SUMMARY OF THE INVENTION

[0007] In one aspect, there is provided an *in vitro* cell culture comprising: human cells, wherein at least 10% of said human cells are immature beta cells that co-express INS and NKX6.1; and an effective amount of Y-27632 to promote survival of the immature beta cells in the cell culture. In another aspect, there is provided a cryopreserved *in vitro* cell culture comprising: human cells, wherein the human cells comprise unipotent human immature beta cell suspension aggregates and wherein at least 10% of cells in the aggregates are human immature beta cells that co-express INS and NKX6.1, and a cryopreservation agent. In another aspect, there is provided a cryopreserved *in vitro* cell culture comprising: human cells, wherein at least 10% of said human cells are immature beta cells that co-express INS and NKX6.1; and a cryopreservation agent. In a further aspect, there is provided a cryopreserved *in vitro* cell culture comprising: human cells, wherein at least 50% of said human cells are immature beta cells that co-express INS and NKX6.1; and a cryopreservation agent. In one example, an in vitro method of producing a cell population including human PDX1 positive pancreatic endoderm cells is provided. The method includes contacting a cell population including definitive endoderm lineage cells with a TGFβ superfamily growth factor and a Wnt family member, thereby generating the cell population including PDX1 positive pancreatic endoderm cells. In examples, the TGFβ superfamily growth factor is selected from the group consisting of Nodal, Activin A, Activin B, BMP2, BMP4, GDF8, GDF-10, GDF-11 and GDF-15. In examples, the method further includes contacting the cell population including definitive endoderm lineage cells with an ERBB receptor kinase activating agent. In examples, the TGFβ superfamily growth factor is Activin A. In examples, the Wnt family member is Wnt3a. In examples, the ERBB receptor kinase activating agent is Heregulin.

[0008] In examples, the cell population including PDX1 positive pancreatic endoderm cells is contacted with at least 25 ng/mL of the TGFβ superfamily growth factor. In examples, the cell population including PDX1 positive pancreatic endoderm cells is contacted with at least 50 ng/mL of the TGFβ superfamily growth factor. In examples, the cell population including PDX1 positive pancreatic endoderm cells is contacted with at least 75 ng/mL of the TGFβ superfamily growth factor.

[0009] In examples, the cell population including PDX1 positive pancreatic endoderm cells is contacted with at least 25 ng/mL of the Wnt family member. In

examples, the cell population including PDX1 positive pancreatic endoderm cells is contacted with at least 50 ng/mL of the Wnt family member. In examples, the cell population including PDX1 positive pancreatic endoderm cells is contacted with at least 75 ng/mL of the Wnt family member.

[00010] In examples, the cell population is contacted with 5-15 fold less of the ERBB receptor kinase activating agent than each of the TGFβ superfamily growth factor and the Wnt family member. In examples, at least about 50% of said cell population including PDX1 positive pancreatic endoderm cells are PDX1-positive pancreatic endoderm cells. In examples, at least about 60% of the cell population including PDX1 positive pancreatic endoderm cells are PDX1-positive pancreatic endoderm cells. In examples, at least about 70% of the cell population including PDX1 positive pancreatic endoderm cells are PDX1-positive pancreatic endoderm cells. In examples, at least about 80% of the cell population

including PDX1 positive pancreatic endoderm cells are PDX1-positive pancreatic endoderm cells. In examples, at least about 90% of the cell population including PDX1 positive pancreatic endoderm cells are PDX1-positive pancreatic endoderm cell.

[00011] In examples, the PDX1-positive pancreatic endoderm cells further express NKX6.1 and do not substantially express NGN3. In examples, the PDX1-positive pancreatic endoderm cells do not express CHGA. In examples, the PDX1-positive pancreatic endoderm cells further express NKX6.1 and PFT1A and do not substantially express NGN3. In examples, the PDX1-positive pancreatic endoderm cells are multipotent cells that can that can differentiate into unipotent immature beta cells.

[00012] In one example, a human pancreatic endoderm cell culture including a definitive endoderm linage cell and a TGFβ superfamily growth factor and a Wnt family member is provided. In examples, the TGFβ superfamily growth factor is selected from the group consisting of Nodal, Activin A, Activin B, BMP2, BMP4, GDF8, GDF-10, GDF-11 and GDF-15. In examples, the method further includes contacting the cell population including definitive endoderm lineage cells with an ERBB receptor kinase activating agent. In examples, the TGFβ superfamily growth factor is Activin A. In embodiments, the Wnt family member is Wnt3a. In examples, the ERBB receptor kinase activating agent is Heregulin.

[00013] In another example, an in vitro unipotent human immature beta cell is provided. In examples, the unipotent human immature beta cell expresses INS and NKX6.1 and does not substantially express NGN3. In examples, the unipotent human immature beta cell is capable of differentiating to a mature beta cell. In examples, the differentiating is in vivo. In examples, the unipotent human immature beta cell forms part of a cell population. In examples, at least 10% of the cell population are immature beta cells. In examples, at least 20% of the cell population are immature beta cells. In examples, at least 30% of the cell population are immature beta cells. In examples, at least 40% of the cell population are immature beta cells. In examples, at least 50% of the cell population are immature beta cells. In examples, at least 60% of the cell population are immature beta cells. In examples, at least 80% of the cell population are immature beta cells. In examples, at least 90% of the cell population are immature beta cells. In examples, at least 98% of the

cell population are immature beta cells. In examples, the human immature beta cell is singly hormonal. In examples, the unipotent human cell expresses MAFB.

[00014] In one example, a method of producing a unipotent human immature beta cell is provided. The method includes contacting human definitive endoderm lineage cells in vitro with a TGFβ superfamily member and Wnt family member thereby generating immature beta cells. In examples, the unipotent human immature beta cell expresses INS, NKX6.1 and does not substantially express NGN3. In examples, the TGFβ superfamily member is selected from the group consisting of Nodal, Activin A, Activin B, BMP2, BMP4, GDF8, GDF-10, GDF-11 and GDF-15. In examples, the method further includes contacting the human definitive endoderm lineage cells in vitro with an ERBB receptor kinase activating agent. In examples, the TGFβ superfamily growth factor is Activin A. In examples, the Wnt family member is Wnt3a. In examples, the ERBB receptor kinase activating agent is Heregulin. In examples, the TGFβ superfamily growth factor is BMP.

