METHODS FOR PREPARING ENDODERM STEM CELLS AND ISLETS DERIVED THEREFROM

TECHNICAL FIELD

[0001] The present application generally relates to methods for preparing endoderm stem cells (EnSCs), EnSC-derived pancreatic progenitor (PP) cells, EnSC-derived endocrine progenitor (EP) cells and EnSC-derived islets (E-islets), as well as therapeutic uses of EnSCs, EnSC-derived PP cells, EnSC-derived EP cells and E-islets in treating diseases or conditions associated with impaired pancreatic islet function.

BACKGROUND OF THE INVENTION

[0002] Human pluripotent stem cells (hPSCs) are able to differentiate into a variety of functional cells or tissues, such as pancreatic progenitor cells (PP) and islet tissues. These cells have been demonstrated to survive, function and reverse hyperglycemia in diabetic animal models. See, e.g., *Pagliuca, F. W. et al. Cell 159, 428-439 (2014); Rezania, A. et al. Nat. Biotechnol. 32, 1121-1133 (2014); and Sneddon, J. B. et al. Cell Stem Cell 22, 810-823 (2018).* In addition, a recent clinical trial has shown that, when subcutaneously implanted into type 1 diabetic patients, the hPSC-derived pancreatic endodermal cells were able to further mature into meal-responsive β-like cells and secrete insulin, albeit at the levels insufficient to achieve the independency of exogenous insulin. See, e.g., *Ramzy, A. et al. Cell Stem Cell 28, 2047-2061 (2021); and Shapiro, A. M. J. et al. Cell Rep. Med. 2, 100466 (2021).*

[0003] Nevertheless, clinical applications of hPSC-derived cells are undermined by the complicated differentiation processes and the risk of having any residual undifferentiated cells in the system that may form teratomas *in vivo*. In addition, the use of hPSCs for laboratory studies and cell-based therapies is hindered by their limited capacity to generate pure populations of differentiated cell types *in vitro*. These limitations would impact the reproducibility and reliability of experiments, as well as the safety and efficacy of potential therapeutic applications.

[0004] Therefore, need exists to develop improved methods for generating nontumorigenic intermediate stem cell types with high purity for safer and more efficient therapeutic applications, especially in treating diseases associated with impaired pancreatic islet function, such as diabetes.

SUMMARY OF THE INVENTION

[0005] An objective of the present application is to provide a method for producing a population of endoderm stem cells, comprising:

a) providing a population of pluripotent stem cells;

b) culturing the population of pluripotent stem cells in a first medium comprising a Nodal signaling agonist and a WNT signaling agonist;

c) culturing the cells in a second medium comprising a Nodal signaling agonist and a fibroblast growth factor (FGF);

d) culturing the cells in a third medium comprising a factor belonging to the TGF-beta superfamily, FGF, a hepatocyte growth factor (HGF) and VEGF; and

e) culturing the cells in a fourth medium comprising a WNT signaling agonist, a TGF-beta inhibitor and an epidermal growth factor (EGF);

thereby producing a population of endoderm stem cells.

[0006] In another aspect, the present disclosure provides a method for producing a population of pancreatic endocrine cells, comprising:

a) providing a population of endoderm stem cells;

b) culturing the population of endoderm stem cells in the presence of a BMP inhibitor, Nodal signaling agonist, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and Nicotinamide to produce a population of pancreatic progenitor (PP) cells;

c) culturing the population of PP cells in the presence of a BMP inhibitor, a TGF-beta inhibitor and a gamma-secretase inhibitor to produce a population of endocrine progenitor (EP) cells; and

d) culturing the population of EP cells in the presence of T3 and Nicotinamide in addition to the factors involved in step c;

thereby producing a population of pancreatic endocrine cells.

[0007] In another aspect, the present disclosure provides a method of treating a disease or condition associated with impaired pancreatic islet function in a subject in need thereof, comprising: administering an effective amount of pancreatic endocrine cells derived from a population of endoderm stem cells (or E-islets) to the subject, thereby treating the disease or condition associated with impaired pancreatic islet function in the subject in need thereof.

[0008] In another aspect, the present disclosure provides a population of EnSCs produced according to the methods provided herein.

[0009] In another aspect, the present disclosure provides a population of pancreatic endocrine cells or E-islets produced according to the methods provided herein.

[0010] In another aspect, the present disclosure provides a pharmaceutical composition comprising the population of EnSCs, EnSC-derived PP cells, EnSC-derived EP cells, the population of pancreatic endocrine cells or E-islets produced according to the methods provided herein.

[0011] In another aspect, the present disclosure provides use of a population of endoderm stem cells in the manufacture of E-islets for treating a disease or condition associated with impaired pancreatic islet function in a subject in need thereof.

[0012] In another aspect, the present disclosure provides use of E-islets in the manufacture of a medicament for treating a disease or condition associated with impaired pancreatic islet function in a subject in need thereof.

[0013] In another aspect, the present disclosure provides a kit for producing a population of EnSCs from a population of pluripotent stem cells, wherein the kit comprises a first set of factors, a second set of factors, a third set of factors and a fourth set of factors, wherein the first set of factors comprises a Nodal signaling agonist and a WNT signaling agonist, the second set of factors comprises a Nodal signaling agonist and a fibroblast growth factor (FGF), the third set of factors comprises a factor belonging to the TGF-beta superfamily, FGF, a hepatocyte growth factor (HGF) and VEGF, and the fourth set of factors comprises a WNT signaling agonist, a TGF-beta inhibitor and an epidermal growth factor (EGF).

[0014] In another aspect, the present disclosure provides a kit for producing a population of pancreatic endocrine cells from a population of EnSCs, wherein the kit comprises a fifth set of factors, a sixth set of factors and a seventh set of factors, wherein the fifth set of factors comprises

a BMP inhibitor, Nodal signaling agonist, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and/or Nicotinamide, the sixth set of factors comprises a BMP inhibitor, a TGF-beta inhibitor and/or a gamma-secretase inhibitor, and the seventh set of factors comprises T3 and/or Nicotinamide.

[0015] In another aspect, the present disclosure provides a kit for producing a population of pancreatic endocrine cells from a population of pluripotent stem cells, wherein the kit comprises first to seventh sets of factors, wherein the first set of factors comprises a Nodal signaling agonist and a WNT signaling agonist, the second set of factors comprises a Nodal signaling agonist and a fibroblast growth factor (FGF), the third set of factors comprises a factor belonging to the TGF-beta superfamily, FGF, a hepatocyte growth factor (HGF) and VEGF, the fourth set of factors comprises a WNT signaling agonist, a TGF-beta inhibitor and an epidermal growth factor (EGF), the fifth set of factors comprises a BMP inhibitor, Nodal signaling agonist, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and Nicotinamide, the sixth set of factors comprises a BMP inhibitor, a TGF-beta inhibitor and a gamma-secretase inhibitor, and the seventh set of factors comprises T3 and Nicotinamide.

[0016] In another aspect, the present disclosure provides a kit for producing a population of pancreatic endocrine cells from a population of PP cells, wherein the kit comprises a set of factors comprising a BMP inhibitor, a TGF-beta inhibitor and a gamma-secretase inhibitor.

BRIEF DESCRIPTION OF DRAWINGS

[0017] FIG. 1 illustrates quality control of EnSCs. (a) Morphology of EnSCs. (b) Result of FACS, revealing the proportion of SOX17+ / FOXA1+ cells in EnSCs. (c) Karyotyping result of WB20 EnSC line. (d) Growth curve of EnSCs. (e) Release criteria and the results of pathogen testing for EnSCs. (f) Circos plots of parental PBMC (left) and EnSC (clone WB20) (right) for genomic variations from WGS analysis. From outside to inside, each of nine circles represents one aspect of genomic variations. Circle 1: chromosome; circle 2: density map of SNV (single nucleotide variation); circle 3: density map of Indel insertion; circle 4: density map of Indel deletion; circle 5: density map of mutation sites occurring in the coding region; circle 6: density map of mutation sites occurring in the non-coding region; circle 7: location map of CNV (copy number variation), with red and blue columns indicating copy number gain and loss, respectively; circle 8: location map of SV (structural variation), with orange and green columns indicating deletion and insertion,

respectively; circle 9: type association map of SV, with blue, red and green lines indicating inversion, interchromosomal translocation and intrachromosomal translocation, respectively.

[0018] FIG. 2 illustrates quality control of intermediate pancreatic differentiation stages of EnSCs. (a) and (b) Morphology and the cell composition of the differentiation culture at the pancreatic progenitor (PP) stage. (a) is a representative phase contrast image of EnSC-derived PP cells. (b) shows the FACS data revealing the proportion of NKX6-1+ / PDX1+ PP cells at this stage. (c) and (d) Morphology and the cell composition of the differentiation culture at the endocrine progenitor (EP) stage. (c) is a representative phase contrast image of EnSC-derived EP cells. (d) shows the FACS data revealing the proportion of NKX6-1+ / PDX1+ and CHGA- / NKX6-1+ PP cells that are differentiating towards the CHGA+ endocrine progenitors at early endocrine progenitor stage (day 3 of EP stage) and the proportion of CHGA+ endocrine progenitors and emerging endocrine (C-peptide + or Glucagon+) cells at late endocrine progenitor stage (day 8 of EP stage). Scale bars, 100 μm.

[0019] FIG. 3 illustrates quality control of E-islets. (a) Morphology of E-islets (scale bar, 100 μm). (b) Results from FACS analysis of pancreatic endocrine cells in E-islets, with Chromogranin A+ population representing the pan-endocrine compartment, C-peptide+ Glucagon- population representing β cells, Glucagon+ population representing α cells and Somatostatin+ population representing δ cells. (c) immunofluorescence staining of E-islets that have C-peptide (CPEP) positive β cells, glucagon (GCG) positive α cells, as well as somatostatin (SST) positive δ cells (scale bars, 50 μm). (d) Expression levels of the indicated genes in E-islets and adult human islets measured by quantitative RT-PCR (qRT-PCR), among which *PDX1, NKX6.1* and Insulin (*INS*) are expressed by adult β cells, while Glucagon (*GCG*) and Somatostatin (*SST*) are typically expressed by adult α cells and δ cells, respectively. (e) and (f) Clustering and gene expression among the subpopulations of E-islets, revealed by single cell transcriptomic analysis (scRNA seq, 10× Genomics). (e) is the tSNE clustering data of scRNA seq, showing the various subpopulations of E-islets (2721 cells with low UMI are excluded from 15244 sequenced cells). (f) shows the expression of representative genes for the cell types (indicated by the boxes with blue dotted lines) in E-islets. (g) C-peptide secretions from human (primary) islets and E-islets in response to low and high glucose stimulations under static conditions (GSIS). (h) FACS data, revealing the nontarget hepatic lineages (Alpha fetoprotein/AFP+ or Albumin/ALB+ cells) are undetectable in E-islets. (i) Release criteria and results of pathogen testing for E-islets.

[0020] FIG. 4 illustrates preclinical studies and clinical outcomes of autologous E-islet transplantation in a T2D patient. (a) Brief scheme of the story, illustrating the major procedures involved in the generation and quality control of autologous E-islets and the post-surgery evaluations of safety and effectiveness of E-islet transplantation. (b)-(d) E-islets reverse hyperglycemia in immunocompromised (SCID Beige) mice with STZ induced diabetes. (b) Schematic illustration of kidney capsule transplantation of E-islets into diabetic mice. (c) Fasting blood glucose dynamics in diabetic mice (blue line representing the sham group, which demonstrates persistent hyperglycemia and death within 2 months; red line representing the E-islet transplanted group, which shows the reversal of hyperglycemia within a month and the abolishment of cure after nephrectomy of the transplanted organ). (d) Secretion of human C-peptide in STZ-induced diabetic mice after fasting and 30 minutes following an i. p. glucose bolus on days 90 and 180 post transplantation of E-islets. (e)-(g) Immunogenicity of E-islets in humanized mice. (e) Schematic illustration of the syngeneic and allogeneic kidney capsule transplantation of patient-specific E-islets into the diabetic mice (NCG hIL 15, the nonobese diabetic mice knocked-in with human IL15, with severe combined immunodeficiency and depletion of the interleukin 2 receptor γ) humanized with the patient's and a volunteer's PBMCs. (f) Fasting blood glucose dynamics of STZ-induced diabetic humanized mice (blue line represents the control group with the patient E-islets transplanted into three diabetic mice humanized with the volunteer's PBMCs; red line represents the group with the patient E-islets transplanted into three diabetic mice humanized with the patient's PBMCs). (g) Secretion of human C-peptide in humanized diabetic mice after fasting and 30 minutes following an i. p. glucose bolus on days 7 and 14 post E-islet transplantation ([U. D.]: undetectable). (h) Clinical measurements of time-in-tight-target-range (TITR), time-in-the-range (TIR) and Hemoglobin A1C (HbA1c), and the insulin (degludec) dosage during 116 weeks. The changes in degludec dosages (yellow line) are shown on the left y axis. The proportions of time-in-tight-target-range (TITR, 3.9-7.8 mM) (green line) and time-in-range (TIR, 3.9-10.0 mM) (blue line) and the serum hemoglobin A1c levels (red line) are shown on the right y-axis. (i) Continuous interstitial glucose fluctuations derived from the CGM measurements at week 52 and week 105 compared with presurgery. The green horizontal lines at 3.9 mM and 7.8 mM demarcate the target glucose range for the healthy. The brown (presurgery) / green (week 52) / navy (week 105) lines, the medium brown (presurgery) / medium green (week 52) / medium blue (week 105) areas, and the pale brown (presurgery) / pale green

(week 52) / pale blue (week 105) areas represent the medians (50%), 25-75% ranges and 5-95% ranges, respectively. (j)-(l) Results of the mixed meal tolerance tests (MMTT) performed to evaluate the islet function by monitoring the serum levels of fasting and meal-stimulated circulating glucose (j), C-peptide (k) and insulin (l).

[0021] FIG. 5 illustrates E-islets ameliorate diabetes in STZ-induced diabetic monkeys. (a) Schematic illustration of the hepatic portal implantation of E-islets into STZ-induced diabetic monkeys. Two diabetic monkeys were transplanted with 6000 (Monkey 1) or 30000 (Monkey 2) E-islets, respectively. Monkey 1 was used to test the feasibility of hepatic portal injection of E-islets without digital subtraction angiography (DSA), while Monkey 2 was used for evaluating the short-term safety and effectiveness of E-islets. The tail tip blood glucose was measured before feeding in the morning and afternoon. The dose of exogenous insulin treatment for animal was determined according to preprandial blood glucose. (b) Daily preprandial blood glucose levels (left) and insulin administration dosage (right) during the 28 day-periods before and after surgery (Pre: pre-surgery; Post: post-surgery; A.M.: before breakfast; P.M.: before dinner.). (c) and (d) Results of the 8-time-point intravenous glucose tolerance tests (IVGTT) of the E-islet-transplanted STZ-induced diabetic monkey. The patterns of blood glucose (c) and human C-peptide (d) were monitored by IVGTT before diabetes modeling / STZ treatments (before diabetes modeling), 2 weeks before transplantation (2wks before Tx), as well as 2, 3 and 4 weeks post-transplantation (2wks, 3wks, and 4wks post Tx).

[0022] FIG. 6 illustrates characterization of humanized mice. (a) and (b) Characterization of NCG-hIL15 mice humanized with either patient (a) or volunteer (b)-derived PBMCs by the presence of hCD45+ human cells in peripheral blood. a Proportions of live cells (by SSC and FSC), human-derived blood cells (hCD45+) and mouse blood cells (mCD45+), among the three patient humanized mice. (b) Proportions of live cells, human-derived and mouse blood cells, among the three volunteer humanized mice. (c) Immunofluorescence staining of the grafts harvested under kidney capsule of the patient humanized mice for the presence of human β cells (C-peptide + and NKX6-1 +), α cells (Glucagon +), and δ cells (Somatostatin +); C- peptide (CPEP, red), Glucagon (GCG, green), Somatostatin (SST, violet) and NKX6-1 (Cyan); the area defined by the white dotted line indicating the mouse kidney tissue; scale bar, 50 μm.

