acid

(EDTA).

10. A method of differentiating cell clusters of pluripotent stem cells in a dynamically agitated suspension culture system comprising:

a. treating pluripotent stem cells cultured in a planar adherent culture with a chelating agent or enzyme to release cell aggregates from the planar adherent culture;
b. transferring the cell aggregates from the planar adherent culture to a dynamic suspension culture in the presence of a Rho-kinase inhibitor;
c. diluting the culture using culture media and a Rho-kinase inhibitor to a concentration of cells from about 1 to about 1.5 million cells/ml;
d. expanding the suspension of cell aggregates to generate pluripotent cell clusters, wherein the cell clusters express CD9, SSEA4, TRA-1-60, and TRA-1-81, and lack expression of CXCR4; and
e. differentiating the pluripotent cell clusters in a dynamic agitated suspension culture system to generate a gut tube cell population, a neural precursor cell population or a cardiomyocyte precursor population.

11. The method of claim 10, wherein the pluripotent stem cells are selected from the group consisting of induced pluripotent stem cells, human umbilical cord tissue-derived cells, parthenotes, human embryonic stem cells (hES) and amniotic fluid derived-cells.

12. The method of claim 10 or claim 11, wherein the method generates a gut tube cell population which expresses β cell transcription factors.

13. The method of claim 12, wherein the transcription factors are PDX1 and/or NKX6.1.

14. The method of any one of claims 10 to 13, wherein the method comprises differentiating the pluripotent cell clusters in a dynamic agitated suspension culture system to generate a gut tube cell population.

15. The method of any one of claims 10 to 13, wherein the method comprises differentiating the pluripotent cell clusters in a dynamic agitated suspension culture system to generate a neural precursor cell population.

16. The method of any one of claims 10 to 13, wherein the method comprises differentiating the pluripotent cell clusters in a dynamic agitated suspension culture system to generate a cardiomyocyte precursor population.

17. The method of any one of claims 10 to 16, wherein the pluripotent stem cells are treated with a chelating agent.

18. The method of claim 17, wherein the chelating agent is EDTA.

19. The method of any one of claims 10 to 18, wherein the pluripotent stem cells are treated with an enzyme.

20. The method of claim 19, wherein the enzyme is a neutral protease or Accutase.

21. The method of any one of claims 10 to 20, wherein the treating in step a. does not completely disaggregate the cells to a single cell suspension.

22. The method of any one of claims 10 to 21, wherein the step of differentiating comprises culturing at an oxygen range from about hypoxia to about 30% of ambient, a lipid in the range of 0.1% to about 2%, or the combination thereof.

23. The method of claim 14, wherein the method comprises differentiating the three-dimensional pluripotent stem cell clusters to definitive endoderm cells in a culture medium supplemented with either (i) a cyclic aniline-pyridinotriazine and GDF8 or (ii) WNT3A and activin A.

24. The method of claim 23, wherein the cyclic aniline-pyridinotriazine 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-non- aen-16-one.

25. The method of any one of claims 1 to 24, wherein the dynamic agitated suspension culture system comprises microcarriers.

26. An *in vitro* method of producing pluripotent stem cell clusters comprising the steps of:

a. treating pluripotent stem cells cultured in a planar adherent culture with an enzymatic or chelating agent at room temperature for a time sufficient to release the pluripotent stem cells as cell aggregates;
b. removing said enzymatic or chelating agent;
c. adding culture media supplemented with a Rho-kinase inhibitor to the cell aggregates
d. transferring the cell aggregates cells to a dynamic suspension culture system; and
e. expanding the suspension of cell aggregates to generate cell clusters, wherein the cells in the cell clusters are pluripotent.

27. The method of claim 26, wherein the method comprises use of chelating agent.

28. The method of claim 27, wherein the chelating agent is EDTA.

29. The method of any one of claims 26 to 28, wherein the method comprises use of an enzymatic agent.

30. The method of claim 29, wherein the enzymatic agent is a neutral protease or Accutase.

31. The method of any one of claims 26 to 30, wherein the pluripotent stem cells are selected from the group consisting of induced pluripotent stem cells, human umbilical cord tissue-derived cells, parthenotes, human embryonic stem cells (hES) and amniotic fluid derived-cells.

32. The method of any one of claims 26 to 31 further comprising differentiating the three-dimensional pluripotent stem cell clusters in the dynamic agitated suspension culture system to generate a gut tube cell population, a neural precursor cell population or a cardiomyocyte precursor population.