[00015] In examples, the method further includes contacting the human definitive endoderm lineage cells in vitro with nicotinamide, a retinoic acid or retinoic acid analogue, a rho kinase inhibitor or a gamma secretase inhibitor. In examples, the retinoic acid is selected from the group consisting of all-trans-retinoic acid (RA), 13-cis-retinoic acid (13-cis-RA), and arotinoid acid (TTNPB). In examples, the retinoic acid is all-trans retinoic acid (RA). In examples, the retinoic acid is 13-cis-retinoic acid (13-cis-RA). In examples, the retinoic acid is arotinoid acid (TTNPB). In examples, the rho kinase inhibitor is selected from the group consisting of Y-27632, Fasudil, H-1152P, Wf-536 and Y-30141. In examples, the rho kinase inhibitor is Y-27632.

[00016] In examples, the gamma secretase inhibitor is selected from the group consisting of gamma secretase inhibitor I (GSI I), gamma secretase inhibitor II (GSI II), gamma secretase inhibitor III (GSI III), gamma secretase inhibitor IV (GSI IV), gamma secretase inhibitor V (GSI V), gamma secretase inhibitor VI (GSI VI), gamma secretase inhibitor VII (GSI VII), gamma secretase inhibitor IX (GSI IX), (DAPT), gamma secretase inhibitor XI (GSI XI), gamma secretase inhibitor XII, (GSI XII), gamma secretase inhibitor XIII (GSI XIII), gamma secretase inhibitor XIV (GSI XIV), gamma secretase inhibitor XVI (GSI XVI), gamma secretase inhibitor XVII (GSI XVII), gamma secretase inhibitor XIX (GSI XIX), gamma secretase inhibitor XX (GSI XX), gamma secretase inhibitor XXI (GSI XXI), gamma40 secretase inhibitor I, gamma40

secretase inhibitor II and RO4929097. In examples, the gamma secretase inhibitor is RO4929097. In examples, the gamma secretase inhibitor is GSI IV.

[00017] In another example, a method for producing mature beta cells in vivo is provided. The method includes, (a) contacting human definitive endoderm lineage cells in vitro with a TGFβ superfamily member and Wnt family member, thereby generating immature beta cells; (b) transplanting the immature beta cells of step (a) into a mammalian subject; and allowing the immature beta cells to differentiate in vivo to produce a population of cells including mature beta cells. In examples, the method further includes allowing the mature beta cells to produce insulin in response to glucose stimulation. In examples, the TGFβ superfamily growth factor is selected from the group consisting of Nodal, Activin A and Activin B, GDF-8, GDF-11 and GDF-15. In examples, the method further includes contacting the human definitive endoderm lineage cells in vitro with an ERBB receptor kinase activating agent. In examples, the TGFβ superfamily growth factor is Activin A. In examples, the Wnt family member is Wnt3a. In examples, the ERBB receptor kinase activating agent is Heregulin.

[00018] In examples, the human definitive endoderm lineage cells are contacted with at least 25 ng/mL of the TGFβ superfamily growth factor. In examples, human definitive endoderm lineage cells are contacted with at least 50 ng/mL of the TGFβ superfamily growth factor. In examples, the human definitive endoderm lineage cells are contacted with at least 75 ng/mL of the TGFβ superfamily growth factor.

[00019] In examples, the human definitive endoderm lineage cells are contacted with at least 25 ng/mL of the Wnt family member. In examples, the human definitive endoderm lineage cells are contacted with at least 50 ng/mL of the Wnt family member. In examples, the human definitive endoderm lineage cells are contacted with at least 75 ng/mL of the Wnt family member.

[00020] In examples, the human definitive endoderm lineage cells are contacted with 5-15 fold less of the ERBB receptor kinase activating agent than the TGFβ superfamily growth factor and the Wnt family member. In examples, at least 10% of the cell population are immature beta cells. In examples, at least 20% of the cell population are immature beta cells. In examples, at least 30% of the cell population are immature beta cells. In examples, at least 40% of the cell population are immature beta cells. In examples, at least 50% of the cell population are immature beta cells. In examples, at least 60% of the cell population are

immature beta cells. In examples, at least 70% of the cell population are immature beta cells. In examples, at least 80% of the cell population are immature beta cells. In examples, at least 90% of the cell population are immature beta cells. In examples, the immature beta cells express INS and NKX6.1 and do not express substantially NGN3. In examples, the immature beta cells express INS, NKX6.1 and MAFB and do not express substantially NGN3.

[00021] In one example, an in vitro unipotent human immature beta cell is provided. In examples, the unipotent human immature beta cell expresses INS and NKX6.1 and does not substantially express NGN3. In examples, the unipotent human immature beta cell is capable of maturing to a mature beta cell.

[00022] In one example, a method for producing mature beta cells in vivo is provided. The method includes: a. contacting human definitive endoderm lineage cells in vitro with a TGFβ superfamily member and Wnt family member thereby generating immature beta cells; b. transplanting the immature beta cells of step (a) into a mammalian subject; and c. allowing the immature beta cells to mature in vivo to produce a population of cells including insulin secreting beta cells.

[00023] In one example, a unipotent human immature beta cell that expresses INS and NKX6.1 and does not substantially express NGN3 is provided. In examples, the unipotent human immature beta cell is capable of maturing to a mature beta cell. In examples, the unipotent human immature beta cell further expresses MAFB.

[00024] In one example, an in vitro unipotent human immature beta cell expressing INS and NKX6.1 and not substantially expressing NGN3 is provided. In examples, the unipotent human immature beta cell is capable of maturing to a mature beta cell. In examples, the unipotent human immature beta cell further expresses MAFB.