[0023] FIG. 7 illustrates clinical assessments and outcomes of glycemic control. (a) Schematic illustration of follow-up time points for routine and disease-specific clinical assessments, as well

as the treatments the patient received during the whole follow-up period. Examinations of endocrine function and diabetes–specific parameters by mixed-meal tolerance tests (MMTT) were performed at baseline and at 4, 8, 12, 16, 20, 24, 36 and 48 weeks and thereafter at specified time points. The glycemic control of the patient was measured with a 24-hour real-time blood glucose monitoring (CGM) system. The baseline and follow-up CGM glucose values were measured throughout the first 52 weeks and thereafter during specified time periods, and the mean duration of CGM device wearing is at least 3 days. The main preconditioning regimen included antihyperglycemic medication and immunosuppressant treatments. The antidiabetic treatment included metformin (0.75 g bid, tapered from week 44 and interrupted from week 56) and acarbose (50 mg tid, tapered from the week 44 and interrupted from week 48). The insulin analog degludec had been administrated since 2021 (20 U once daily at bedtime) but interrupted right after E-islet transplantation, and had been resumed and tapered from week 2, and was stopped at the end of week 11. Graft-versus-host disease was treated with mycophenolate mofetil (administered since kidney transplantation at 0.5 g bid) and tacrolimus (administered orally after kidney transplantation at 1~3 mg bid, depending on the serum FK506 concentrations). ☆ represents the follow-up time points of clinical assessment, and △ represents the CGMS monitoring periods. The checkerboard design represents the tapering period. (b) Areas under the curves (AUCs) derived from the 5-point (0, 30, 60, 120, 180 min) intravenous glucose (FIG. 4(j)), C- peptide (FIG. 4(k)) and insulin (FIG. 4(l)) values in the results of the mixed meal tolerance tests (MMTT). (c) Results of continuous glucose monitoring during MMTTs (0-240 minutes) at various follow-up time points, measured by CGM device at intervals of every 5 minutes. The horizontal dotted lines at 3.9 and 10.0 mM demarcate the target glucose range. Fold of AUCs (right panel) are the areas under the curves of each follow-up time points, normalized to the AUC of week 2 (2W). (d) Prandial glucose excursions (mean amplitude glucose excursion, MAGE), the gold standard of blood glucose variability, represented by mean glucose values (within 95% ranges) of 1.5 hours before and 2 hours after each meal at baseline, week 52 and week 105. The green horizontal lines at 3.9 and 7.8 mM demarcate the target glucose excursion for healthy individuals.

[0024] FIG. 8 illustrates continuous glucose monitoring at various follow-up time points. (a)-(l) Continuous glucose monitoring (CGM) traces at presurgery (a) and various follow- up time points (b)-(l). For each follow-up time point, GCM data were collected from continuous 72 hours. The median (navy lines) the 25-75% (medium blue areas) and 5-95% (pale blue areas) ranges are

shown, with the green horizontal lines demarcating the target glucose range (3.9 to 10.0 mM) for diabetic patients. The prandial glucose excursions (MAGE), calculated from mean glucose values (within 95% ranges), are listed on each panel in red.

[0025] FIG. 9 shows teratoma formation assay.

[0026] FIG. 10 shows quality control release criteria of EnSCs and E-islets.

[0027] FIG. 11 shows primer list.

[0028] FIG. 12 shows antibody list.

[0029] FIG. 13 shows key laboratory values before and after transplantation.

[0030] FIG. 14 shows primary follow-up objectives.

[0031] FIG. 15 shows exploratory objectives.

DETAILED DESCRIPTION OF THE INVENTION

[0032] The following description of the disclosure is merely intended to illustrate various embodiments of the disclosure. As such, the specific modifications discussed are not to be construed as limitations on the scope of the disclosure. It will be apparent to one skilled in the art that various equivalents, changes, and modifications may be made without departing from the scope of the disclosure, and it is understood that such equivalent embodiments are to be included herein. All references cited herein, including publications, patents and patent applications, are incorporated herein by reference in their entirety.

[0033] I. General Definitions

[0034] The singular terms “a”, “an”, and “the” include plural referents unless context clearly indicates otherwise. By way of example, reference to “a cell” refers to one or more cells, and reference to “the method” includes reference to equivalent steps and methods disclosed herein and/or known to those skilled in the art, and so forth. Similarly, the word "or" is intended to include “and” unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below.

[0035] As used herein, the term “about” or “approximately” means within 20%, preferably within 10%, and more preferably within 5% of a given value or range.

[0036] As used herein, the term "cell" as used herein refers to individual cells, cell lines, or cultures derived from such cells.

[0037] As used herein, the term "Nodal signaling agonist" refers to a substance that stimulates or enhances the Nodal signaling pathway. The Nodal signaling pathway plays a pivotal role in pattern formation and differentiation during the early stages of chordate development, particularly before and during gastrulation. This pathway is essential for establishing the primary body axes and for the formation of mesoderm and endoderm.

[0038] As used herein, the term "Notch signaling pathway" refers to a pathway that plays a pivotal role in regulating cell fates, cell proliferation, and cell death during development. This pathway is unique in that it primarily involves interactions between neighboring cells, as the ligands that activate the Notch receptor are mostly transmembrane proteins. Inhibition of the Notch signaling pathway can be achieved by substances that disrupt the function of any protein within the pathway or prevent the functional interaction between two pathway proteins. Exemplary inhibitors for the Notch signaling pathway include, without limitation, γ-secretase inhibitors, RBPJ Inhibitor-1. See, e.g., *Hurtado, C. et al. Disruption of NOTCH signaling by a small molecule inhibitor of the transcription factor RBPJ. Sci Rep 9, 10811 (2019).*

[0039] As used herein, the term "WNT signaling agonist" refers to a substance that stimulates or enhances the WNT signaling pathway. The WNT signaling pathway is a fundamental cell-to-cell communication mechanism that plays a crucial role in numerous physiological processes, including stem cell behavior, cell polarity, and tissue development.

[0040] As used herein, the term "fibroblast growth factor" or "FGF" refers to a member from the FGF family, which is a diverse group of secreted proteins that signal through receptor tyrosine kinases and regulate various cellular processes.

[0041] As used herein, the term "TGF-beta superfamily" refers to a superfamily of factors involved in the TGF-beta signaling pathway. The TGF-beta signaling pathway is a conserved mechanism that regulates many aspects of physiological development and tissue homeostasis. TGF-β family members include secreted polypeptides that play critical roles in embryogenesis and adult tissue maintenance, but also contribute to the development of various diseases. BMP, or Bone Morphogenetic Protein, constitutes a subset of signaling molecules within the TGF-β superfamily. Specifically, "BMP4" refers to a protein encoded by the *BMP4* gene in humans, which is a member of the BMP family and, by extension, part of the broader TGF-β superfamily.

[0042] As used herein, the term "TGF-beta inhibitor" refers to an agent which can decrease the expression and/or activity of the TGF-beta or receptors thereof, e.g., by at least 10% or more, e.g., by 10% or more, 50% or more, 70% or more, 80% or more, 90% or more, 95% or more, or 98% or more. The efficacy of an inhibitor, e.g., its ability to decrease the level and/or activity of TGF-beta or receptors thereof, can be determined, e.g., by measuring the level of an expression product of and/or the activity of TGF-beta or receptors thereof. In some embodiments, the inhibitor can be an inhibitory nucleic acid; an aptamer; an antibody or its binding fragment; or a small molecule.

[0043] As used herein, the term "gamma-secretase inhibitor" refers to an agent which can decrease the expression and/or activity of the gamma-secretase, e.g., by at least 10% or more, e.g., by 10% or more, 50% or more, 70% or more, 80% or more, 90% or more, 95% or more, or 98% or more. The efficacy of an inhibitor, e.g., its ability to decrease the level and/or activity of gamma-secretase, can be determined, e.g., by measuring the level of an expression product of and/or the activity of gamma-secretase. In some embodiments, the inhibitor can be an inhibitory nucleic acid; an aptamer; an antibody or its binding fragment; or a small molecule. Gamma secretase is a protease complex that cleaves single-pass transmembrane proteins within their transmembrane domain. It is an integral membrane protein and belongs to the intramembrane protease class.

[0044] As used herein, the term "BMP inhibitor" refers to an agent which can decrease the expression and/or activity of the BMP, e.g., by at least 10% or more, e.g., by 10% or more, 50% or more, 70% or more, 80% or more, 90% or more, 95% or more, or 98% or more. The efficacy of an inhibitor, e.g., its ability to decrease the level and/or activity of BMP, can be determined, e.g. by measuring the level of an expression product of and/or the activity of BMP. In some embodiments, the inhibitor can be an inhibitory nucleic acid; an aptamer; an antibody or its binding fragment; or a small molecule.

[0045] As used herein, the term "Activin A" refers to a member of the TGF-beta superfamily, which is structurally similar to TGF-beta 1 and signals through the common SMAD2/3 (mothers against decapentaplegic homologues 2 and 3) pathway.

[0046] As used herein, the term "CHIR99021" is a GSK-3a and GSK-3b inhibitor with a CAS number 252917-06-9.

[0047] As used herein, the term "bFGF" used interchangeably with the term "FGF2" refers to a growth factor and signaling protein encoded by the *FGF2* gene.

[0048] As used herein, the term "Wnt3A" refers to a protein that in humans is encoded by the *WNT3A* gene.

[0049] As used herein, the term "A83-01" is a TGF-beta type I receptor inhibitor with a CAS number 909910-43-6.

[0050] As used herein, the term "Rspondin1" refers to is a secreted protein that in humans is encoded by the *RSPO1* gene.

[0051] As used herein, the term "LDN-193189" is a selective BMP signaling inhibitor with a CAS number 1062368-24-4.

[0052] As used herein, the term "TPPB" is a protein kinase C activator with a CAS number 497259-23-1.

[0053] As used herein, the term "Noggin" refers to a protein that is involved in the development of many body tissues, including nerve tissue, muscles, and bones. In humans, noggin is encoded by the *NOG* gene.

[0054] As used herein, the term "GSI-XX" is a gamma-secretase inhibitor with a CAS number 209984-56-5.

[0055] As used herein, the term "epidermal growth factor" or "EGF" is a protein that stimulates cell growth and differentiation by binding to its receptor, epidermal growth factor receptor (EGFR). The term "TGF-alpha" refers to a protein that in humans is encoded by the *TGFA* gene. TGF-alpha is a member of the epidermal growth factor (EGF) family.

[0056] As used herein, the term "hepatocyte growth factor" or "HGF" refers to a mitogen produced by stromal cells that stimulates epithelial cell proliferation, motility, morphogenesis, and angiogenesis in various organs. It exerts its effects through tyrosine phosphorylation of its receptor, c-Met. HGF plays essential roles in organ development, self-repair of injured tissues, and protection of epithelial and non-epithelial organs via anti-apoptotic and anti-inflammatory signals. See, e.g., *Nakamura T et al., The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. Proc Jpn Acad Ser B Phys Biol Sci. 2010;86(6):588-610.*

[0057] As used herein, the term "vascular endothelial growth factor" or "VEGF" refers to a growth factor with significant pro-angiogenic activity that exerts mitogenic and anti-apoptotic effects on endothelial cells. It enhances vascular permeability, promotes cell migration, and actively contributes to the regulation of both normal and pathological angiogenic processes. The VEGF

family in humans comprises several members, including VEGF-A (with different isoforms), VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, placenta growth factor (PlGF), and endocrine gland-derived vascular endothelial growth factor (EG-VEGF). VEGF binds to tyrosine kinase cell receptors (VEGFRs) such as VEGFR-1, VEGFR-2, and VEGFR-3, which are expressed predominantly on vascular and lymphatic endothelial cells. VEGFR-2 possesses the strongest pro-angiogenic activity. VEGF and its receptors are also expressed on non-endothelial cells. Anti-VEGF and anti-VEGFRs therapies are currently considered crucial for blocking angiogenesis in cancer and other pathological processes. See, e.g., *Melincovici CS et al., Vascular endothelial growth factor (VEGF) - key factor in normal and pathological angiogenesis. Rom J Morphol Embryol. 2018;59(2):455-467.*

[0058] As used herein, the term "endoderm stem cells" or "EnSCs" refers to a particular type of cells derived from pluripotent stem cells that are specific to germ layers and non-tumorigenic. See, e.g., *Cheng, X. et al. Cell Stem Cell 10, 371-384 (2012).*

[0059] As used herein, the term "pancreatic islet" refers to a small cluster of specialized cells in the pancreas, also known as the islets of Langerhans. These pancreatic islets are responsible for secreting hormones that regulate blood glucose levels. The pancreatic islets contain different types of cells, including B cells (β cells) that secrete insulin, A cells (α cells) that secrete glucagon, D cells (δ cells) that secrete somatostatin, and pancreatic polypeptide-secreting cells that secrete pancreatic polypeptide. The pancreatic islets function as the endocrine part of the pancreas and play a crucial role in maintaining blood glucose levels.

[0060] As used herein, the term "E-islet" refers to the clusters of pancreatic endocrine cells that are obtained through the optimized differentiation culture of EnSCs using the methods described in the present disclosure. These E-islets have a similar morphology, endocrine cell composition, gene expression patterns, and/ or functionality to natural pancreatic islets.

[0061] As used herein, the term "treatment," "treat" or "treating", with regard to a disorder, refers to managing, eliminating, reducing or ameliorating a disorder and/or a symptom associated therewith. Although not excluded, treatment of a disorder does not require that the disorder, or symptoms associated therewith be completely eliminated. The term "treatment" as used herein may include "prophylactic treatment" that is applied before development of any symptom or manifestation of a disorder to reduce the possibility of or block the occurrence or recurrence of a disorder, or reducing the possibility of relapse of a previously controlled disorder, in a subject who

is not afflicted with a disorder but at risk, or who is susceptible to recurrence of the disorder, or who is at risk or susceptible to relapse of the disorder. Within the meaning of the invention, "treatment" also includes prevention of relapse or prevention stages, as well as treatment of acute or chronic signs, symptoms and/or dysfunction. Treatment can target symptoms, for example, to suppress symptoms. It can function in a short period of time, for a medium period of time, or can be a long-term treatment, such as in the case of maintenance therapy.

[0062] As used herein, "autologous" cells refer to any cells derived from the same subject into which they are later to be re-introduced.

[0063] As used herein, "allogeneic" cells refer to any cells derived from a different subject of the same species.

[0064] As used herein, the term "effective amount" refers broadly to the amount of a compound or cells that, when administered to a patient for treating a disease, is sufficient to affect such treatment for the disease. The effective amount may be an amount effective for prophylaxis, and/or an amount effective for prevention. The effective amount may be an amount effective to reduce, an amount effective to prevent the incidence of signs/symptoms, to reduce the severity of the incidence of signs/symptoms, to eliminate the incidence of signs/symptoms, to slow the development of the incidence of signs/symptoms, to prevent the development of the incidence of signs/symptoms, and/or effect prophylaxis of the incidence of signs/symptoms. The "effective amount" may vary depending on the disease and its severity and the age, weight, medical history, susceptibility, and preexisting conditions, of the patient to be treated. The term "effective amount" is synonymous with "therapeutically effective amount" for purposes of this invention.

[0065] As used herein, the term "subject" is not limited to a specific species or sample type. For example, the term "subject" may refer to a patient, and frequently a human patient. However, this term is not limited to humans and thus encompasses a variety of mammalian species, such as non-human veterinarian mammal, such as dogs, cats, rabbits, pigs, rodents, horses, or monkeys.

[0066] II. Endoderm Stem Cells

[0067] In one aspect, the present disclosure provides a method for producing a population of endoderm stem cells (EnSCs), comprising: a) providing a population of pluripotent stem cells; b) culturing the population of pluripotent stem cells in a first medium comprising a Nodal signaling agonist and a WNT signaling agonist; c) culturing the cells in a second medium comprising a

Nodal signaling agonist and a fibroblast growth factor (FGF); d) culturing the cells in a third medium comprising a factor belonging to the TGF-beta superfamily, FGF, a hepatocyte growth factor (HGF) and VEGF; and e) culturing the cells in a fourth medium comprising a WNT signaling agonist, a TGF-beta inhibitor and an epidermal growth factor (EGF); thereby producing a population of endoderm stem cells.

[0068] In some embodiments, the culturing period of step b is 1-2 days. In some embodiments, the culturing period of step b is 1 day. In some embodiments, the culturing period of step c is 2-6 days. In some embodiments, the culturing period of step c is 4 days. In some embodiments, the culturing period of step d is 2-6 days. In some embodiments, the culturing period of step d is 4 days.

[0069] In some embodiments, steps b-d are performed under less than 10% O_2, such as less than 8% O_2, less than 6% O_2, less than 4% O_2 or less than 2% O_2. In some embodiments, steps b-d are performed under 5% O_2.