[00025] In one example a method of producing a unipotent human immature beta cell is provided. The method includes contacting human definitive endoderm lineage cells in vitro with a TGFβ superfamily member and a Wnt family member thereby producing a unipotent human immature beta cell. In examples, the unipotent human immature beta cell expresses INS, NKX6.1 and does not substantially express NGN3. In examples, the method further includes contacting the human definitive endoderm lineage cells in vitro with an ERBB receptor kinase activating

agent. In examples, the TGFβ superfamily growth factor is Activin A. In examples, the Wnt family member is Wnt3a. In examples, the ERBB receptor kinase activating agent is Heregulin.

[00026] In one example, a method for producing mature beta cells in vivo is provided. The method includes: a. contacting human definitive endoderm lineage cells in vitro with a TGFβ superfamily member and Wnt family member thereby generating immature beta cells; b. transplanting the immature beta cells of step (a) into a mammalian subject; and c. allowing the immature beta cells to mature in vivo to produce a population of cells including insulin secreting beta cells. In examples, the method further includes contacting the human definitive endoderm lineage cells in vitro with an ERBB receptor kinase activating agent. In examples, the TGFβ superfamily growth factor is Activin A. In examples, the Wnt family member is Wnt3a. In examples, the ERBB receptor kinase activating agent is Heregulin. In examples, the human definitive endoderm lineage cells are contacted with at least 50 ng/mL of the TGFβ superfamily growth factor. In examples, the human definitive endoderm lineage cells are contacted with at least 25 ng/mL of the Wnt family member. In examples, the human definitive endoderm lineage cells are contacted with 5-15 fold less of the ERBB receptor kinase activating agent than the TGFβ superfamily growth factor and the Wnt family member. In examples, the immature beta cells express INS and NKX6.1 and do not substantially express NGN3. In examples, the immature beta cells express INS, NKX6.1 and MAFB and do not substantially express NGN3.

[00027] One example provides a composition and method for differentiating pluripotent stem cells or precursor cells derived from pluripotent stem cells to endocrine and non-endocrine populations. In one example, endocrine cells express at least CHGA (or CHGA+) and non-endocrine do not express CHGA (or CHGA-). In one example, these cell populations are referred to as endocrine (CHGA+) and non-endocrine (CHGA-) sub-populations, or just CHGA+ or CHGA- sub-populations, or simply endocrine and non-endocrine sub-populations. In another example, these endocrine and non-endocrine sub-populations may be multipotent progenitor/precursor sub-populations such as non-endocrine multipotent pancreatic progenitor sub-populations or endocrine multipotent pancreatic progenitor sub-populations; or they may be unipotent sub-populations such as immature endocrine cells, preferably immature beta cells, immature glucagon cells and the like.

[00028] One example provides a composition and method for differentiating pluripotent stem cells *in vitro* to pancreatic endoderm cell (PEC) cultures comprising both endocrine and non-endocrine sub-populations, wherein expression of NGN3 is suppressed. In one example, expression of NKX2.2 is also suppressed in the PEC culture. In one example, expression of CHGA is also suppressed in the PEC culture. In one example, expression of PDX1 and NKX6.1 are co-expressed on a single cell in the PEC culture. In one example, less than 50% preferably less than 40%, 30%, more preferably less than 20%, 10%, 5%, 2%, 1% of the cells in the PEC culture express NGN3, NKX2.2 or CHGA. In one example, the PEC culture is enriched for non-endocrine multipotent pancreatic progenitor sub-population (CHGA-). In one example, the PEC culture expresses markers (PDX1 and/or NKX6.1) for the non-endocrine multipotent pancreatic progenitor sub-population (CHGA-). In one example, the PEC culture with abundant non-endocrine multipotent progenitor sub-population (CHGA-) is implanted in a mammalian host and the graft matures and produces insulin *in vivo* in response to glucose. In one example, the PEC culture with abundant non-endocrine multipotent pancreatic progenitor sub-population (CHGA-) matures *in vivo* at a faster rate than PEC cultures without an abundant non-endocrine multipotent progenitor sub-population (CHGA-). In one example, the implanted PEC culture with abundant non-endocrine multipotent progenitor sub-population (CHGA-) has at least a 2X improvement in *in vivo* function as measured by C-peptide (insulin) production in response to glucose stimulation at about 10 weeks or about 12 weeks post implant than implanted PEC cultures without an abundant non-endocrine multipotent pancreatic progenitor sub-population (CHGA-).

[00029] One example provides a composition and method for differentiating pluripotent human stem cells *in vitro* wherein expression of genes typical of endocrine differentiation and development such as NGN3 and NKX2.2 are suppressed until after non-endocrine multipotent pancreatic progenitor (CHGA- and PDX1 / NKX6.1 co-expression) production.

[00030] One example provides a method for differentiating pluripotent stem cells *in vitro* to PEC cultures comprising the steps of: (a) obtaining a PDX1-negative foregut endoderm cell population; (b) contacting the cell population in step (a) with an agent that activates a TGFβ receptor family member thereby generating a cell culture comprising PEC wherein the PEC do not substantially express a marker selected from the group comprising CHGA, NGN3 or

NKX2.2. In one example, the PEC comprises a sup-population of cells that co-expresses PDX1 and NKX6.1. In one example, the PEC comprises a sub-population of non-endocrine multipotent pancreatic progenitor (CHGA-) cells. In one example the agent that activates a TGFβ receptor

family member is selected from the group consisting of Nodal, Activin A, Activin B, BMP2, BMP4, GDF8, GDF-10, GDF-11, GDF-15 and a mixture thereof. In one example, Activin A is added at 100 ng/mL, 75 ng/mL, 50 ng/mL, 25 ng/mL or 10 ng/mL at stage 3; and 15 ng/mL, 10 ng/mL or 5 ng/mL at stage 4. In one example, the PEC culture formed by adding Activin A to a PDX1-negative foregut endoderm cell population has at least a 2X improvement *in vivo* function as measured by C-peptide (insulin) production in response to glucose stimulation than PEC cultures without an abundant non-endocrine multipotent progenitor sub-population (CHGA-).

[00031] In one example, the PEC culture with a non-endocrine multipotent pancreatic progenitor sub-population (CHGA-) has at least a 2X improvement in *in vivo* function as measured by insulin production in response to glucose stimulation than PEC cell cultures without abundant non-endocrine multipotent progenitor sub-populations (CHGA-).

[00032] One example provides a method for differentiating pluripotent stem cells *in vitro* to a cell population comprising PEC comprising the steps of: (a) obtaining a PDX1-negative foregut endoderm cell population (b) contacting the cell population in step (a) with an agent that activates a TGFβ receptor family member thereby generating a PEC population wherein the PEC population does not substantially express a marker selected from the group comprising CHGA, NGN3 or NKX2.2. In one example, the PEC population comprises cells that co-express PDX1 and NKX6.1. In one example, the PEC population comprises non-endocrine multipotent pancreatic progenitor cells (CHGA-). In one example the agent that activates a TGFβ receptor family member is selected from the group consisting of Nodal, Activin A, Activin B, BMP2, BMP4, GDF8, GDF-10, GDF-11, GDF-15 and a mixture thereof. In one example the agent that activates a TGFβ receptor family member is Activin A. In one example, Activin A is added at 100 ng/mL, 75 ng/mL, 50 ng/mL, 25 ng/mL or 10 ng/mL at stage 3; and 15 ng/mL, 10 ng/mL or 5 ng/mL at stage 4. In one example the EGF family ligand is selected from the group comprising Heregulin (also known as Neuregulin1 (NRG1)), Neuregulin2 (NRG2), Neuregulin3 (NRG3), Neuregulin4 (NRG4), EGF (epidermal growth factor) and betacellulin. In one example the EGF family ligand is Heregulin. In one example, Heregulin is added at 2ng/mL, 5ng/mL, 10ng/mL, 20ng/mL, 15ng/mL, 30ng/mL, 40ng/mL, 50ng/mL, 60ng/mL, 70ng/mL or 80ng/mL. In one example, the PEC culture formed by adding Activin A and Heregulin to a PDX1-negative foregut endoderm cell population has at least a 2X improvement *in vivo* function as measured by C-peptide (insulin) production in response to glucose stimulation than PEC cultures without abundant non-endocrine multipotent progenitor sub-populations (CHGA-).

[00033] One example provides a method for differentiating pluripotent stem cells *in vitro* to a cell culture comprising PEC comprising the steps of: (a) obtaining a PDX1-negative foregut endoderm cell population (b) contacting the cell population in step (a) with at least an agent that activates a TGFβ receptor family member, an EGF family ligand or an agent that activates a Wnt signaling pathway, alone or in combination, thereby generating a cell culture comprising PEC wherein the PEC do not substantially express a marker selected from the group comprising CHGA, NGN3 or NKX2.2. In one example, the PEC culture comprises cells that co-express PDX1 and NKX6.1. In one example, the PEC cell culture comprises a non-endocrine multipotent pancreatic progenitor sub-population (CHGA-). In one example the agent that activates a TGFβ receptor family member is selected from the group consisting of Nodal, Activin A, Activin B, BMP2, BMP4, GDF8, GDF-10, GDF-11, GDF-15 and a mixture thereof. In one example the agent that activates a TGFβ receptor family member is Activin A. In one example, an agent that activates a Wnt signaling pathway is a Wnt family member selected from the group comprising WNT1, WNT2, WNT2B, WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A, WNT9B, WNT10A, WNT10B, WNT11, WNT16 and Wnt3A. In one example, the Wnt family member is Wnt3A. In one example, Activin A is added at 100 ng/mL, 75 ng/mL, 50 ng/mL, 25 ng/mL or 10 ng/mL at stage 3; and 15 ng/mL, 10 ng/mL or 5 ng/mL at stage 4. In one example, Heregulin is added at 2 ng/mL, 5 ng/mL, 10ng/mL, 20 ng/mL, 15 ng/mL, 30 ng/mL, 40 ng/mL, 50 ng/mL, 60 ng/mL, 70 ng/mL or 80 ng/mL. In one example, WNT is added at 5 ng/mL, 25 ng/mL, 50 ng/mL to 75 ng/mL more preferably at 50 ng/mL.

[00034] In one example, Activin A is added alone or combined with WNT at stage 3. In another example, Activin A is added with WNT or WNT and Heregulin at stage 3. In still one more example, Active A is added alone or combined with Heregulin at stage 4.

[00035] In one example, more than 10% preferably more than 20%, 30%, 40% and more preferably more than 50%, 60%, 70%, 80%, 90%, 95%, 98% or 100% of the cells in the cell population are the non-endocrine multipotent progenitor sub-population (CHGA-). In one example, the PEC cell culture formed by adding Activin A, Heregulin and WNT to a PDX1-negative foregut endoderm cell population has at least a 2X improvement *in vivo* function as measured by insulin production in response to glucose stimulation than PEC cell cultures without an abundant non-endocrine multipotent progenitor sub-population (CHGA-).

[00036] One example provides a composition and method for differentiating pluripotent stem cells *in vitro* to PEC cultures comprising minimal cells committed to the endocrine lineage (CHGA+). In one example, the cells committed to the endocrine lineage are characterized by the expression of CHGA. In one example, less than 50%, preferably less than 40%, 30%, more preferably less than 20%, 10%, 5%, 2%, 1% of the cells in the cells in the PEC culture are cells committed to the endocrine lineage (CHGA+).