[0070] In certain embodiments, the method further comprises washing cells obtained from step b before starting step c, washing cells obtained from step c before starting step d, and washing cells obtained from step d before starting step e. In certain embodiments, there is no washing step among steps b-e.

[0071] In some embodiments, at least 80% (e.g., at least 85%, at least 90%, at least 95%, at least 98% or at least 99%) of the population of endoderm stem cells express SOX17, CDX2, SOX9, GATA6 and/or FOXA1.

[0072] Expression of the markers may be detected by any method known in the art, including but not limited to, Fluorescence-activated cell sorting (FACS), Western Blotting, mRNA amplification-based methods (e.g., PCR, isothermal amplification, etc., which may include reverse transcription and may be applied to detect expression from single cells or multiple cells), Northern blotting, immunostaining, etc. Additionally, expression of said markers may be inferred by expression of a reporter construct (such as a fluorescent protein whose expression may be visually detected, an antibiotic resistance gene whose expression may be detected by cell survival in the presence of the antibiotic, etc.) under the control of a genetic element that confers cell type specific expression, such as the promoter of one of the foregoing markers or a fragment thereof.

[0073] II-1. Pluripotent Stem Cells

[0074] In certain embodiments, the population of pluripotent stem cells are autologous or allogeneic. In certain embodiments, the pluripotent stem cells are selected from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

[0075] As used herein, the terms "embryonic stem cells" and "ESCs" are used interchangeably and refer to the pluripotent stem cells of the inner cell mass of the embryonic blastocyst (see U.S. Pat. Nos. 5,843,780, 6,200,806, which are incorporated herein by reference). The distinguishing characteristics of an embryonic stem cell define an embryonic stem cell phenotype. Accordingly, a cell has the phenotype of an embryonic stem cell if it possesses one or more of the unique characteristics of an embryonic stem cell such that the cell can be distinguished from other cells. Exemplary distinguishing embryonic stem cell characteristics include, without limitation, gene expression profile, proliferative capacity, differentiation capacity, karyotype, responsiveness to particular culture conditions, and the like.

[0076] As used herein, the terms "induced pluripotent stem cells" and "iPSCs" are used interchangeably and refer to pluripotent cells artificially derived (e.g., induced by complete or partial reversal) from differentiated somatic cells (i.e. from non-pluripotent cells). A pluripotent cell can differentiate to cells of all three developmental germ layers.

[0077] Ectoderm, mesoderm, and endoderm are the three germ layers formed during the embryo development with the mesoderm as the middle layer, the ectoderm as the outside layer and the endoderm as the inside layer. The ectoderm forms surface ectoderm, neural crest, and neural tube, wherein the surface ectoderm develops into epidermis, hair, nails, lens of the eye, sebaceous glands, cornea, tooth enamel, the epithelium of mouth and nose; the neural crest of the ectoderm develops into peripheral nervous system, adrenal medulla, melanocytes, facial cartilage, and dentin of teeth; and the neural tube of the ectoderm develops into brain, spinal cord, posterior pituitary, motor neurons, and retina. The mesoderm forms mesenchyme, mesothelium, non-epithelial blood cells and coelomocytes that constitute muscle (smooth and striated), bone, cartilage, connective tissue, adipose tissue, circulatory system, lymphatic system, dermis, genitourinary system, serous membranes, and notochord. The endoderm forms pharynx, esophagus, stomach, small intestine, colon, liver, pancreas, bladder, epithelial parts of the trachea and bronchi, lungs, thyroid, and parathyroid.

[0078] Induced pluripotent stem cells (iPSCs) can be generated using methods known in the art. For example, the iPSCs provided in the methods of the present disclosure can be generated from

peripheral blood mononuclear cells (PBMCs). In certain embodiments, the PBMCs are obtained from a human subject who will be subject to the EnSC-derived therapies. In certain embodiments, iPSCs are generated by culturing PBMCs in the presence of human SCF, FLT-3, IL-3 and IL-6, followed by transducing the PBMCs with four reprogramming factors OCT4, SOX2, KLF4 and L-MYC.

[0079] II-2. Media for Preparing EnSCs

[0080] The basal medium used for culturing in steps b to e of the methods provided herein are not particularly limited. Any medium may be used as the basal medium as long as it enables the generation of endoderm stem cells. In certain embodiments, the basal medium is serum-free and/or stromal-free. In certain embodiments, the basal medium is selected from the group consisting of mTeSR™1, TeSR™-AOF, Essential 8™, NutriStem® hPSC XF Medium (Sartorius), RPMI/B27™, SFD-based medium, MCDB131, Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12 medium), StemPro™ 34-SFM, RPMI-1640, Iscove's Modified Dulbecco's Medium (IMDM), Ham's F-12 and CMRL-1066.

[0081] For the culture temperature, culture at a temperature of 35.0°C or more that has been confirmed to promote cell differentiation. The culture temperature is a temperature that does not damage the cells, such as preferably 35.0°C to 42.0°C, or more preferably 36.0°C to 40.0°C, or still more preferably 37.0°C to 39.0°C.

[0082] *First medium*

[0083] In certain embodiments, the first medium comprises a Nodal signaling agonist and a WNT signaling agonist. Exemplary Nodal signaling agonists include, but not limited to, Activin A, Nodal and GDF-8. In certain embodiments, the Nodal signaling agonist is Activin A.

[0084] In certain embodiments, the Nodal signaling agonist has a concentration ranging from about 20 ng/mL to about 200 ng/mL, such as, 40 ng/mL, 60 ng/mL, 80 ng/mL, 100 ng/mL, 120 ng/mL, 140 ng/mL, 160 ng/mL, 180 ng/mL or 200 ng/mL.

[0085] Exemplary WNT signaling agonists include, but not limited to, CHIR99021, Wnt3A, Wnt3a-AFM, R-Spondin-1 and BIO (6-bromoindirubin-3'-oxime), SKL2001, BML-284, CP21R7 and SB 216763. In certain embodiments, the WNT signaling agonist is CHIR99021 or Wnt3A.

[0086] In certain embodiments, the WNT signaling agonist has a concentration ranging from about 0.2 μM to about 4 μM, such as, 0.4 μM, 0.6 μM, 0.8 μM, 1 μM, 1.2 μM, 1.4 μM, 1.6 μM, 1.8 μM, 2.0 μM, 2.2 μM, 2.4 μM, 2.6 μM, 2.8 μM, 3.0 μM, 3.2 μM, 3.4 μM, 3.6 μM, 3.8 μM or 4.0 μM.

[0087] In certain embodiments, the first medium comprises Activin A and CHIR99021. In certain embodiments, the first medium comprises a medium supplemented with Activin A and CHIR99021.

[0088] *Second medium*

[0089] In certain embodiments, the second medium comprises a Nodal signaling agonist and a fibroblast growth factor (FGF). Exemplary FGF include, but not limited to, basic FGF (bFGF), FGF4 and chimeric fibroblast growth factor (FGFC), FGF1, FGF10 and FGF7. In certain embodiments, the FGF is bFGF.

[0090] In certain embodiments, the Nodal signaling agonist is Activin A.

[0091] In certain embodiments, the Nodal signaling agonist has a concentration ranging from about 20 ng/mL to about 200 ng/mL, such as, 40 ng/mL, 60 ng/mL, 80 ng/mL, 100 ng/mL, 120 ng/mL, 140 ng/mL, 160 ng/mL, 180 ng/mL or 200 ng/mL.

[0092] In certain embodiments, the FGF has a concentration ranging from about 1 ng/mL to 10 ng/mL, such as 2 ng/mL, 3 ng/mL, 4 ng/mL, 5 ng/mL, 6 ng/mL, 7 ng/mL, 8 ng/mL, 9 ng/mL or 10 ng/mL.

[0093] In certain embodiments, the second medium comprises Activin A and bFGF. In certain embodiments, the second medium comprises a medium supplemented with Activin A and bFGF.

[0094] Those skilled in the art would appreciate that further necessary supplements as described can be added to the medium. In certain embodiments, the second medium further comprises VEGF, ascorbic acid, and/or glutaMAX.

[0095] *Third medium*

[0096] In certain embodiments, the third medium comprises a factor belonging to the transforming growth factor-β (TGF-beta) superfamily, FGF, a hepatocyte growth factor (HGF) and Vascular Endothelial Growth Factor (VEGF).

[0097] Exemplary factors belonging to the TGF-beta superfamily include, but not limited to, BMP4, BMP2 and BMP7. In certain embodiments, the factor belonging to the TGF-beta superfamily is BMP4.

[0098] In certain embodiments, the factor belonging to the TGF-beta superfamily has a concentration ranging from about 20 ng/mL to about 200 ng/mL, such as, 40 ng/mL, 50 ng/mL, 60 ng/mL, 80 ng/mL, 100 ng/mL, 120 ng/mL, 140 ng/mL, 160 ng/mL, 180 ng/mL or 200 ng/mL.

[0099] In certain embodiments, the FGF is bFGF. In certain embodiments, the FGF has a concentration ranging from about 1 ng/mL to 20 ng/mL, such as 2 ng/mL, 3 ng/mL, 4 ng/mL, 5 ng/mL, 6 ng/mL, 7 ng/mL, 8 ng/mL, 9 ng/mL, 10 ng/mL, 11 ng/mL, 12 ng/mL, 13 ng/mL, 14 ng/mL, 15 ng/mL, 16 ng/mL, 17 ng/mL, 18 ng/mL, or 19 ng/mL.

[0100] In certain embodiments, the VEGF has a concentration ranging from about 1 ng/mL to 20 ng/mL, such as 2 ng/mL, 3 ng/mL, 4 ng/mL, 5 ng/mL, 6 ng/mL, 7 ng/mL, 8 ng/mL, 9 ng/mL, 10 ng/mL, 11 ng/mL, 12 ng/mL, 13 ng/mL, 14 ng/mL, 15 ng/mL, 16 ng/mL, 17 ng/mL, 18 ng/mL, or 19 ng/mL.

[0101] In embodiments, the HGF has a concentration ranging from about 1 ng/mL to 40 ng/mL, such as 2 ng/mL, 3 ng/mL, 4 ng/mL, 5 ng/mL, 6 ng/mL, 7 ng/mL, 8 ng/mL, 9 ng/mL, 10 ng/mL, 11 ng/mL, 12 ng/mL, 13 ng/mL, 14 ng/mL, 15 ng/mL, 16 ng/mL, 17 ng/mL, 18 ng/mL, 19 ng/mL, 20 ng/mL, 25 ng/mL, 30 ng/mL, 35 ng/mL, 36 ng/mL, 37 ng/mL, 38 ng/mL, or 39 ng/mL.

[0102] In certain embodiments, the third medium comprises BMP4, bFGF, HGF and VEGF. In certain embodiments, the third medium comprises a medium supplemented with BMP4, bFGF, HGF and VEGF.

[0103] Those skilled in the art would appreciate that further necessary supplements as described can be added to the medium. In certain embodiments, the third medium further comprises TGF-alpha and/or dexamethasone.

[0104] *Fourth medium*

[0105] In certain embodiments, the fourth medium, the fourth medium comprises a WNT signaling agonist, a TGF-beta inhibitor and an epidermal growth factor (EGF). Exemplary TGF-beta inhibitors include, but not limited to, A83-01, SB431542, ALK5 inhibitor, LDN-193189, Galunisertib (LY2157299), LY2109761, SB525334, SB505124, GW788388, LY364947, RepSox, LDN-193189, K02288, LDN-214117, SD-208, Vactosertib (TEW-7197), ML347, LDN-212854,

DMH1, Pirfenidone, Alantolactone, SIS3 and Hesperetin and Dorsomorphin. In certain embodiments, the TGF-beta inhibitor is A83-01.

[0106] In certain embodiments, the WNT signaling agonists is Wnt3A.

[0107] In certain embodiments, the WNT signaling agonist has a concentration ranging from about 0.2 μM to about 4 μM, such as, 0.4 μM, 0.6 μM, 0.8 μM, 1 μM, 1.2 μM, 1.4 μM, 1.6 μM, 1.8 μM, 2.0 μM, 2.2 μM, 2.4 μM, 2.6 μM, 2.8 μM, 3.0 μM, 3.2 μM, 3.4 μM, 3.6 μM, 3.8 μM or 4.0 μM.

[0108] In certain embodiments, the TGF-beta inhibitor has a concentration ranging from about 0.1 mM to about 2.0 mM, such as, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM.

[0109] In certain embodiments, the EGF has a concentration ranging from about 2 ng/mL to about 40 ng/mL, such as about 4 ng/mL, about 6 ng/mL, about 8 ng/mL, about 10 ng/mL, about 12 ng/mL, about 14 ng/mL, about 16 ng/mL, about 18 ng/mL, about 20 ng/mL, about 22 ng/mL, about 24 ng/mL, about 26 ng/mL, about 28 ng/mL, about 30 ng/mL, about 32 ng/mL, about 34 ng/mL, about 36 ng/mL, about 38 ng/mL or about 40 ng/mL.

[0110] In certain embodiments, the fourth medium does not comprise FGF2 and/or CHIR99021. In certain embodiments, the fourth medium does not comprise FGF.

[0111] In certain embodiments, the fourth medium comprises a Wnt3A, A83-01 and EGF. In certain embodiments, the fourth medium comprises a medium (e.g., MCDB131) supplemented with Wnt3A, A83-01 and EGF.

[0112] Those skilled in the art would appreciate that further necessary supplements as described can be added to the medium. In certain embodiments, the fourth medium further comprises Rspondin1, ascorbic acid, and/or glutamine.

[0113] In certain embodiments, the method for producing a population of endoderm stem cells comprises: a) providing a population of induced pluripotent stem cells; b) culturing the cells in a first medium comprising Activin A and CHIR99021; c) culturing the cells in a second medium comprising Activin A and bFGF; d) culturing the cells in a third medium comprising BMP4, bFGF, HGF and VEGF; and e) culturing the cells in a fourth medium comprising a Wnt3A, A83-01 and EGF; thereby producing a population of endoderm stem cells.

[0114] In certain embodiments, the method for producing a population of endoderm stem cells comprises: a) providing a population of induced pluripotent stem cells; b) culturing the cells in a

first medium comprising Activin A from about 50 ng/mL to about 150 ng/mL and CHIR99021 from about 1 μM to 3 μM; c) culturing the cells in a second medium comprising Activin A from about 50 ng/mL to about 150 ng/mL and bFGF from about 4 ng/mL to about 6 ng/mL; d) culturing the cells in a third medium comprising BMP4 from 40 ng/mL to about 60 ng/mL, bFGF from 5 ng/mL to about 15 ng/mL, HGF from 15 ng/mL to about 35 ng/mL and VEGF from 5 ng/mL to about 15 ng/mL; and e) culturing the cells in a fourth medium comprising a Wnt3A from 0.5 μM to about 1.5 μM, A83-01 from about 0.1 mM to about 1.0 mM and EGF from 10 ng/mL to about 30 ng/mL; thereby producing a population of endoderm stem cells.

[0115] In certain embodiments, the method for producing a population of endoderm stem cells comprises: a) providing a population of induced pluripotent stem cells; b) culturing the cells in a first medium comprising Activin A from about 50 ng/mL to about 150 ng/mL and CHIR99021 from about 1 μM to 3 μM for 12 hours to 48 hours (e.g., 24 hours); c) culturing the cells in a second medium comprising Activin A from about 50 ng/mL to about 150 ng/mL, bFGF from about 4 ng/mL to about 6 ng/mL, VEGF from about 5 ng/mL to about 15 ng/mL, ascorbic acid from about 0.1 mM to 1.0 mM and glutaMAX from about 1 mM to about 3 mM for about 2-6 days (e.g., 4 days); d) culturing the cells in a third medium comprising BMP4 from about 40 ng/mL to about 60 ng/mL, bFGF from about 5 ng/mL to about 15 ng/mL, HGF from about 15 ng/mL to about 35 ng/mL, VEGF from about 5 ng/mL to about 15 ng/mL, TGF-alpha from about 10 ng/mL to about 30 ng/mL and dexamethasone from about 20 ng/mL to about 60 ng/mL for 2-6 days (e.g., 4 days); and e) culturing the cells in a fourth medium comprising a Wnt3A from 0.5 μM to about 1.5 μM, A83-01 from about 0.1 mM to about 1.0 mM, EGF from 10 ng/mL to about 30 ng/mL, Rspondin1 from about 20 ng/mL to about 80 ng/mL, ascorbic acid from about 0.1 mM to 1.0 mM and glutamine from about 1 mM to about 3 mM; thereby producing a population of endoderm stem cells (EnSCs).