[00037] One example provides a composition and method for differentiating pluripotent stem cells *in vitro* to substantially PEC cultures and further differentiating the PEC culture to endocrine cells *in vitro*. In one example, the endocrine cells express CHGA. In one example, the endocrine cells can produce insulin *in vitro*. In one example, the *in vitro* endocrine insulin secreting cells may produce insulin in response to glucose stimulation. In one example, more than 10% preferably more than 20%, 30%, 40% and more preferably more than 50%, 60%, 70%, 80%, 90%, 95%, 98% or 100% of the cells in the cells population are endocrine cells.

[00038] One example provides a method for differentiating pluripotent stem cells *in vitro* to endocrine cells comprising the steps of: (a) obtaining a cell population comprising PEC (b) contacting the cell population in step (a) with growth factors selected from the group consisting of a bone morphogenetic protein (BMP), a hedgehog inhibitor, Platelet-derived growth factor (PDGF), member of the fibroblast growth factor family, a retinoid, insulin growth factor 1 and 2 (IGF1 and IGF2) and nicotinamide thereby generating endocrine cells. In one example the endocrine cells express CHGA. In one example, the endocrine cells are immature insulin-producing beta-cells *in vitro*. In one example, the endocrine cells are loaded into an implantable semipermeable encapsulation device, mature and are capable of producing insulin in response to glucose stimulation *in vivo*. In one example, more than 10% preferably more than 20%, 30%, 40% and more preferably more than 50%, 60%, 70%, 80%, 90%, 95%, 98% or 100% of the cells in the cell population are endocrine cells. In one aspect, SSH is added at 10g/mL to 100ng/mL. In one example, PDGF is added at 10ng/mL to 100ng/mL. In one example, BMP is added at 10g/mL to 20ng/mL. In one example, FGF2 is added at 2ng/mL to 20ng/mL. In one example, IGF1 or IGF2 are added at 25ng/mL to 100ng/mL. In one example, the above compounds are added in combination with each other. In one example the member of the fibroblast growth factor family is FGF2. In one example the retinoid is retinoic acid and/or retinoic acid analogs such as TTNPB or TT3, Insulin-like growth factor I (IGF-I or IGF1) and -II (IGF-II or IGF2). In one example the bone morphogenetic protein is BMP4. In one example the hedgehog inhibitor is sonic Hedgehog,

KAAD-cyclopamine, KAAD-cyclopamine analogs, jervine, jervine analogs, hedgehog pathway blocking antibodies and any other inhibitors of hedgehog pathway function known to those of ordinary skill in the art.

[00039] One example provides a method for differentiating pluripotent stem cells *in vitro* to endocrine cells comprising the steps of: (a) obtaining a cell population comprising PEC (b) contacting the cell population in step (a) with a gamma secretase inhibitor thereby making endocrine cells *in vitro*. In one example the endocrine cells express CHGA. In one example, the endocrine cells are immature insulin-producing beta-cells *in vitro*. In one example, the endocrine cells are insulin secreting cells that produce insulin in response to glucose stimulation *in vitro*. In one example, the endocrine cells are loaded into an implantable semipermeable encapsulation device, mature and are capable of producing insulin in response to glucose stimulation *in vivo*. In one example, the gamma secretase inhibitor is selected from the group comprising N-[N-(3,5-Diflurophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester (DAPT), RO44929097, 1-(S)-endo-N-(1,3,3)-Trimethylbicyclo[2.2.1]hept-2-yl)-4-fluorophenyl Sulfonamide, WPE-III31C, S-3-[N'-(3,5-difluorophenyl-alpha-hydroxyacetyl)-L-alanilyl]amino-2,3-dih- ydro-1-methyl-5-phenyl-1H-1,4-benzodiazepin-2-one, (N)-[(S)-2-hydroxy-3-methyl-butyryl]-1-(L-alaninyl)-(S)-1-amino-3-methyl-- 4,5,6,7-tetrahydro-2H-3-benzazepin-2-one, BMS-708163 (Avagacestat), BMS-708163, Semagacestat (LY450139), Semagacestat (LY450139), MK-0752, MK-0752, YO-01027, YO-01027 (Dibenzazepine, DBZ), LY-411575, LY-411575, or LY2811376. In some examples, the gamma secretase inhibitor is present in the cell culture or cell population at a concentration ranging from about 0.01 μM to about 1000 μM. In preferred examples, the gamma secretase inhibitor is present in the cell culture or cell population at a concentration ranging from about 0.1μM to about 100 μM. In one example, the gamma secretase inhibitor is removed subsequent to its addition.

[00040] Examples described herein provide for compositions and methods of differentiating pluripotent human stem cells *in vitro* to endocrine cells. In one example, the endocrine cells express CHGA. In one example, the endocrine cells can produce insulin *in vitro*. In one example, the endocrine cells are immature endocrine cells such as immature beta cells. In one example, the *in vitro* insulin producing cells may produce insulin in response to glucose stimulation.

[00041] One example provides a method for producing insulin *in vivo* in a mammal, said method comprising: (a) loading an endocrine cell population into an implantable semi-permeable

device; (b) implanting the device with the endocrine cell into a mammalian host; and (c) maturing the endocrine cell population in said device *in vivo* wherein at least some of the endocrine cells are insulin secreting cells that produce insulin in response to glucose stimulation *in vivo*, thereby producing insulin *in vivo* to the mammal. In one example the endocrine cell is derived from a cell composition comprising PEC with a higher non-endocrine multipotent pancreatic progenitor sub-population (CHGA-). In another example, the endocrine cell is derived from a cell composition comprising PEC with a reduced endocrine sub-population (CHGA+). In another example, the endocrine cell is an immature endocrine cell, preferably an immature beta cell.