[0116] In certain embodiments, the EnSCs are harvested, for example, every 3-4 days and dissociated into single cells for subsequent expansion, subcloning or differentiation.

[0117] In certain embodiments, the EnSCs (e.g., at passage 20) are selected for quality control tests including those of morphology, viability, purity, sterility, karyotype, as well as whole genome sequencing. Clinically acceptable EnSCs require those, for example, free of known cancer-related mutations, with the lowest overall mutational burden compared to the PBMCs from which the

EnSCs are derived, and with minimal tumor-forming potential. The clinically acceptable EnSCs can be frozen and stored for future uses.

[0118] **III. Pancreatic Endocrine Cells**

[0119] In another aspect, the present disclosure provides a method for producing a population of pancreatic endocrine cells, comprising: a) providing a population of endoderm stem cells; b) culturing the population of endoderm stem cells in the presence of a BMP inhibitor, Nodal signaling agonist, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and/or Nicotinamide to produce a population of pancreatic progenitor (PP) cells; c) culturing the population of PP cells in the presence of a BMP inhibitor, a TGF-beta inhibitor and/or a gamma-secretase inhibitor to produce a population of endocrine progenitor (EP) cells; and d) culturing the population of EP cells in the presence of T3 and Nicotinamide; thereby producing a population of pancreatic endocrine cells. In certain embodiments, the population of PP cells in step c refer to homogeneous cell clusters formed by the population of PP cells.

[0120] In certain embodiments, the culturing period of step b is 2-6 days. In certain embodiments, the culturing period of step c is 3-8 days. In certain embodiments, the culturing period of step d is 7-21 days.

[0121] III-1. Endoderm Stem Cells as Starting Cells

[0122] In certain embodiments, the EnSCs are produced by the methods described under section "*II. Endoderm Stem Cells*".

[0123] In certain embodiments, clinically acceptable EnSCs have one or more of the following characteristics: 1) having a typical epithelial morphology with clear cell boundary and a diameter of 3-5 um as detected by microscopy (e.g., phase contrast observation); 2) having at least 90% of the cells positive for FOXA1 as measured by flow cytometry; 3) having intact karyotype as assayed by karyotype test; 4) devoid of known cancer-related mutations and the lowest overall mutational burden compared to the original PBMC as detected by the whole genome sequencing; 5) having at least 90% live cells before frozen and at least 60% live cells after thaw as detected by flow cytometry; 6) having undetectable mycoplasma, sterility and negative for virus as assayed by the pathogen test; 7) with no teratoma found as assayed by the teratoma formation test.

[0124] III-2. Generation of Pancreatic Progenitor (PP) Cells

[0125] For the induction of pancreatic endoderm and subsequently generation of pancreatic progenitor (PP) cells, EnSCs were treated in a basal medium (e.g., MCDB) with a cocktail containing a variety of factors. In certain embodiments, a population of pancreatic progenitor (PP) cells are produced by culturing a population of endoderm stem cells in the presence of a BMP inhibitor, Nodal signaling agonist, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and/or Nicotinamide. Exemplary BMP inhibitors include, but not limited to, Noggin, dorsomorphin and LDN-193189. In certain embodiments, the BMP inhibitor comprises Noggin.

[0126] In certain embodiments, the BMP inhibitor has a concentration ranging from about 2 ng/mL to about 40 ng/mL, such as about 4 ng/mL, about 6 ng/mL, about 8 ng/mL, about 10 ng/mL, about 12 ng/mL, about 14 ng/mL, about 16 ng/mL, about 18 ng/mL, about 20 ng/mL, about 22 ng/mL, about 24 ng/mL, about 26 ng/mL, about 28 ng/mL, about 30 ng/mL, about 32 ng/mL, about 34 ng/mL, about 36 ng/mL, about 38 ng/mL or about 40 ng/mL.

[0127] In certain embodiments, the Nodal signaling agonist is Activin A. In certain embodiments, the Nodal signaling agonist has a concentration ranging from about 0.1 ng/mL to about 1.0 ng/mL, such as 0.2 ng/mL, 0.3 ng/mL, 0.4 ng/mL, 0.5 ng/mL, 0.6 ng/mL, 0.7 ng/mL, 0.8 ng/mL, 0.9 ng/mL or 1.0 ng/mL.

[0128] In certain embodiments, the FGF10 has a concentration ranging from about 2 ng/mL to about 40 ng/mL, such as about 4 ng/mL, about 6 ng/mL, about 8 ng/mL, about 10 ng/mL, about 12 ng/mL, about 14 ng/mL, about 16 ng/mL, about 18 ng/mL, about 20 ng/mL, about 22 ng/mL, about 24 ng/mL, about 26 ng/mL, about 28 ng/mL, about 30 ng/mL, about 32 ng/mL, about 34 ng/mL, about 36 ng/mL, about 38 ng/mL or about 40 ng/mL.

[0129] In certain embodiments, the EGF has a concentration ranging from about 2 ng/mL to about 40 ng/mL, such as about 4 ng/mL, about 6 ng/mL, about 8 ng/mL, about 10 ng/mL, about 12 ng/mL, about 14 ng/mL, about 16 ng/mL, about 18 ng/mL, about 20 ng/mL, about 22 ng/mL, about 24 ng/mL, about 26 ng/mL, about 28 ng/mL, about 30 ng/mL, about 32 ng/mL, about 34 ng/mL, about 36 ng/mL, about 38 ng/mL or about 40 ng/mL.

[0130] In certain embodiments, the SANT1 has a concentration ranging from about 0.1 μM to about 1.0 μM, such as 0.2 μM, 0.3 μM, 0.4 μM, 0.5 μM, 0.6 μM, 0.7 μM, 0.8 μM, 0.9 μM or 1.0 μM.

[0131] In certain embodiments, the retinoic acid has a concentration ranging from about 0.5 μM to about 4 μM, such as 1 μM, 1.5 μM, 2 μM, 2.5 μM, 3 μM, 3.5 μM or 4 μM.

[0132] In certain embodiments, the ascorbic acid has a concentration ranging from about 0.1 mM to about 1.0 mM, such as 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM or 1.0 mM.

[0133] In certain embodiments, the Nicotinamide has a concentration ranging from about 1 mM to about 20 mM, such as 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM or 20 mM.

[0134] In certain embodiments, a population of pancreatic progenitor (PP) cells are produced by culturing a population of endoderm stem cells in the presence of Noggin, Activin A, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and/or Nicotinamide.

[0135] In certain embodiments, a population of pancreatic progenitor (PP) cells are produced by culturing a population of endoderm stem cells in the presence of Noggin, Activin A, FGF10, EGF, SANT1, retinoic acid, ascorbic acid, Nicotinamide, Rspondin1, LDN-193189 and/or TPPB.

[0136] In certain embodiments, a population of pancreatic progenitor (PP) cells are produced by culturing a population of endoderm stem cells in the presence of Noggin, Activin A, FGF10, LDN 193189, Rspondin1, TPPB and/or EGF for 1-3 days (e.g., 2 days), followed by culturing the cells in the presence of LDN 193189, FGF10, EGF, SANT1, ascorbic acid and/or retinoic acid (Sigma) for 1-3 days (e.g., 2 days), followed by culturing the cells in the presence of FGF10, EGF, SANT1, retinoic acid, Nicotinamide and/or ascorbic acid for 1-3 days (e.g., 2 days).

[0137] In certain embodiments, the EnSC-derived PP cells are evaluated for microorganism contamination, morphology, purity and viability. In certain embodiments, clinically acceptable PP cells have one or more of the following characteristics: 1) having a typical epithelial morphology with unclear cell boundary and a diameter of no more than 3 um as detected by microscopy (e.g., phase contrast observation); 2) having at least 60% of the cells positive for PDX1 as measured by flow cytometry; 3) having at least 90% live cells as detected by flow cytometry; and 4) having undetectable mycoplasma, sterility, negative for virus and no more than 1 EU/mL Endotoxin as assayed by the pathogen test.

[0138] In certain embodiments, at the end of this stage, pancreatic progenitor (PP) cells are single cell dispersed and suspended, for example, in a three-dimensional environment (e.g., in a AggreWell (STEMCELL), Corning® Elplasia® culture plates, Nunclon™ Sphera™ 96-well, or Corning® round-bottom ultra-low attachment microplates with spherical wells) to form

homogeneous cell clusters for a certain period of time (e.g., 1-5 days, such as 3 days) for further islet tissue reconstruction and maturation.

[0139] III-3. Generation of Endocrine Progenitor (EP) Cells

[0140] For endocrine progenitor (EP) induction, triple inhibition of BMP, TGF-β and Notch signaling pathways can be manipulated for several days. In certain embodiments, the homogeneous cell clusters obtained using the method described under the section " *Generation of Pancreatic Progenitor (PP) Cells*" are further cultured in the presence of a BMP inhibitor, a TGF-beta inhibitor and/or a gamma-secretase inhibitor. In certain embodiments, the BMP inhibitor comprises Noggin. In certain embodiments, the TGF-beta inhibitor is A83-01. Exemplary gamma-secretase inhibitors include, but not limited to, Compound E, GSI-XX and GSI-XXI, DAPT, LY-411575, RO4929097 and DBZ (Dibenzazepine). In certain embodiments, the gamma-secretase inhibitor comprises GSI-XX.

[0141] In certain embodiments, the BMP inhibitor has a concentration ranging from about 1 ng/mL to about 40 ng/mL, such as 2 ng/mL, 3 ng/mL, 4 ng/mL, 5 ng/mL, 6 ng/mL, 7 ng/mL, 8 ng/mL, 9 ng/mL, 10 ng/mL, 11 ng/mL, 12 ng/mL, 13 ng/mL, 14 ng/mL, 15 ng/mL, 16 ng/mL, 17 ng/mL, 18 ng/mL, 19 ng/mL, 20 ng/mL, 22 ng/mL, 24 ng/mL, 26 ng/mL, 28 ng/mL, 30 ng/mL, 32 ng/mL, 34 ng/mL, 36 ng/mL, 38 ng/mL or 40 ng/mL.

[0142] In certain embodiments, the TGF-beta inhibitor has a concentration ranging from about 0.1 mM to about 2.0 mM, such as, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM, 1.1 mM, 1.2 mM, 1.3 mM, 1.4 mM, 1.5 mM, 1.6 mM, 1.7 mM, 1.8 mM, 1.9 mM, 2.0 mM.

[0143] In certain embodiments, the gamma-secretase inhibitor has a concentration ranging from about 0.5 μM to about 4 μM, such as 1 μM, 1.5 μM, 2 μM, 2.5 μM, 3 μM, 3.5 μM or 4 μM.

[0144] In certain embodiments, the sixth medium comprises a Noggin, A83-01 and/or GSI-XX.

[0145] In certain embodiments, the homogeneous cell clusters are further cultured in the presence of Noggin, A83-01 and/or GSI-XX. In certain embodiments, the homogeneous cell clusters are further cultured in the presence of Noggin, A83-01, GSI-XX, retinoic acid and/or SANT1.

[0146] In certain embodiments, the EnSC-derived EP cells are evaluated for microorganism contamination, morphology, purity and viability. In certain embodiments, clinically acceptable EP cells have one or more of the following characteristics: 1) having at least 60% of the cells positive

for PDX1 and NKX6-1 as measured by flow cytometry; 2) having at least 90% live cells as detected by flow cytometry; and 3) having undetectable mycoplasma, sterility, negative for virus and no more than 1 EU/mL Endotoxin as assayed by the pathogen test.

[0147] III-4. Generation of E-islets (maturation of endocrine cells)

[0148] For the maturation of endocrine cells, additional factors can be added to the medium for generation of endocrine progenitor cells described under section "*III-3. generation of endocrine progenitor (EP)*". In certain embodiments, additional factors are selected from the group consisting of 3,3',5-Triiodo-L-thyronine (T3) and Nicotinamide. In certain embodiments, additional factors comprise T3 and Nicotinamide.

[0149] In certain embodiments, the T3 has a concentration ranging from about 0.5 μM to about 4 μM, such as 1 μM, 1.5 μM, 2 μM, 2.5 μM, 3 μM, 3.5 μM or 4 μM.

[0150] In certain embodiments, the Nicotinamide has a concentration ranging from about 1 mM to about 20 mM, such as 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM or 20 mM.

[0151] In certain embodiments, the additional factors further comprise BMP4.

[0152] In certain embodiments, the method for producing a population of pancreatic endocrine cells comprises: a) providing a population of endoderm stem cells; b) culturing the population of endoderm stem cells in the presence of a Noggin, Activin A, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and/or Nicotinamide to produce a population of pancreatic progenitor (PP) cells; c) culturing the population of PP cells in the presence of a Noggin, A83-01 and/or GSI-XX to produce a population of endocrine progenitor (EP) cells; and d) culturing the population of EP cells in the presence of T3 and/or Nicotinamide; thereby producing a population of pancreatic endocrine cells.

[0153] In certain embodiments, the method for producing a population of pancreatic endocrine cells comprises: a) providing a population of endoderm stem cells; b) culturing the population of endoderm stem cells in the presence of a Noggin from about 10 ng/mL to about 30 ng/mL, Activin A from about 0.1 ng/mL to about 1.0 ng/mL, FGF10 from about 10 ng/mL to about 30 ng/mL, EGF from about 10 ng/mL to about 30 ng/mL, LDN 193189 from about 100 nM to about 300 nM, Rspondin1 from about 10 ng/mL to about 30 ng/mL and/or TPPB from about 300 nM to about 800 nM for 1-3 days (e.g., 2 days), followed by further culturing in the presence of LDN 193189 from about 100 nM to about 300 nM, FGF10 from about 10 ng/mL to about 30 ng/mL, EGF from about

10 ng/mL to about 30 ng/mL, SANT1 from about 0.3 uM to about 0.8 uM, ascorbic acid from about 0.3 mM to about 0.8 mM and/or retinoic acid from about 0.2 uM to about 3 uM for 1-3 days (e.g., 2 days), further followed by culturing in the presence of FGF10 from about 10 ng/mL to about 60 ng/mL, EGF from about 10 ng/mL to about 30 ng/mL, SANT1 from about 0.1 uM to about 0.4 uM, retinoic acid from about 0.1 uM to about 0.3 uM, Nicotinamide from about 5 mM to about 15 mM and/or ascorbic acid from about 0.3 mM to about 0.8 mM for 1-3 days (e.g., 2 days) to produce a population of pancreatic progenitor (PP) cells; c) culturing a population of PP cells in the presence of a Noggin from about 10 ng/mL to about 30 ng/mL, A83-01 from about 0.1 mM to about 1.0 mM, GSI-XX from about 1 uM to about 3 uM, retinoic acid from about 0.05 uM to about 0.2 uM and/or SANT1 from about 0.05 uM to about 0.2 uM to produce a population of endocrine progenitor (EP) cells; and d) culturing the population of EP cells in the presence of T3 from about 0.5 uM to about 1.5 uM, Nicotinamide from about 5 mM to about 15 mM and/or BMP4 from about 1 ng/mL to about 3 ng/mL in addition to the factors involved in step c; thereby producing a population of pancreatic endocrine cells.

[0154] In certain embodiments, the EnSCs, EnSC-derived PP cells, EnSC-derived EP cells, or EnSC-derived matured endocrine cells described herein are capable of forming three-dimensional cellular aggregates, which mimics the natural islet structure and facilitates intercellular interactions. Subsequently, the additional three-dimensional incubation of the cellular aggregates result in functional islet-like structures, namely EnSC-derived islets, i.e., E-islets. The resulting E-islets are then assessed for maturity and functionality, including marker expression, endocrine cell composition, hormone secretion, and glucose responsiveness.

[0155] In certain embodiments, the E-islets are evaluated for microorganism contamination, morphology, purity and viability, and are also analyzed for endocrine cell composition, such as Insulin+NKX6-1+ β cells, Glucagon+ α cells, Somatostatin+ δ cells and Chromogranin A+ endocrine cells by, for example, flow cytometry and single-cell transcriptomic analyses (scRNA-seq).

[0156] In certain embodiments, the *in vitro* functionality of E-islets can be assayed by glucose-stimulated insulin secretion (GSIS) as human cadaveric islets, and the *in vivo* functionality can be evaluated by kidney capsule or hepatic portal transplantation into a streptozotocin-induced diabetic animal (e.g., mouse or monkey) models. In certain embodiments, the nontarget hepatocytes (HNF4A+Albumin+), cholangiocytes (SOX9+CK7+), intestinal epithelial cells (CDX2+) and the

pancreatic ductal cells (SOX9+PTF1A+PDX1+) can be estimated from, for example, scRNA-seq data.

[0157] In certain embodiments, clinically acceptable E-islets have one or more of the following characteristics: 1) having a dense spherical cell mass with unclear cell boundary with an average diameter of about 150 um as detected by microscopy (e.g., phase contrast observation); 2) having at least 85% of the cells positive for PDX1 (i.e., the pancreatic lineage), at least 60% of the cells positive for CHGA+ (i.e., the endocrine lineage), at least 40% of cells positive for C-peptide (i.e., β cell), at least 20% of the cells positive for Glucagon (i.e., α cell), at least 15% of the cells positive for Somatostatin (i.e., δ cell), and less than 2% of the cells positive for AFP+ (i.e., untargeted hepatic lineage) as measured by flow cytometry; 3) having at least 90% live cells as measured by flow cytometry; 4) having at least 1 fold (e.g., 1.5 fold) changes of insulin or C-peptide as detected by static glucose stimulated insulin (C-peptide) secretion assay, 5) having undetectable mycoplasma, sterility, negative for virus and no more than 1 EU/mL Endotoxin as assayed by the pathogen test.

[0158] In certain embodiments, at least 50% (e.g., at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95%) of the E-islets provided herein are positive for C-peptide, Glucagon, PDX1 and/or NKX6-1.

[0159] C-peptide is a byproduct of insulin production, that is secreted by the beta cells of the pancreatic islets along with insulin. The secretion of human C-peptide represents the activity of the beta cells within the islets and their ability to produce and release insulin. Insulin is a key hormone responsible for regulating blood glucose levels, and C-peptide is often used as a marker for insulin secretion due to its co-secretion with insulin in equimolar amounts. See, e.g., *Wilcox G. Insulin and insulin resistance. Clin Biochem Rev. 2005 May;26(2):19-39.*

[0160] Glucagon serves as a marker for islets due to its specific secretion from α-cells within the pancreatic islets and its crucial role in regulating blood glucose levels, particularly during fasting states. This hormone counteracts the action of insulin, promoting gluconeogenesis and glycogenolysis in the liver, which raises blood glucose levels.

[0161] PDX1, known as pancreas duodenal homeobox 1, is a marker for pancreatic islets due to its essential role in pancreas development and β-cell function. As a homeodomain transcription factor, PDX1 regulates the expression of genes critical for pancreatic development and the maintenance of islet cell identity.

[0162] NKX6-1 is a pancreatic-specific marker that is expressed in pancreatic progenitor (PP) cells, endocrine progenitor (EP) cells, and certain subpopulations of pancreatic endocrine cells.

[0163] III-5. Media for Preparing Pancreatic Endocrine Cells

[0164] Any cell culture system known in the art can be used in the present disclosure. In certain embodiments, adherent culture system is used in the method for producing a population of pancreatic endocrine cells. The term "adherent culture" refers to a cell culture system whereby cells are cultured on a solid surface, which may in turn be coated with a substrate. The cells may or may not tightly adhere to the solid surface or to the substrate. The substrate for the adherent culture may further comprise, for example, any one or combination of polystyrene, polyester, polycarbonate, poly(N-isopropylacrylamide), polyornithine, laminin, polylysine, purified collagen, gelatin, cellulose, extracellular matrix, fibronectin, tenacin, vitronectin, poly glycolytic acid (PGA), poly lactic acid (PLA), poly lactic-glycolic acid (PLGA), matrigel, hydroxyapatite, and amniotic membrane.

[0165] In certain embodiments, suspension culture can be used in the method for producing a population of pancreatic endocrine cells. The term "suspension culture" as used herein refers to the culture of cells such that the cells do not adhere to the solid support or the culture vessel. To transfer cells into a suspension culture, they are for example removed from the culture receptacle by a cell scraper and transferred to sterile low attachment plates containing culture medium, which do not allow adhesion of the cells to the surface of the plate. Thus, the cells are cultured in suspension without adherence to a matrix or the bottom of the dish.

[0166] Media suitable for culturing cells are any media suitable for growing a certain cell type in a dish. The media includes, such as MCDB, Ham's F10 (Sigma), Ham's F12 medium, Minimal Essential Medium (MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM), Sigma), IMDM medium, Medium 199, Eagle's Minimum Essential Medium (EMEM), aMEM medium, CMRL1066, DMEM/F12, and mixtures of these. Any of these media may be supplemented as necessary with salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCINTM drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), glucose or an equivalent energy source, albumin, insulin, transferrin, selenium, fatty acids, 2-mercaptoethanol, thiol glycerol, lipids, amino

acids, L-glutamine, non-essential amino acids, vitamins, growth factors, low-molecular-weight compounds, antioxidants, pyruvic acid, cytokines and the like as necessary. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used for cell culture, and will be apparent to the ordinarily skilled artisan.

[0167] **IV. Pharmaceutical Compositions and Therapeutic Uses**

[0168] IV-1. Pharmaceutical Compositions

[0169] In another aspect, the present disclosure provides a population of EnSCs produced according to the methods provided herein, for example, under the section "*II. Endoderm Stem Cells*".

[0170] In another aspect, the present disclosure provides a population of PP cells, EP cells, and pancreatic endocrine cells, produced according to the methods provided herein, for example, under the section "*III. Pancreatic Endocrine Cells*".

[0171] In another aspect, the present disclosure provides a pharmaceutical composition comprising the population of EnSCs, the population of PP cells, the population of EP cells, or the population of pancreatic endocrine cells provided herein, and a pharmaceutically acceptable medium.

[0172] The term "pharmaceutically acceptable" indicates that the designated carrier, vehicle, diluent, excipient(s), and/or salt is generally chemically and/or physically compatible with the other ingredients comprising the formulation, and physiologically compatible with the recipient thereof. Pharmaceutical acceptable carriers for use in the pharmaceutical compositions disclosed herein may include, for example, pharmaceutically acceptable liquid, gel, or solid carriers, aqueous vehicles, nonaqueous vehicles, antimicrobial agents, isotonic agents, buffers, antioxidants, anesthetics, suspending/dispending agents, sequestering or chelating agents, diluents, adjuvants, excipients, or non-toxic auxiliary substances, other components known in the art, or various combinations thereof.

[0173] The composition described herein can also include components that facilitate engraftment. The composition described herein may be pyrogen-free or essentially pyrogen-free, and pathogen-free, wherein the pathogen comprises bacterial contaminants, mycoplasmal contaminants, and viruses.

[0174] In certain embodiments, the pharmaceutical composition may further comprise an immunosuppressive agent or immune tolerizing agent.

[0175] IV-2. Therapeutic Uses

[0176] In a further aspect, the present disclosure is directed to therapeutic uses of the EnSCs, EnSC-derived PP cells, EnSC-derived EP cells, EnSC-derived pancreatic endocrine cells or E-islets provided herein.

[0177] In one aspect, the present disclosure provides a method of treating a disease or condition associated with impaired pancreatic islet function in a subject in need thereof, comprising: administering an effective amount of pancreatic endocrine cells derived from a population of EnSCs or E-islets to the subject, thereby treating the disease or condition associated with impaired pancreatic islet function in the subject in need thereof. In certain embodiments, the population of EnSCs are produced according to the methods provided herein, for example, under the section "*II. Endoderm Stem Cells*". In certain embodiments, the population of pluripotent stem cells are autologous or allogeneic.

[0178] The "disease or condition associated with impaired pancreatic islet function" includes any disease or condition amenable to treatment by administration of pancreatic endocrine cells or E-islets, including diseases in which a subject's pancreatic endocrine cells decrease in numbers or die, decrease in density, or otherwise become dysfunctional. As used herein, the term "impaired pancreatic islet function" encompasses not only a decrease in the optimal functioning of the islets of Langerhans in the pancreas, leading to reduced production or secretion of insulin and glucagon, but also includes partial or complete pancreatic islet functional failure. This wide range of dysfunction can result in abnormal blood glucose levels, potentially leading to diabetes or other metabolic disorders. In the case of complete functional failure, the body loses its ability to regulate blood sugar effectively, which can lead to severe hyperglycemia and associated health complications if left untreated.

[0179] In certain embodiments, the disease or condition associated with impaired pancreatic islet function is diabetes. Diabetes is a chronic metabolic disease that occurs when the body either cannot produce enough insulin or cannot effectively use the insulin it produces. Insulin is a hormone that plays a crucial role in regulating blood sugar levels. Diabetes is a leading cause of blindness, kidney failure, heart disease, stroke, and lower limb amputations.

[0180] In certain embodiments, the disease or condition associated with impaired pancreatic islet function is Type 1 diabetes (T1D), Type 2 diabetes (T2D), Type 3c diabetes or Maturity-onset diabetes of the young (MODY).

[0181] Type 1 diabetes (T1D), also known as juvenile diabetes, is an autoimmune disease where the body's immune system attacks and destroys the insulin-producing cells in the pancreas.

[0182] Type 2 diabetes (T2D) typically starts with insulin resistance in peripheral tissues and proceeds with gradual loss of islet function due to the reduction in β-cell mass or dedifferentiation of β cells under progressively worsening pathological conditions. See, e.g., *Talchai, C. et al. Cell 150, 1223-1234 (2012); and Chatterjee, S. et al. Lancet 389, 2239-2251 (2017)*. More than 30% of T2D patients eventually rely on exogenous insulin treatment. Cadaveric islet transplantation is a safe and effective treatment for insulin-dependent diabetes. See, e.g., *Shapiro, A. M. et al. N. Engl. J. Med. 355, 1318-1330 (2006); and Marfil-Garza, B. A. et al. Lancet Diabetes Endocrinol. 10, 519-532 (2022)*. Notably, improved metabolic control after islet transplantation is associated with better kidney allograft function and long-term survival. See, e.g., *Lablanche, S. et al. Lancet Diabetes Endocrinol. 6, 527-537 (2018); and Markmann, J. F. et al. Am. J. Transplant. 21, 1477-1492 (2021)*. However, the application of islet transplantation is severely hampered due to the critical shortage of healthy donor organs and the complicated isolation procedure.

[0183] Type 3c diabetes is a type of diabetes that is secondary to diseases of the exocrine pancreas. It is also known as pancreatogenic or pan-creatogenous diabetes mellitus. The most common cause of type 3c diabetes is chronic pancreatitis, which is an inflammation of the pancreas that causes damage to the exocrine and endocrine tissues. Other causes include pancreatic ductal adenocarcinoma, haemochromatosis, cystic fibrosis, and previous pancreatic surgery. The pathogenesis of type 3c diabetes is not fully understood, but it is thought to be related to the loss of functional β-cells in the pancreas due to the underlying pancreatic disease. Type 3c diabetes is less common than other forms of diabetes, but it is still relatively prevalent, particularly among patients with chronic pancreatitis or pancreatic ductal adenocarcinoma. The diagnosis of type 3c diabetes can be challenging because the symptoms are similar to those of other forms of diabetes, and the disease can be masked by the presence of other pancreatic disorders. See, e.g., *Hart PA et al., Lancet Gastroenterol Hepatol. 2016 Nov;1(3):226-237.*

[0184] Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes that is characterized by early-onset diabetes. It is caused by a single, pathogenic genetic alteration that

results in dysfunction or altered development of the pancreatic beta cells, which secrete insulin. The common denominator of MODY is early-onset diabetes, which usually occurs during childhood, adolescence, or young adulthood. Patients with MODY typically have a strong family history of diabetes, and the condition often runs in families. The symptoms of MODY are similar to those of type 2 diabetes, including hyperglycemia and insulin resistance. The diagnosis of MODY can be challenging because it is often confused with type 1 or type 2 diabetes. See, e.g., *Bonnefond, A. et al. Nat Rev Dis Primers 9, 12 (2023).*

[0185] Administration routes may include any suitable means, including, but not limited to, percutaneous transhepatic infusion, hepatic portal infusion, kidney capsule transplantation, rectus abdominis injection, subcutaneous implantation, mesentery injection, retroperitoneal injection, hepatic artery injection, iliac fossa injection, and the like. In some embodiments, the particular mode of administration selected will depend upon the particular treatment, disease state or condition of the patient, the nature or administration route of other drugs or therapeutics administered to the subject, etc.

[0186] In certain embodiments, the subject is human.

[0187] In certain embodiments, the effective amount is about 0.5-3.0 million Islet equivalent (IEQ) units. IEQ measurements are the standard estimate of islet volume with one IEQ equaling a single spherical islet of 150 um in diameter. See, e.g., *Lembert N et al., Cell Transplant. 2003;12(1):33-41.*

[0188] In certain embodiments, the method further comprises administering an immunosuppressant to the subject in need thereof. When immunosuppressants are used, they may be administered systemically or locally, and they may be administered prior to, concomitantly with, or subsequent to administration of the E-islets.

[0189] In another aspect, the present disclosure provides uses of a population of endoderm stem cells in the manufacture of E-islets for treating a disease or condition associated with impaired pancreatic islet function in a subject in need thereof.

[0190] In certain embodiments, the manufacture comprises the steps described herein, for example, under the section "*III. Pancreatic Endocrine Cells*".

[0191] In another aspect, the present disclosure provides a first-in-human tissue replacement therapy for treating a disease or condition associated with impaired pancreatic islet function, such

as diabetes, for example, type I diabetes (T1D), type II diabetes (T2D). In certain embodiments, the therapy provided herein offers several significant advantages over the existing options.

[0192] First, unlike traditional cadaveric islet transplantation, the provided therapy utilizes patient's own cells (such as PBMCs) to produce functional islets. This not only enhances tolerance but also provides a more accessible source compared to donor cadaveric islet transplantation.

[0193] Second, in contrast to hPSC-derived islets that are currently under clinical trials, the endoderm stem cells (EnSCs) used for generating functional islets in the present disclosure are nontumorigeneic in vivo and are more suitable precursors for the efficient mass production of islets. Their endoderm-specific nature and closer developmental relationship with pancreatic lineages make them a superior choice as an islet precursor compared to hPSCs (e.g., hiPSCs).

[0194] Third, while other trials have involved T1D patients, therapies of the present disclosure can expand the indication to T2D while allowing assessment of the engraftment and functionality of EnSC-derived islets (E-islets) without autoimmune interference. Furthermore, this therapy has the potential to treat late-stage diseases, including advanced T2D.

[0195] The above-mentioned advantages can be achieved by the novel methods for producing EnSCs as well as EnSC-derived islets (E-islets) used for the therapies of the present disclosure.

[0196] **V. Kits**

[0197] In one aspect, the present disclosure provides a kit for producing a population of endoderm stem cells from a population of pluripotent stem cells, wherein the kit comprises a first set of factors, a second set of factors, a third set of factors and a fourth set of factors, wherein the first set of factors comprises a Nodal signaling agonist and a WNT signaling agonist, the second set of factors comprises a Nodal signaling agonist and a fibroblast growth factor (FGF), the third set of factors comprises a factor belonging to the TGF-beta superfamily, FGF, a hepatocyte growth factor (HGF) and VEGF, and the fourth set of factors comprises a WNT signaling agonist, a TGF-beta inhibitor and an epidermal growth factor (EGF).

[0198] In certain embodiments, the fourth set of factors does not comprise FGF2 and/or Chir99021. In certain embodiments, the fourth medium does not comprise FGF.

[0199] In another aspect, the present disclosure provides a kit for producing a population of pancreatic endocrine cells from a population of endoderm stem cells, wherein the kit comprises a fifth set of factors, a sixth set of factors and a seventh set of factors, wherein the fifth set of factors

comprises a BMP inhibitor, Nodal signaling agonist, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and/or Nicotinamide, the sixth set of factors comprises a BMP inhibitor, a TGF-beta inhibitor and/or a gamma-secretase inhibitor, and the seventh set of factors comprises T3 and/or Nicotinamide.

[0200] In another aspect, the present disclosure provides a kit for producing a population of pancreatic endocrine cells from a population of pluripotent stem cells, wherein the kit comprises first to seventh sets of factors, wherein the first set of factors comprises a Nodal signaling agonist and a WNT signaling agonist, the second set of factors comprises a Nodal signaling agonist and a fibroblast growth factor (FGF), the third set of factors comprises a factor belonging to the TGF-beta superfamily, FGF, a hepatocyte growth factor (HGF) and VEGF, the fourth set of factors comprises a WNT signaling agonist, a TGF-beta inhibitor and an epidermal growth factor (EGF), the fifth set of factors comprises a BMP inhibitor, Nodal signaling agonist, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and Nicotinamide, the sixth set of factors comprises a BMP inhibitor, a TGF-beta inhibitor and a gamma-secretase inhibitor, and the seventh set of factors comprises T3 and Nicotinamide.