[00042] In one example the endocrine cells made *in vitro* from pluripotent stem cells express more PDX1 and NKX6.1 as compared to PDX-1 positive pancreatic endoderm populations, or the non-endocrine (CHGA-) subpopulations which are PDX1/NKX6.1 positive. In one example, the endocrine cells made *in vitro* from pluripotent stem cells express PDX1 and NKX6.1 relatively more than the PEC non-endocrine multipotent pancreatic progenitor sub-population (CHGA-). In one example, a Bone Morphogenic Protein (BMP) and a retinoic acid (RA) analog alone or in combination are added to the cell culture to obtain endocrine cells with increased expression of PDX1 and NKX6.1 as compared to the PEC non-endocrine multipotent progenitor sub-population (CHGA-). In one example BMP is selected from the group comprising BMP2, BMP5, BMP6, BMP7, BMP8 and BMP4 and more preferably BMP4. In one example the retinoic acid analog is selected from the group comprising all-trans retinoic acid and TTNPB (4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2- naphthalenyl)-l-propenyl]benzoic acid Arotinoid acid), or 0.1-10μM AM-580 (4-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid) and more preferably TTNPB.

[00043] One example provides a method for differentiating pluripotent stem cells *in vitro* to endocrine and immature endocrine cells, preferably immature beta cells, comprising dissociating and re-associating the aggregates. In one example the dissociation and re-association occurs at stage 1, stage 2, stage 3, stage 4, stage 5, stage 6 or stage 7 or combinations thereof. In one example the definitive endoderm, PDX1-negative foregut endoderm, PDX1-positive foregut endoderm, PEC, and / or endocrine and endocrine progenitor/precursor cells are dissociated and re-associated. In one example, the stage 7 dissociated and re-aggregated cell aggregates consist of fewer non-endocrine (CHGA-) sub-populations as compared to endocrine (CHGA+) sub-populations. In one example, more than 10% preferably more than 20%, 30%, 40% and more

preferably more than 50%, 60%, 70%, 80%, 90%, 95%, 98% or 100% of the cells in the cell population are endocrine (CHGA+) cells.

[00044] One example provides a method for differentiating pluripotent stem cells *in vitro* to endocrine cells by removing the endocrine cells made during stage 4 PEC production thereby enriching for non-endocrine multipotent pancreatic progenitor (CHGA-) sub-population which is PDX1+ and NKX6.1+.

[00045] One example provides a method for differentiating pluripotent stem cells *in vitro* to endocrine cells wherein stage 6 and stage 7 cells as defined in Table 17 are cultured in low levels of Matrigel from about 0.02% to 2%, preferably about 0.02% to 0.05%, preferably about 0.05% to 1%, preferably 0.05%.

[00046] One example provides a method for improving cell adhesion of PEC and endocrine cells aggregates by treating cell cultures with Matrigel and a rho-kinase or ROCK inhibitor. In one example the rho-kinase or ROCK inhibitor is Y-27632.

[00047] In one example, PEC cultures enriched for the non-endocrine multipotent progenitor sub-population (CHGA-) are made by not adding a Noggin family member at stage 3 and / or stage 4. In one example, PEC cultures which are relatively replete of cells committed to the endocrine lineage (CHGA+) are made by not adding a Noggin family member at stage 3 and / or stage 4. In one example the Noggin family member is a compound selected from the group comprising Noggin, Chordin, Follistatin, Folistatin-like proteins, Cerberus, Coco, Dan, Gremlin, Sclerostin, PRDC (protein related to Dan and Cerberus).

[00048] One example provides a method for maintaining endocrine cells in culture by culturing them in a media comprising exogenous high levels of glucose, wherein the exogenous glucose added is about 1mM to 25mM, about 1mM to 20mM, about 5mM to 15mM, about 5mM to 10mM, about 5mM to 8mM. In one example, the media is a DMEM, CMRL or RPMI based media.

[00049] One example provides a method for differentiating pluripotent stem cells *in vitro* to endocrine cells with and without dissociating and re-associating the cell aggregates. In one example the non-dissociated or the dissociated and re-associated cell aggregates are cryopreserved or frozen at stage 6 and/or stage 7 without affecting the *in vivo* function of the endocrine cells.

In one example, the cryopreserved endocrine cell cultures are thawed, cultured and, when transplanted, function *in vivo*.

[00050] Another example provides a culture system for differentiating pluripotent stem cells to endocrine cells, the culture system comprising of at least an agent capable of suppressing or inhibiting endocrine gene expression during early stages of differentiation and an agent capable of inducing endocrine gene expression during later stages of differentiation. In one example, an agent capable of suppressing or inhibiting endocrine gene expression is added to the culture system consisting of pancreatic PDX1 negative foregut cells. In one example, an agent capable of inducing endocrine gene expression is added to the culture system consisting of PDX1-positive pancreatic endoderm progenitors or PEC. In one example, an agent capable of suppressing or inhibiting endocrine gene expression is an agent that activates a TGFbeta receptor family, preferably it is Activin, preferably, it is high levels of Activin, followed by low levels of Activin. In one example, an agent capable of inducing endocrine gene expression is a gamma secretase inhibitor selected from a group consisting of N-[N-(3,5-Diflurophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester (DAPT), RO44929097, DAPT (N--[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester), 1-(S)-endo-N-(1,3,3)-Trimethylbicyclo[2.2.1]hept-2-yl)-4-fluorophenyl Sulfonamide, WPE-III31C, S-3-[N'-(3,5-difluorophenyl-alpha-hydroxyacetyl)-L-alanilyl]amino-2,3-dih- ydro-1-methyl-5-phenyl-1H-1,4-benzodiazepin-2-one, (N)-[(S)-2-hydroxy-3-methyl-butyryl]-1-(L-alaninyl)-(S)-1-amino-3-methyl-- 4,5,6,7-tetrahydro-2H-3-benzazepin-2-one, BMS-708163 (Avagacestat), BMS-708163, Semagacestat (LY450139), Semagacestat (LY450139), MK-0752, MK-0752, YO-01027, YO-01027 (Dibenzazepine, DBZ), LY-411575, LY-411575, or LY2811376. In one example, high levels of Activin is meant levels greater than 40 ng/mL, 50 ng/mL, and 75ng/mL. In one example, high levels of Activin are used during stage 3 or prior to production of pancreatic foregut endoderm cells. In one example, low levels of Activin means less than 30 ng/mL, 20 ng/mL, 10 ng/mL and 5 ng/mL. In one example, low levels of Activin are used during stage 4 or for production of PEC. In one example, the endocrine gene that is inhibited or induced is NGN3. In another example, Activin A and Wnt3A are used alone or in combination to inhibit endocrine expression, preferably to inhibit NGN3 expression prior to production of pancreatic foregut endoderm cells, or preferably during stage 3. In one example, a gamma secretase inhibitor, preferably RO44929097 or DAPT, is used in the culture system to induce expression of endocrine gene expression after production of PEC, or preferably during stages 5, 6 and/or 7.