[0201] In certain embodiments, the fourth set of factors does not comprise FGF2 and/or Chir99021. In certain embodiments, the fourth medium does not comprise FGF.

[0202] In another aspect, the present disclosure provides a kit for producing a population of pancreatic endocrine cells from a population of PP cells, wherein the kit comprises a set of factors comprising a BMP inhibitor, a TGF-beta inhibitor and a gamma-secretase inhibitor.

[0203] All publications and patents cited in this specification are herein incorporated by reference to their entirety.

EXAMPLES

[0204] Example 1. Generation of induced pluripotent stem cells (iPSCs) from human peripheral blood mononuclear cells (PBMCs)

[0205] This example illustrates the generation of induced pluripotent stem cells (iPSCs) from the peripheral blood mononuclear cells (PBMCs) of a T2D patient with impaired insulin secretion.

[0206] PBMCs were harvested and tested for microorganisms, including bacteria, fungi, mycoplasma, HIV, HAV, HBV, HCV, HTLV, EBV, HCMV and TP to ensure their safety for

subsequent uses, and then used to generate iPSC lines with the Sendai Viral reprogramming system under GMP conditions. PBMCs of the patient (WB as the donor code) were isolated from whole blood using Ficoll gradient. The whole blood was sampled with BD Vacutainer® EDTA Tubes. The blood was diluted with Dulbecco's Phosphate-Buffered Saline (DPBS) and poured onto the Ficoll solution, and were centrifuged for 30 minutes at 400 g at room temperature. The layer of PBMCs was collected and washed with DPBS. PBMCs were frozen or proceeded to iPSCs generation.

[0207] Sendai virus reprogramming kit (Invitrogen, GMP grade) containing four reprogramming factors (OCT4, SOX2, KLF4, L-MYC) was used for iPSCs generation. Two million PBMCs were first cultured in the presence of human SCF, FLT-3, IL-3 and IL-6 in StemPro™-34 SFM (Gibco) SP34 for 7 days according to the manufacturer's instruction. On the day of transduction, PBMCs were washed and counted, and the appropriate volumes of virus vectors were calculated according to the cell count and the titers of virus. The PBMCs and virus vectors were mixed for the transduction. Two days post transduction, cells were plated onto culture dishes and gradually transitioning cells to mTeSR1 medium in the next 7 days. iPSCs colonies were picked and transferred onto individual culture plates in about 2-3 weeks, and were maintained in a 37°C incubator with a 5% CO_2, 5% O_2, 90% N_2 environment.

[0208] Ten iPSCs clones at Passage 10 were tests for in vitro differentiation potential (data not shown), and two of them (designated WB20 and WB34) were selected for further characterization and the establishment of EnSC lines under GMP conditions.

[0209] Example 2. Generation of EnSCs from human iPSCs

[0210] This example illustrates the generation of EnSCs from human iPSCs (hiPSCs).

[0211] 2.1 Cell resource of human endoderm stem cells

[0212] Human endoderm stem cell lines were generated from patient-specific hiPSCs and maintained in serum-free and stromal-free conditions. The hiPSCs were generated from the experiment described in Example 1 above.

[0213] 2.2 Generation of EnSCs from hiPSCs

[0214] EnSC lines were established from patient-specific hiPSC lines WB20 and WB34 and maintained in a 37°C incubator with a 5% CO_2, 5% O_2, 90% N_2 environment. Endodermal cells were differentiated from hiPSCs by dual activation of Nodal and WNT signaling pathways with

Activin A (100 ng/mL) and CHIR99021 (2 μM) for 24 hours, and then cultured in the presence of bFGF (5 ng/mL), Activin A (100 ng/mL), VEGF (10 ng/mL), ascorbic acid (0.5 mM, Wako) and glutaMAX (2 mM, Invitrogen) for 4 days, followed by culturing in the presence of bFGF (10 ng/ml), TGF-alpha (20 ng/mL), VEGF (10 ng/mL), BMP4 (50 ng/mL), HGF (25 ng/mL), dexamethasone (40 ng/mL, Sigma) for 4 days. Thereafter EnSCs were established by replating the foregoing processed endodermal cells at $1x10^6$ cells per milliliter and maintaining in MCDB131 supplemented with Wnt3A (1 μM), Rspondin1 (50 ng/mL), EGF (20 ng/mL), A83-01 (0.5 mM), ascorbic acid (0.5 mM, Wako) and glutamine (2 mM, Corning).

[0215] EnSCs were harvested every 3-4 days and dissociated into single cells for subsequent expansion, subcloning or differentiation. Typically, EnSCs at passage 20 were selected for quality control tests including those of morphology, viability, purity, sterility, karyotype, as well as whole genome sequencing, using conventional techniques, such as flow cytometry and microscopic morphological examination.

[0216] As shown in FIGs. 1 and 10-11, the produced EnSCs were subject to quality control under the criteria shown in FIG. 10. Specifically, whole-genome sequencing (WGS) was performed on EnSC lines to confirm the absence of newly emerged cancer or diabetes-associated mutations that were not detected in the original harvested PBMCs (FIG. 1). EnSCs were further tested in immunocompromised mice (SCID Beige) for tumor-forming potential for 6 months and confirmed that the generated EnSCs did not form teratoma whereas human pluripotent stem cells (hPSC) exhibited 100% teratoma forming percentage (FIG. 9).

[0217] Collectively, the EnSCs produced using the methods described herein have the following characteristics: 1) having a typical epithelial morphology with clear cell boundary and a diameter of 3-5 um as detected by microscopy (e.g., phase contrast observation); 2) having at least 90% of the cells positive for FOXA1 as measured by flow cytometry; 3) having intact karyotype as assayed by karyotype test; 4) devoid of known cancer-related mutations and the lowest overall mutational burden compared to the original PBMC as detected by the whole genome sequencing; 5) having at least 90% live cells before frozen and at least 60% live cells after thaw as detected by flow cytometry; 6) having undetectable mycoplasma, sterility and negative for virus as assayed by the pathogen test; 7) with no teratoma found as assayed by the teratoma formation test. Detailed description of the quality control experiments and results thereof for EnSCs is provided in Example 4 below.

[0218] WB20 EnSC line was finally selected as a clinical grade clone as it was devoid of known cancer-related mutations and with the lowest overall mutational burden compared to the patient PBMCs.

[0219] Example 3. Generation of E-islets from EnSCs

[0220] This example relates to generation of E-islets from EnSCs through two intermediate stages under GMP conditions.

[0221] 3.1 Scalable differentiation of EnSCs into E-islets

[0222] EnSCs from Example 2 above were thawed and expanded with T225 flasks at a starting concentration of $2x10^6$/flask. Sterility was tested before the initiation of differentiation.

[0223] For the induction of pancreatic endoderm (1st stage), EnSCs were treated in MCDB (Gibco) with a cocktail containing LDN 193189 (Stemgent) (200 nM), Noggin (R&D) (20 ng/mL), ActivinA (R&D) (0.5 ng/mL), FGF10 (R&D) (20 ng/mL), Rspondin1 (R&D) (20 ng/mL), EGF (R&D) (20 ng/mL) and TPPB (Calbiochem) (500 nM) for 2 days; during day 2-4 of induction, cells were further differentiated in MCDB supplemented with LDN 193189 (Stemgent) (200 nM), FGF10 (20 ng/mL), EGF (20 ng/mL), SANT1 (Tocris) (0.5 μM), ascorbic acid (WAKO) (0.5 mM) and retinoic acid (Sigma) (2 μM); during day 4-6 of differentiation, cells were cultured in the presence of FGF10 (50 ng/mL), EGF (20 ng/mL), SANT1 (0.3 μM), retinoic acid (0.2 μM), Nicotinamide (Sigma) (10 mM) and ascorbic acid (0.5 mM).

[0224] For the formation of homogeneous cell clusters (2nd stage), pancreatic progenitor (PP) cells produced in the 1st stage were single cell dispersed and suspended in AggreWell (STEMCELL) to form homogeneous cell clusters for 3 days and then transferred to orbital shakers (90~110 rpm) for further islet tissue reconstruction and maturation.

[0225] For endocrine progenitor (EP) induction (3rd stage), triple inhibition of BMP, TGF-β and Notch signaling pathways was manipulated in the homogeneous cell clusters formed by the PP cells in the 2nd stage for 10 days in the presence of Noggin (20 ng/mL), A83-01 (Stemgent) (0.5 mM), gamma-secretase inhibitor XX (GSI-XX) (2 μ M, MERCK), retinoic acid (0.1 μM) and SANT1 (0.1 μM).

[0226] For the maturation of endocrine cells (4th stage, 8-10 days), T3 (Sigma) (1 μM), Nicotinamide (10 mM) and BMP4 (R&D) (2 ng/mL) were added to the 3rd stage recipe. MCDB was routinely supplemented with glucose (22.5mM, Sigma), sodium bicarbonate (Sigma) and ITS-

X (Invitrogen), GlutaMAX (Invitrogen) and ascorbic acid (0.5 mM, Wako). All cytokines were purchased from R&D Systems, with GMP grade if applicable.

[0227] Three batches of E-islets were generated through two intermediate (pancreatic progenitor / PP and endocrine progenitor / EP) stages under GMP conditions at scales that met the dose requirement for each patient (1.2×10^6 islet equivalents [IEQs] / patient).

[0228] 3.2 Characterization of PP cells, EP cells, and islets derived from EnSCs

[0229] The PP cells produced at the end of the 1st stage were evaluated for microorganism contamination, morphology, purity and viability, and were subject to quality control under the criterial shown in FIGs. 2 and 10. Collectively, the PP cells produced using the method described herein have the following characteristics: 1) having a typical epithelial morphology with unclear cell boundary and a diameter of no more than 3 um as detected by microscopy (e.g., phase contrast observation); 2) having at least 60% of the cells positive for PDX1 as measured by flow cytometry; 3) having at least 90% live cells as detected by flow cytometry; and 4) having undetectable mycoplasma, sterility, negative for virus and no more than 1 EU/mL Endotoxin as assayed by the pathogen test.

[0230] The EP cells produced at the end of the 3rd stage were evaluated for microorganism contamination, morphology, purity and viability and were subject to quality control under the criterial shown in FIGs. 2 and 10. Collectively, the EP cells produced using the method described herein have the following characteristics: 1) having a dense spherical cell mass with unclear cell boundary with an average diameter of about 150 um as detected by microscopy (e.g., phase contrast observation); 2) having at least 85% of the cells positive for PDX1 (i.e., the pancreatic lineage), at least 60% of the cells positive for CHGA+ (i.e., the endocrine lineage), at least 40% of cells positive for C-peptide (i.e., β cell), at least 20% of the cells positive for Glucagon (i.e., α cell), at least 15% of the cells positive for Somatostatin (i.e., δ cell), and less than 2% of the cells positive for AFP+ (i.e., untargeted hepatic lineage) as measured by flow cytometry; 3) having at least 90% live cells as measured by flow cytometry; 4) having at least 1 fold (e.g., 1.5 fold) changes of insulin or C-peptide as detected by static glucose stimulated insulin (C-peptide) secretion assay, 5) having undetectable mycoplasma, sterility, negative for virus and no more than 1 EU/mL Endotoxin as assayed by the pathogen test.

[0231] The E-islets produced using the method described herein were evaluated for microorganism contamination, morphology, purity and viability. E-islets were further analyzed for

endocrine cell composition (Insulin+NKX6-1+ β cells; Glucagon+ α cells; Somatostatin+ δ cells; Chromogranin A+ endocrine cells) by flow cytometry and single-cell transcriptomic analyses (scRNA-seq). The *in vitro* functionality of E-islets was assayed by glucose-stimulated insulin secretion (GSIS) as human cadaveric islets, and the *in vivo* functionality was evaluated by kidney capsule or hepatic portal transplantation into the streptozotocin-induced diabetic mouse or monkey models. The nontarget hepatocytes (HNF4A+Albumin+), cholangiocytes (SOX9+CK7+), intestinal epithelial cells (CDX2+) and the pancreatic ductal cells (SOX9+PTF1A+PDX1+) were estimated from the scRNA-seq data.

[0232] The E-islets produced at the end of the 3rd stage were evaluated for microorganism contamination, morphology, purity and viability and were subject to quality control under the criterial shown in FIGs. 3 and 10. Collectively, the EP cells produced using the method described herein have the following characteristics: 1) having a dense spherical cell mass with unclear cell boundary with an average diameter of about 150 um as detected by microscopy (e.g., phase contrast observation); 2) having at least 85% of the cells positive for PDX1 (i.e., the pancreatic lineage), at least 60% of the cells positive for CHGA+ (i.e., the endocrine lineage), at least 40% of cells positive for C-peptide (i.e., β cell), at least 20% of the cells positive for Glucagon (i.e., α cell), at least 15% of the cells positive for Somatostatin (i.e., δ cell), and less than 2% of the cells positive for AFP+ (i.e., untargeted hepatic lineage) as measured by flow cytometry; 3) having at least 90% live cells as measured by flow cytometry; 4) having at least 1.5 fold changes of insulin as detected by static glucose stimulated insulin (C-peptide) secretion assay, 5) having undetectable mycoplasma, sterility, negative for virus and no more than 1 EU/mL Endotoxin as assayed by the pathogen test.

[0233] Detailed description of the quality control experiments and results thereof for PP cells, EP cells and E-islets is provided in Example 4 below. In addition, functional analysis of E-islets produced using the method described herein is also provided in Example 4 below.

[0234] Example 4. Quality control for EnSCs, PP cells, EP cells and E-islets and functional analysis of E-islets

[0235] This example describes methods and results of quality control of EnSCs, PP cells, EP cells and E-islets produced using the methods described herein.

[0236] 4.1 Release criteria

[0237] Release criteria for EnSCs, PP cells, EP cells and E-islets produced using the methods described herein is summarized in FIG. 10.

[0238] 4.2 RNA extraction and quantitative real-time PCR

[0239] The reverse transcription and qRT-PCR reactions were performed as reported previously (Cheng et al., 2012). The RNAs was prepared with an RNA kit (TIANGEN) according to the manufacturer's directions and reverse-transcribed into cDNAs using random hexamers and oligo (dT) primers with GoScript Reverse Transcriptase (Promega). The qRT-PCR reactions were performed using an ABI Q6 (Life Technology) system and SYBR Green Master Mix (Roche). The expression levels were normalized to the housekeeping gene TBP. The primer information is provided in FIG. 11.

[0240] 4.3 Flow cytometry

[0241] Cell samples were collected as single cells. The staining of surface markers was performed in PBS (Gibco) with 0.2% BSA (Sigma). The cells were incubated with antibodies for 30 minutes on ice. For intracellular proteins, cells were fixed with 1.6% PFA (Servicebio) at 37 ℃ for 30 minutes and washed with the Permeabilization Wash buffer (BioLegend). The antibodies were incubated for 30 minutes at room temperature. Finally, cells were analyzed using a flow cytometer Celesta or Fortessa (BD). For cell viability test, calcein blue dye (Invitrogen) was used to mark live cell. Incubate cells and analyze using a flow cytometer Celesta or Fortessa (BD). See antibody information in FIG. 12.

[0242] 4.4 Immunofluorescence

[0243] E-islets were fixed with 4% PFA for 15 minutes at 4 ℃ and permeabilized with 0.5% Triton-100 (Sigma) before blocking. E-islets were washed three times with PBST (0.05% Tween 20 in PBS) for 10 minutes at room temperature (RT) both before and after each staining step, and blocked with 2% BSA at 4 ℃ for 2 hours. E-islets were stained with diluted primary antibodies at 4 ℃ overnight, and the samples were then incubated in the diluted secondary antibodies for 2 hours at 4 ℃. All antibodies were diluted in 2% BSA in PBS. Prolong Gold Antifade reagent with 4,6-diamino-2-phenyl indole (DAPI) (Invitrogen) was used to counterstain the nuclei. E-islets were analyzed using confocal fluorescence microscopes (Olympus FV3000). The images of E-islets were captured and 3D-projected using the Olympus software. The antibody information is listed in FIG. 12.

[0244] 4.5 *In vitro* static glucose stimulated insulin (C-peptide) secretion assay

[0245] Before glucose stimulation, E-islets prepared using the methods described in Example 3 above or primary islets provided and isolated by Shanghai Changzheng Hospital were rinsed and starved in Krebs- Ringer buffer supplemented with 2 mM glucose for 2 hours at 37 °C. For glucose stimulation, E-islets were treated alternately by Krebs-Ringer buffer with low (2 mM) or high (20 mM) glucose. Supernatants were collected after 30 minutes of each stimulation. C-peptide was measured by a human C-peptide ELISA kit (Mercodia, 10-1141-01) according to the manufacturer's instruction.

[0246] 4.6 Mycoplasma, sterility and endotoxin tests

[0247] Culture supernatant samples were sent to a certified laboratory, Shanghai Simple Gene Medical Laboratory, for testing.

[0248] 4.7 Karyotype analysis

[0249] EnSC samples were sent to a certified laboratory, Shanghai Simple Gene Medical Laboratory, for standard G-banded Chromosome analysis.

[0250] 4.8 Whole genome sequencing

[0251] *DNA preparation*

[0252] DNA degradation and contamination was monitored on 0.8% agarose gels. DNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). DNA concentration was measured using Qubit® DNA Assay Kit in Qubit® 3.0 Flurometer (Life Technologies, CA, USA).

[0253] *DNA Library preparation and sequencing*

[0254] The NEB Next® Ultra DNA Library Prep Kit for Illumina® (NEB, USA) was used to construct the libraries for sequencing as per the manufacturer's instructions. DNA was fragmented into ~200 base pair pieces. The end of the DNA fragment was subjected to an end repair process that included the addition of a single "A" base, followed by ligation of the adapters. Products were purified and enriched by polymerase chain reaction (PCR) to amplify the library DNA. The final libraries were quantified using KAPA Library Quantification kit (KAPA Biosystems, South Africa) and an Agilent 2100 Bioanalyzer. Paired-end sequencing (2 × 150 base pair) was performed on an Illumina NovaSeq 6000 sequencer (Illumina, USA).

[0255] 4.9 Single cell RNA sequencing and data processing

[0256] *Cell capture and cDNA synthesis*

[0257] E-islets produced in the method described in Examples 1-3 were dissociated into single cells with 0.25% trypsin and resuspended at 1×10^6 cells per milliliter in 1 × PBS. Using single cell 3' Library and Gel Bead Kit V3.1 (10x Genomics, 1000121) and Chromium Single Cell G Chip Kit (10x Genomics, 1000120), the cell suspension (300-600 living cells per microliter determined by Count Star) was loaded onto the Chromium single cell controller (10x Genomics) to generate single-cell gel beads in the emulsion according to the manufacturer's protocol. In short, single cells were suspended in PBS containing 0.04% BSA. About 10,000 cells were added to each channel, and the target cells recovered were estimated to be about 15,000 cells. Captured cells were lysed and the released RNA were barcoded through reverse transcription in individual GEMs. Reverse transcription was performed on a S1000TM Touch Thermal Cycler (Bio Rad) at 53°C for 45 min, followed by 85°C for 5 min, and hold at 4°C. The cDNA was generated and then amplified, and quality assessed using an Agilent 4200 (performed by CapitalBio Technology, Beijing).

[0258] *Single cell RNA-Seq library preparation and sequencing*

[0259] According to the manufacture's introduction, Single-cell RNA-seq libraries were constructed using Single Cell 3' Library and Gel Bead Kit V3.1. The libraries were finally sequenced using an Illumina Novaseq6000 sequencer with a sequencing depth of at least 30,000 reads per cell with pair-end 150 bp (PE150) reading strategy (performed by CapitalBio Technology, Beijing). 15,244 cells were sequenced and 2,721 cells with low UMI (UMI counts <5000) were excluded from sequenced cells during tSNE clustering analysis.

[0260] 4.10 Quality control results

[0261] The morphology, purity, viability and microorganism contamination of EnSC-derived pancreatic progenitor cells (PPs), endocrine progenitor cells (EPs) and E-islets were proven to meet the release criteria (FIGS. 1-3, and 10). E-islets displayed similar morphology (FIG. 3 (subpanel a)), endocrine cell composition (FIG. 3 (subpanels b and c), gene expression patterns (FIG. 3 (subpanels d-f)) and *in vitro* functionality (glucose-stimulated insulin secretion assay, GSIS) (FIG. 3 (subpanel g)) to human cadaveric islets, and showed functional efficacy in Streptozotocin (STZ)-induced diabetic mouse (FIG. 4 (subpanels b-d)) and monkey (FIG. 5) models. The nontarget hepatic or intestinal lineages, when examined either by scRNA-seq (FIG. 3 (subpanel f)) or by FACS (FIG. 3 (subpanel h)), were not detected. Neither tumor formation nor cystic/ ductal structures that indicate cell proliferation were detected in the immunocompromised

animals transplanted with either EnSCs or E-islets during the experiments (FIG. 9) as described in Examples 5-6 below.

[0262] Example 5. Transplantation of E-islets into Streptozodocin (STZ)-induced diabetic model mice

[0263] This example demonstrated that neither tumor formation nor cystic/ ductal structures that indicate cell proliferation were detected in STZ-induced diabetic model mice transplanted with either EnSCs or E-islets.

[0264] 5.1 Resource of rodent strains

[0265] SCID Beige mice were obtained from Shanghai Lingchang Biotech company. All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Shanghai Institute of Biochemistry and Cell Biology.

[0266] NCG-hIL15 mice were obtained from GemPharmatech Co., Ltd.

[0267] All animals were males and were housed in individually ventilated cages (IVC) in specific pathogen-free (SPF) animal facility with temperature and light controlled (12-h light/dark cycle).

[0268] 5.2 Teratoma formation test

[0269] SCID Beige (4~6 weeks) male mice were transplanted with 1×10^5 hiPSCs or 1×10^7 EnSCs intramuscularly or cervical subcutaneously. The formation of teratoma was monitored during the period of 6 months.

[0270] 5.3 Transplantation of E-islets into Streptozodocin (STZ)-induced diabetic model mice

[0271] STZ was dissolved immediately in 50 mM sodium citrate buffer (pH 4.5) to a final concentration of 20 mg/mL, and kept in dark and low temperature before injection. The administrations of STZ were completed within 5 minutes after the dissolution. SCID Beige male mice (4~6 weeks) were treated with 170 mg/kg STZ (Sigma-Aldrich, S0130) by intraperitoneal injection after 4 hours of starvation. The fasting blood glucose were measured at days 5 and 8, in a tail bleed using a hand-held blood glucose meter (Roche) to ensure hyperglycemia. E-islets (1000~2000 IEQ) were transplanted under left kidney capsules of diabetic mice. Glucose-stimulated human C-peptide secretion was measured by collecting mouse serum from the eye socket after 16 hours of fasting and at 25 minutes after glucose intraperitoneal injection (3 g/kg, 30% solution).

[0272] As shown in FIG. 9, neither tumor formation nor cystic/ ductal structures that indicate cell proliferation were detected in the immunocompromised STZ-induced diabetic model mice transplanted with either the EnSCs or E-islets produced using the methods provided herein during the experiments.

[0273] As shown in Fig. 4, the E-islets produced using the methods provided herein reverse hyperglycemia in immunocompromised (SCID Beige) mice with STZ induced diabetes in terms of blood glucose dynamics and human C-peptide secretion (subpanels b-d). This suggested that the E-islets produced using the methods provided herein could function as pancreatic islets.

[0274] Example 6. Transplantation of E-islets into STZ-induced diabetic monkey

[0275] This example demonstrated that neither tumor formation nor cystic/ ductal structures that indicate cell proliferation were detected in STZ-induced diabetic monkey transplanted with either EnSCs or E-islets.

[0276] 6.1 Resource of cynomolgus monkey model resource

[0277] Cynomolgus monkeys were obtained and housed in WUXI Biologics company. All animals were housed in a separate stainless-steel cage with temperature (18-26℃), relative humidity (40-70%) and light (12-h light/dark cycle) controlled. All animals provided with a continuous water supply and were fed a regular primate diet supplemented with fresh fruits twice daily (9-11 a.m. and 3-4 p.m.). All animal care and handling were performed in accordance with the guidelines established by IACUC at WUXI Biologics company.

[0278] 6.2 Induction of diabetes in monkey

[0279] To induce hyperglycemia, male Cynomolgus monkey (3~6 years) were fasted overnight and treated twice (with an interval of 2 weeks), with a dose of 50 mg/kg STZ intravenous injection. STZ was freshly dissolved before injection in sodium citrate buffer (pH 4.5) to final concentration of 25 mg/ml. The tail tip blood glucose was measured four times a day, before and 2 hours after feeding in the morning and afternoon. The dose of exogenous insulin treatment for animal was determined according to pre-prandial blood glucose.

[0280] 6.3 Immunosuppression strategy

[0281] The immunosuppression treatment was started 2 days before transplantation (day -2). Sirolimus (Pfizer) (0.5 mg, every day (quaque die (q.d.)), by mouth (per os (p.o.))) and mycophenolate mofetil dispersible tablet (Roche) (62.5 mg, twice a day (bis in die (b.i.d.)), p.o.)

were administered daily since day -2. Diclofenac sodium suppository (Hubei Qianjiang) (50 mg, by rectum (per rectus (p.r.))) was used 30 minutes before transplantation. ATG (Genzyme) (12.5 mg, intravenous (i.v.)) was injected 1 hour before and 48 hours after transplantation. Etanercept (Pfizer) (25 mg, hypodermic injection (i.h.)) was used 1 hour before transplantation and days 3 and 7 after transplantation.

[0282] 6.4 Transplant surgeries

[0283] The animal was fasted for at least 4 hours and anaesthetized with intramuscular injection of Zoletil®50 (VIRBAC) at 3-5 mg/kg. Heart rate, temperature, blood oxygenation and blood pressure were monitored in real time during the surgical procedure. E-islets (6000 or 30000 IEQ) were transplanted through B-ultrasound-mediated percutaneous hepatic portal vein injection. Antibiotic treatment was continued for 7 days post transplantation.

[0284] Two diabetic monkeys were transplanted with 6,000 (Monkey 1) or 30,000 (Monkey 2) E-islets, respectively. Monkey 1 was used to test the feasibility of hepatic portal injection of E-islets without DSA, while Monkey 2 was used for evaluating the short-term safety and effectiveness of E-islets.

[0285] As shown in FIG. 9, neither tumor formation nor cystic/ ductal structures that indicate cell proliferation were detected in the immunocompromised STZ-induced diabetic monkey transplanted with either EnSCs or E-islets during the experiments.

[0286] Example 7. Transplantation of E-islets into diabetic humanized mice

[0287] This example illustrates the functionality of the E-islets when transplanted into diabetic humanized mice.

[0288] 7.1 Generation of humanized mice by engraftment of human PBMCs

[0289] PBMCs of the same patient from Example 1 or an unrelated volunteer were isolated from whole blood using Ficoll gradient, respectively. NCG-hIL15 female mice (6 weeks) were treated with 250 cGy of radiation 4 hours before PBMCs infusion. Five million PBMCs were injected into lateral tail vein for each mouse. Efficiency of PBMC engraftment was evaluated by flow cytometry weekly following the injection, by proportion of mice CD45/human CD45 cells in mice blood. The percentage of human CD45 cells increased to > 40% within two weeks.

[0290] As shown in FIG. 6, the proportions of live cells (by SSC and FSC), human-derived blood cells (hCD45+) and mouse blood cells (mCD45+), among the three patient humanized mice and

the among the three volunteer humanized mice were comparable (subpanels a and b), indicating the successful generation of humanized mouse models.

[0291] As shown in FIG. 6, the E-islets grafts harvested under kidney capsule of the patient humanized mice included human β cells (C-peptide + and NKX6-1 +), α cells (Glucagon +), and δ cells (Somatostatin +) as demonstrated by the immunofluorescence staining of C- peptide (CPEP, red), Glucagon (GCG, green), Somatostatin (SST, violet) and NKX6-1 (Cyan), indicating the successful transplantation of E-islets into the humanized mouse models.

[0292] 7.2 Induction of diabetes, transplantation and evaluation

[0293] After identification of engraftment of human PBMCs, humanized mice were treated with 170 mg/kg STZ (Sigma-Aldrich, S0130) by intraperitoneal injection after 4 hours of starvation. One thousand E-islets generated from patient's EnSCs using the method described in Example 3 were transplanted under the left kidney capsule of 1) NCG-hIL15 mice humanized with the patient's PBMCs, or 2) NCG-hIL15 mice humanized with PBMCs from an unrelated volunteer. Fasting blood glucose were measured every two days. Glucose-stimulated human C-peptide secretion was performed as described above. Animals were sacrificed at 28 days post transplantation and were examined for graft survival by immunofluorescence of islet markers (C-peptide, Glucagon, PDX1, NKX6-1) in tissue sections.

[0294] As shown in FIG. 4, the patient-specific E-islets survived and function under the kidney capsules of the diabetic immunocompromised mice humanized with patient's own PBMCs, but rejected by the ones humanized with PBMCs from an unrelated volunteer (subpanels e-g), which suggests that the autologous E-islets are likely tolerated by patient's immune system.

[0295] As shown in FIG. 4, fasting blood glucose levels of STZ-induced diabetic humanized mice transplanted with the patient E-islets were significantly lower than that of the diabetic mice humanized with the volunteer's PBMCs transplanted with the patient E-islets (subpanel f), which suggests that the autologous E-islets could significantly reduce fasting blood glucose levels in a diabetic subject.

[0296] As shown in FIG. 4, the secretion of human C-peptide in STZ-induced diabetic humanized mice transplanted with the patient E-islets were significantly higher than that in the diabetic mice humanized with the volunteer's PBMCs transplanted with the patient E-islets both after fasting and 30 minutes following an intraperitoneal injection (i.p.) glucose bolus on days 7 and 14 post E-

islet transplantation (subpanel g). This suggests that the autologous E-islets could function as pancreatic islets to secret insulin.

[0297] Example 8. Clinical studies

[0298] This example relates to clinical studies of E-islets differentiated *in vitro* from autologous EnSCs in a T2D patient who had impaired insulin secretion.

[0299] 8.1 Patient information

[0300] The patient was a 59-year-old man with a 25-year history of T2D who developed end-stage diabetic nephropathy and underwent kidney transplantation in June of 2017. His estimated glomerular filtration rate (eGFR) and serum creatinine (SCr) level were maintained at 90 to 105 ml/(min·1.73 cm^2) and 45-72 μmol/L, respectively, indicating good survival and functioning of the donor organ. He had been receiving anti-rejection drugs (tacrolimus (Astellas) 1 mg b.i.d. and mycophenolate mofetil (Roche) 0.5 mg b.i.d.) and subcutaneous insulin injection at a dose of 20 U once daily at bedtime and oral antidiabetic medications (acarbose (Bayer) 50 mg three times a day (t.i.d.) and metformin (Merck) 0.75 g b.i.d.) (FIG. 7 (subpanel a)). However, he reported poor glycemic control since November 2019, characterized by an average blood glucose level of 7.8 ± 2 mmol/L (measured by continuous glucose monitoring system / CGMS, ranging between 3.66-14.60 mmol/L, Mean Amplitude of Glycemic Excursions / MAGE of 5.54 mmol/L), the time-in-the-range (TIR, 3.9-10.0 mM) of 87.7% and the time-in-the-tight-target-range (TITR, 3.9-7.8 mM) of 56.7%, with daily hyperglycemic events (> 10.0 mmol/L) of 0.7/d and hypoglycemic events (< 3.9 mmol/L) of 0.3/d (FIG. 15). Due to the major concerns of hypoglycemia induced by insulin administration, the adverse effect of antirejection drugs on glycemic control, and the detrimental effect of poor glycemic control on the long-term survival of the donor kidney, the patient and the study team agreed to pursue transplantation with autologous E-islets.

[0301] 8.2 E-islet Transplantation

[0302] FIG. 4 (subpanel a) shows the process from obtaining the PBMCs from the patient to finally transplanting the E-islets into the patient.

[0303] Conforming to the regulatory guidance from the clinical islet transplantation registration (CITR), the patient had an image-guided percutaneous transhepatic islet infusion with a local anesthetic, into the main portal circulation with heparinization. Patency of the main portal vein was assessed by monitoring portal pressure during infusion of islets, and doppler ultrasonography

after the infusion. A total of 1.2 million IEQs of E-islets that were generated as a single batch and passed the release criteria were directly delivered without prior cryopreservation. Portal venography and portal vein pressure were monitored throughout the whole procedure to ensure that there was no portal embolization or portal hypertension. No glucocorticoids were used at any time. After the surgery, the patient was monitored overnight and was allowed out of bed the following day. Patient compliance with scheduled appointments was 100% (some visits were either cancelled or relocated to local hospitals due to the COVID-19 pandemic).