[00060] It is preferred that the TGFbeta-receptor family member is selected from a group consisting of Activin A, Activin B, Nodal, GDF-8, GDF-10, GDF-11 and GDF15.

[00061] It is preferred that the TGFbeta-receptor family member is Actin A, GDF-8 and GDF-11.

[00062] It is preferred that the TGFbeta-receptor family member is Activin A.

[00063] It is preferred that the Wnt family member is selected from a group consisting of WNT1, WNT2, WNT2B, WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A, WNT9B, WNT10A, WNT10B, WNT11, WNT16 and WNT3A.

[00064] It is preferred that the Wnt family member is Wnt3a.

[00065] It is preferred that the Heregulin (HRG) family member is HRG1, HRG 2, HRG 3 and HRG4.

[00066] It is preferred that the HRG family member is HRG1 and HRG2.

[00067] A method for producing insulin *in vivo*, said method comprising the steps of: a) contacting a PDX1-negative foregut endoderm cell population with a factor that activates a TGFβ receptor family member, thereby producing PDX1-positive pancreatic endoderm cell population; and b) contacting the PDX-positive pancreatic endoderm cell population with a gamma secretase inhibitor, thereby producing an immature beta cells that express INS and NKX6.1; c) implanting the immature beta cells from step (b); and d) maturing the cells into mature beta cells that secrete insulin *in vivo* in response to glucose stimulation.

[00068] Preferred features and examples of the disclosure are as follows.

[00069] At stage 7 agents can still modulate gene expression. It is preferred that a member of the FGF superfamily is added at stage 7. It is preferred that FGF2 is added at stage 7. FGF2 may increase SST expression and/or suppress INS, GCG or GHRL expression. It is preferred that BMP is added at stage 7. BMP may increase INS, PDX1 or IDI expression.

[00070] An immature beta cell population that gives rise to *in vivo* function. It is preferred that the immature beta population is CHGA+. It is preferred that the immature beta cell population

are capable of functioning *in vivo, i.e.,* when transplanted secrete insulin in response to blood glucose. It is preferred that the endocrine cell population, when transplanted, may develop and mature to functional pancreatic islet cells. It is preferred that the immature beta cell population is enriched for endocrine cells (or depleted of non-endocrine cells). In a preferred example, greater than about 50% of the cells in the immature beta cell population are CHGA+. In a more preferred example greater than about 60% or 70% or 80% or 90% or 95% or 98% or 100% of the cells in the immature beta cell population are CHGA+. In a preferred example less than about 50% of the cells in the immature beta cell population are CHGA-. In a more preferred example less than about 15% of the cells in the immature beta cell population are CHGA-. In a more preferred example less than about 10% or 5% or 3% or 2% or 1% or 0.5% or 0% of the cells in the immature beta cell population are CHGA-. Further, expression of certain markers may be suppressed during production of the immature beta cells such as NGN3 expression during stage 3. It is preferred that the immature beta cell population is singly-hormonal (e.g. INS only). It is preferred that the immature beta cell population co-expresses other cell markers including NKX6.1 and PDX1. It is preferred that the immature beta cell population is both singly-hormonal and co-express other cell markers including NKX6.1 and PDX1. It is preferred that the immature beta cell population has more singly hormone expressing INS cells as a percentage of the total INS population. It is preferred that the immature beta cell population has at least 50% singly hormone expressing INS cells as a percentage of the total INS population. It is preferred that the immature beta cell population is CHGA+/INS+/NKX6.1+ (triple positive). In a preferred embodiment greater than about 25% of the cells in the immature beta cell population are CHGA+/INS+/NKX6.1+ (triple positive). In a preferred example greater than about 30% or 40% or 50% or 60% or 70% or 80% or 90% or 95% 100% of the cells in the immature beta cell population are CHGA+/INS+/NKX6.1+ (triple positive).

[00071] An enriched immature beta cell population made by dissociating and re-aggregating the cell aggregates. It is preferred that the enriched immature beta cell population is dissociated and re-aggregated at stage 7. It is preferred that the immature beta cell population is dissociated and re-aggregated at about day 25 day 26, or day 27 or day 28 or day 29 or day 30 or more.

[00072] Complex media is important for endocrine cell maintenance. It is preferred that the complex media is CMRL or RPMI. It is preferred that the complex media is used at stage 6 or

beta that greater than 80% of the total *in vitro* cell population comprises immature beta cells that are CHGA+, INS+ and NKX6.1+.

[00085] Purified immature beta cells. Purified immature beta cell precursors. It is preferred that immature beta cells or beta cell precursors are purified from stage 7 cell populations using a Zinc sensor. It is preferred that dim florescence due to the presence of Py 1 sensor bound to Zinc is enriched for beta cells. It is preferred that bright florescence due to the presence of Py 1 sensor bound to Zinc is enriched for alpha cells. A method for purifying beta cells using a Zinc sensor. A method for purifying alpha cells using a Zinc sensor. Purified alpha cells. Purified alpha cell precursors.

[00086] An enriched cell population that expresses markers of the beta cell lineage such as INS, IAPP, PDX1, NKX6.1, PAX4, PCSK1, G6PC2, GCK, or SLC30A8.

[00087] An enriched cell population that expresses markers of the alpha cell lineage such as GCG, ARX, or SLC30A8.

[00088] An enriched cell population, wherein greater than 50% of the cells are INS positive. It is preferred that the cell population has greater than 90% INS positive cells. In preferred examples 50%-99% of the cells are INS positive cells.

[00089] A method for producing insulin *in vivo* comprising: a) obtaining a population of CHGA+ cells in vitro; and b) transplanting the CHGA+ cells thereby producing insulin *in vivo*.

[00090] It is preferred that the CHGA+ cells are dissociated and re-aggregated at stage 7.

[00091] It is preferred that the CHGA+ cells express PDX1 and NKX6.1

[00092] It is preferred that the CHGA+ cells express INS and NKX6.1.

[00093] It is preferred that the CHGA+ cells are cultured in CMRL or RPMI.

[00094] It is preferred that the CHGA+ cells are cryopreserved and thawed prior to transplantation.

[00095] It is preferred that the CHGA+ cells are cultured in media containing high glucose.

[000108] In examples, the immature beta cells express INS and NKX6.1 and do not express substantially NGN3. In examples, the immature beta cells express INS, NKX6.1 and MAFB and do not substantially express NGN3.

BRIEF DESCRIPTION OF THE DRAWINGS

[000109] **FIG. 1** is a photographic image of an aggregate suspension culture of dedifferentiated reprogrammed cells or, also referred to herein, as iPS cells. See Example 1.

[000110] **FIGs. 2A-L** are bar graphs showing the relative gene expression levels of OCT4 (FIG.2A), BRACHYURY (FIG.2B), CER1 (FIG.2C), GSC (FIG.2D), FOXA2 (FIG.2E), FOXA1 (FIG.2F), HNF6 (FIG.2G), PDX1 (FIG.2H), PTF1A (FIG.2I), NKX6.1 (FIG.2J), NGN3 (FIG.2K) and INS (FIG.2L). Expression levels are normalized to the average expression levels of housekeeping genes, cyclophilin G and TATA Binding Protein (TBP) expression. The graphs depict fold upregulation over the lowest data point in the data set. See Example 1.

[000111] **FIGs. 3** are photomicrographs of immunocytochemistry (ICC) of human iPS cell (E2021 using G4 hIPS cell line) cultures from Stage 4 differentiation (PDX1-positive pancreatic endoderm cells) using antibodies specific for (panel A) PDX-1; (panel B) NKX6.1; (panel C) PTF1A; and (panel D) Dapi. See Example 2.

[000112] **FIG. 4** are pictures of immunocytochemistry (ICC) of iPS cell cultures (E2021 using G4 hIPS cell line) from Stage 5 differentiation using ligands specific for (panel A) Glucagon; (panel B) Insulin; (panel C) Somatostatin; and (panel D) Dapi. See Example 2.

[000113] **FIGs. 5A-B** is an array location map and array key provided in the Proteome Profiler™ human phospho-RTK antibody arrays from R&D Systems. The location map in FIG.5A shows the coordinates or location of the RTK antibodies. The identity or name of the RTK family and antibodies are described in the key, FIG.5B. The positive signals observed on the developed film can therefore be identified by overlaying a transparency as in FIG.5A and identifying the signals by referring to the coordinates on the overlay (FIG.5A) with the name of the RTK in FIG.5B. See Example 4.

[000114] **FIG. 6** are an RTK array analyses of iPS cell–derived pancreatic endoderm cells (PEC) under four different conditions (panels A, B, C and D described in Example 5. Tyrosine

CLAIMS

What is claimed is:

1. An *in vitro* cell culture comprising:

human cells, wherein at least 10% of said human cells are immature beta cells that co-express INS and NKX6.1; and

an effective amount of Y-27632 to promote survival of the immature beta cells in the cell culture.

2. The cell culture of claim 1, wherein the cell culture is in a semipermeable encapsulation device.

3. The cell culture of claim 1, wherein said human immature beta cells do not substantially express NGN3.

4. A cryopreserved *in vitro* cell culture comprising:

human cells, wherein the human cells comprise unipotent human immature beta cell suspension aggregates and wherein at least 10% of cells in the aggregates are human immature beta cells that co-express INS and NKX6.1, and

a cryopreservation agent.

5. The cell culture of claim 4, wherein the unipotent human immature beta cell suspension aggregates are in a semipermeable encapsulation device.

6. The cell culture of claim 4, wherein the suspension aggregates are about 50 to 200 microns in size.

7. A cryopreserved *in vitro* cell culture comprising:

human cells, wherein at least 10% of said human cells are immature beta cells that co-express INS and NKX6.1; and

a cryopreservation agent.

8. The cell culture of claim 7, wherein said human immature beta cells do not substantially express NGN3.

9. The cell culture of claim 7, wherein the immature beta cells are in a semipermeable encapsulation device.

10. The cell culture of claim 7, wherein the immature beta cells are in a perforated encapsulation device.

11. The cell culture of claim 7, wherein said human immature beta cells further co-expresses pancreatic-duodenal homeobox factor-1 (PDX1).

12. The cell culture of claim 7, wherein said human immature beta cells are capable of maturing to a mature beta cells.

13. The cell culture of claim 7, wherein said human immature beta cells further expresses MAFB.

14. The cell culture of claim 7, further comprising Chromogranin A (CHGA)-negative cells.

15. The cell culture of claim 7, further comprising a compound selected from the group consisting of bone morphogenetic protein, sonic hedgehog, platelet-derived growth factor, a member of the FGF superfamily, retinoic acid, retinoic acid analogs, insulin-like growth factor, a vitamin B3 derivative, and Nicotinamide.

16. The cell culture of claim 7, further comprising a matrix.

17. A cryopreserved *in vitro* cell culture comprising:

human cells, wherein at least 50% of said human cells are immature beta cells that co-express INS and NKX6.1; and

a cryopreservation agent.

18. The cell culture of claim 17, wherein the immature beta cells are in a semipermeable encapsulation device.