[0304] *Dosage Design*

[0305] The rationale behind the dosage of 1.2 million IEQ units for E-islet transplantation of this T2D patient is based on the following facts: 1) there are approximately 4~6 million IEQs of islets in a healthy person, and it is estimated that only 1/3 of the islets function upon glucose stimulation under physiological condition, which means ~1.5 million IEQs of islets might be enough for glycemic control. This is confirmed by the clinical observation that transplantation of 800,000 IEQs of cadaveric islets would normally lead to independency of exogenous insulin in most T1D patients (see reference s below); 2) the patient's endogenous beta cell mass was significantly diminished (estimated at 50% reduction at least), judging from the pre and the post- prandial c-peptide levels; and 3) a significant number of E-islets are likely lost during the vascularization process, as E-islets contain only pancreatic endodermal cells but not vascular endothelial cells that are important for the post-transplantation revascularization of cadaveric islets. We chose the dosage of 1.2 million IEQs, as we speculated that 50% of E-islets might be lost during the engraftment, and that the residual 0.6 million IEQs might be enough to supplement the endogenous islet function.

[0306] 8.3 Mixed Meal Tolerance Test (MMTT)

[0307] After overnight ($\geq$10 hours) fasting, at 7:30 AM, the patient was asked to consume within 5 minutes a standard mixed meal including 200 g steamed buns and 50 mL water at a constant speed. Blood samples were collected before (0 min) and at 15, 30, 60, 120, 180 and 240 minutes after ingesting. Degludec was not administered 24 hours before MMTT, and oral antidiabetic medications were not administrated 20 hours prior to MMTT.

[0308] 8.4 Assessments of clinical outcomes

[0309] At designated visits, the patient was weighed and reported his Clark hypoglycemia awareness score, and was subjected to routine and disease-specific assessments. Examinations of

endocrine function and diabetes-specific parameters by mixed-meal tolerance tests (MMTT) were performed at baseline and at 4, 8, 12, 16, 20, and 24 weeks and thereafter every 12 weeks (FIG. 7 (subpanel a)). The glycemic control of the patient was measured with a 24-hour real-time blood glucose monitoring system (Medtronic GuardianTM Connect Subcutaneous Continuous Glucose Monitoring System/CGMS). All information from the CGMS device was centrally assessed. The baseline and follow-up CGM glucose values were measured throughout the first 52 weeks, and the mean duration of CGM device wearing was at least 3 days.

[0310] *Safety Monitoring*

[0311] Safety endpoints included treatment-emergent adverse events (TEAEs), early discontinuation of treatment due to adverse events and adjudicated adverse events. The tumor formation was monitored every three months by enhanced magnetic resonance imaging performed on upper abdomen and by measurements of serum cancer-related antigens.

[0312] The three major clinical outcomes (i.e. the glycemic targets, the reduction of exogenous insulin and the levels of fasting and meal-stimulated circulating C-peptide/insulin) were monitored throughout the first 116 week (a list of follow-up assessment results is provided in FIGs. 13 and 14). Marked changes in patient's glycemic control were observed as early as week 2 post-transplantation, as the MAGE declined from 5.50 mmol/L to 3.60 mmol/L, and remarkably, the TITR increased rapidly from 56.7% to 77.8% (FIGs. 4 (subpanel h) and 16). Over the same period, the time-above-range (TAR) decreased by 55% from baseline, while the events of severe hyperglycemia (> 13.9 mM) and hypoglycemia (<3.9 mM) completely disappeared (FIGs. 8 (subpanels a-b) and 15).

[0313] During the period between week 4 and 12, a significant reduction in ambulatory mean glucose fluctuations (from 5.50 (baseline) to 2.6 mmol/L) (FIG. 15) and the steady rise in TITR (from 81% to 90%) were observed (FIGs. 4 (subpanel h), 8 (subpanels c-e) and 15). After the 32nd week, the patient's TITR had readily reached 99% and was maintained thereafter (FIGs. 4 (subpanels h and I) and 15), while prandial glucose excursion / MAGE, the gold standard of blood glucose variability, was reduced from 5.50 mM (baseline) to 1.60 mM (FIGs. 7 (subpanel d), 8 (subpanels h-l) and 15). Importantly, no episodes of hypoglycemia or severe hyperglycemia were observed during the whole follow-up period of 116 weeks post-surgery (FIGs. 8 and 15).

[0314] Additionally, MMTT revealed a trend of stabilization in glycemic variability after surgery, as manifested by the stable fasting glucose concentrations and the significant reductions in the

post-meal glucose concentrations (maximum of 21.3 mM at baseline vs. maximum of 9.1 mM at week 105) (FIGs. 4 (subpanel j) and 14). Consistently, the area under the curve (AUC) derived from the 5-point intravenous glucose values decreased to 40% of baseline (FIG. 7 (subpanel b)), which was further confirmed by the AUCs of the values acquired from the continuous glucose monitoring system (CGM) (FIG. 7 (subpanel c)). The hemoglobin A1c levels decreased from 6.6% (baseline) to 5.5% (week 85) and 4.6% (week 113) (FIGs. 4 (subpanel h) and 16).

[0315] Notably, the insulin requirements were reduced gradually until complete withdrawal at the end of week 11 (FIG. 4 (subpanel h)), and the oral antidiabetic medications were tapered since week 44 and discontinued at weeks 48 (Acarbose) and 56 (Metformin) (FIG. 7 (subpanel a)).

[0316] The average post-surgery fasting C-peptide level (0.68 nmol/L) increased by 3-fold when compared to that of pre-surgery (FIG. 4 (subpanel k) and 15). Notably, the secretions of C-peptide (FIG. 4 (subpanel k)) and insulin (FIG. 4 (subpanel l) measured by MMTT revealed significant elevations compared to those of the pre-surgery tests, confirmed by the AUCs (FIG. 7 (subpanel b)).

[0317] During the 116-week follow-up period, no tumor formation was detected either by MRI on upper abdomen or by the measurements of serum tumor-related antigen markers. The treatment emergent adverse events (TEAEs) included: 1) temporary abdominal distension and loss of appetite within 4 to 8 weeks, relieved with methionyl-trichloride; 2) restorable weight loss < 5% (80 kg to 76 kg).

[0318] The data of the first 116 weeks revealed significant improvements in both glycemic control and islet function. The grafts were well tolerated with no tumor formation or severe graft-related adverse events. These data indicates that stem cell-derived islet tissues can rescue islet function in late-stage T2D patients.

[0319] Although the present disclosure has been particularly shown and described with reference to specific embodiments, it will be understood by those skilled in the art that various changes with respect to the form and details can be made without departing from the spirit and scope of the present disclosure as disclosed herein.

Claims

1. A method for producing a population of endoderm stem cells, comprising:

a) providing a population of pluripotent stem cells;

b) culturing the population of pluripotent stem cells in a first medium comprising a Nodal signaling agonist and a WNT signaling agonist;

c) culturing the cells in a second medium comprising a Nodal signaling agonist and a fibroblast growth factor (FGF);

d) culturing the cells in a third medium comprising a factor belonging to the TGF-beta superfamily, FGF, a hepatocyte growth factor (HGF) and VEGF; and

e) culturing the cells in a fourth medium comprising a WNT signaling agonist, a TGF-beta inhibitor and an epidermal growth factor (EGF);

thereby producing a population of endoderm stem cells.

2. The method of claim 1, wherein the fourth medium does not comprise FGF2 and/or Chir99021.

3. The method of any one of the preceding claims, wherein the Nodal signaling agonist is selected from the group consisting of Activin A, Nodal and GDF-8.

4. The method of claim 3, wherein the Nodal signaling agonist is Activin A.

5. The method of any one of the preceding claims, wherein the WNT signaling agonist is selected from the group consisting of: CHIR99021, Wnt3A, Wnt3a-AFM, R-Spondin-1 and BIO (6-bromoindirubin-3'-oxime), SKL2001, BML-284, CP21R7 and SB 216763.

6. The method of claim 5, wherein the WNT signaling agonist is CHIR99021 or Wnt3A.

7. The method of any one of the preceding claims, wherein the FGF is selected from the group consisting of: basic FGF (bFGF), FGF4 and chimeric fibroblast growth factor (FGFC), FGF1, FGF10 and FGF7.

8. The method of claim 7, wherein the FGF is bFGF.

9. The method of any one of the preceding claims, wherein the factor belonging to the TGF-beta superfamily is selected from the group consisting of BMP4, BMP2 and BMP7.

10. The method of claim 9, wherein the factor belonging to the TGF-beta superfamily is BMP4.

11. The method of any one of the preceding claims, wherein the TGF-beta inhibitor is selected from the group consisting of A83-01, SB431542, ALK5 inhibitor, LDN-193189, Galunisertib

(LY2157299), LY2109761, SB525334, SB505124, GW788388, LY364947, RepSox, LDN-193189, K02288, LDN-214117, SD-208, Vactosertib (TEW-7197), ML347, LDN-212854, DMH1, Pirfenidone, Alantolactone, SIS3 and Hesperetin and Dorsomorphin.

12. The method of claim 11, wherein the TGF-beta inhibitor is A83-01.

13. The method of any one of the preceding claims, wherein the pluripotent stem cell is selected from embryonic stem cells (ESC) and induced pluripotent stem cell.

14. The method of any one of the preceding claims, comprising:

a) providing a population of induced pluripotent stem cells;

b) culturing the cells in a first medium comprising Activin A and CHIR99021;

c) culturing the cells in a second medium comprising Activin A and bFGF;

d) culturing the cells in a third medium comprising BMP4, bFGF, HGF and VEGF; and

e) culturing the cells in a fourth medium comprising a Wnt3A, A83-01 and EGF; thereby producing a population of endoderm stem cells.

15. The method of any one of the preceding claims, wherein the second medium further comprises VEGF, ascorbic acid, and/or glutaMAX.

16. The method of any one of the preceding claims, wherein the third medium further comprises TGF-alpha and/or dexamethasone.

17. The method of any one of the preceding claims, wherein the fourth medium further comprises Rspondin1, ascorbic acid, and/or glutamine.

18. The method of any one of the preceding claims, wherein the culturing period of step b is 1-2 days (preferably, 1 day).

19. The method of any one of the preceding claims, wherein the culturing period of step c is 2-6 days (preferably, 4 days).

20. The method of any one of the preceding claims, wherein the culturing period of step d is 2-6 days (preferably, 4 days).

21. The method of any one of the preceding claims, wherein steps b-d are performed under less than 10% O_2 (preferably, less than 8%, less than 6%, less than 4% or less than 2%; more preferably 5%).

22. The method of any one of the preceding claims, wherein at least 80% (e.g., at least 85%, at least 90%, at least 95%, at least 98% or at least 99%) of the population of endoderm stem cells express SOX17, CDX2, SOX9, GATA6 and/or FOXA1.

23. The method of any one of the preceding claims, wherein the first medium, second medium, third medium and fourth medium independently contain a medium selected from the group consisting of mTeSR1, TeSR-AOF, Essential 8, NutriStem hPSC XF Medium (Sartorius), RPMI/B27, SFD-based, MCDB131, DMEM/F12 medium, StemPro34-SFM, , RPMI1640, IMDM, DMEM, Ham's F12 and CMRL1066.

24. The method of any one of the preceding claims, wherein the 1^{st}~4^{th} media are serum-free and/or stromal-free.

25. A population of endoderm stem cells, produced according to the method of any one of the preceding claims.

26. A method for producing a population of pancreatic endocrine cells, comprising:

a) providing a population of endoderm stem cells;

b) culturing the population of endoderm stem cells in the presence of a BMP inhibitor, Nodal signaling agonist, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and Nicotinamide to produce a population of pancreatic progenitor (PP) cells;

c) culturing the population of PP cells in the presence of a BMP inhibitor, a TGF-beta inhibitor and a gamma-secretase inhibitor to produce a population of endocrine progenitor (EP) cells; and

d) culturing the population of EP cells in the presence of T3 and Nicotinamide in addition to the factors involved in step c;

thereby producing a population of pancreatic endocrine cells.

27. The method of claim 26, wherein the population of endoderm stem cells comprises the population of endoderm stem cells of claim 25.

28. The method of claim 26 or 27, wherein the BMP inhibitor is selected from the group consisting of Noggin, dorsomorphin and LDN-193189.

29. The method of claim 28, wherein the BMP inhibitor comprises Noggin.

30. The method of any one of claims 26-29, wherein the TGF-beta inhibitor is selected from the group consisting of A83-01, SB431542, ALK5 inhibitor, LDN-193189, Galunisertib (LY2157299), LY2109761, SB525334, SB505124, GW788388, LY364947, RepSox, LDN-

193189, K02288, LDN-214117, SD-208, Vactosertib (TEW-7197), ML347, LDN-212854, DMH1, Pirfenidone, Alantolactone, SIS3 and Hesperetin and Dorsomorphin.

31. The method of claim 30, wherein the TGF-beta inhibitor comprises A83-01.

32. The method of any one of claims 26-31, wherein the gamma-secretase inhibitor is selected from the group consisting of Compound E, GSI-XX and GSI-XXI, DAPT, LY-411575, RO4929097 and Dibenzazepine (DBZ).

33. The method of any one of claims 26-32, wherein the Nodal signaling agonist is selected from the group consisting of Activin A, Nodal and GDF-8.

34. The method of claim 33, wherein the Nodal signaling agonist is Activin A.

35. The method of any one of claims 26-34, comprising:

a) providing a population of endoderm stem cells;

b) culturing the population of endoderm stem cells in the presence of a Noggin, Activin A, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and Nicotinamide to produce a population of pancreatic progenitor (PP) cells;

c) culturing a population of PP cells in the presence of a Noggin, A83-01 and GSI-XX to produce a population of endocrine progenitor (EP) cells; and

d) culturing the population of EP cells in the presence of T3 and Nicotinamide;

thereby producing a population of pancreatic endocrine cells.

36. The method of any one of claims 26-35, wherein the step b comprises culturing the population of endoderm stem cells in the presence of a Noggin, Activin A, FGF10, EGF, SANT1, retinoic acid, ascorbic acid, Nicotinamide and at least one of Rspondin1, LDN-193189 and TPPB to produce a population of pancreatic progenitor (PP) cells.

37. The method of any one of claims 26-36, wherein the step c comprises culturing a population of PP cells in the presence of a Noggin, A83-01, GSI-XX and at least one of retinoic acid and SANT1 to produce a population of endocrine progenitor (EP) cells.

38. The method of any one of claims 26-37, wherein the step d comprises culturing the population of EP cells in the presence of T3, Nicotinamide and BMP4.

39. The method of any one of claims 26-38, wherein the culturing period of step b is 2-6 days.

40. The method of any one of claims 26-39, wherein the culturing period of step c is 3-8 days.

41. The method of any one of claims 26-40, wherein the culturing period of step d is 7-21 days.

42. The method of any one of claims 26-41, wherein the steps b-d are independently performed in a two-dimensional (2D) or three-dimensional (3D) environment.

43. A population of pancreatic endocrine cells, produced according to the method of any one of claims 26-42.

44. A pharmaceutical composition comprising the population of pancreatic endocrine cells of any one of claims 26-43, and a pharmaceutically acceptable medium.

45. A method of treating a disease or condition associated with impaired pancreatic islet function in a subject in need thereof, comprising:

administering an effective amount of pancreatic endocrine cells derived from a population of endoderm stem cells (i.e., E-islets) to the subject,

thereby treating the disease or condition associated with impaired pancreatic islet function in the subject in need thereof.

46. The method of claim 45, wherein the population of endoderm stem cells are produced according to the method of any one of claims 1-25.

47. The method of claim 46, wherein the population of pluripotent stem cells are autologous or allogeneic.

48. The method of any one of claims 45-47, wherein the administration comprises hepatic portal infusion, kidney capsule transplantation, rectus abdominis injection, subcutaneous implantation, mesentery injection, retroperitoneal injection, hepatic artery injection or iliac fossa injection.

49. The method of any one of claims 45-48, wherein the subject is human.

50. The method of any one of claims 45-48, wherein the effective amount is about 0.5-3.0 million Islet equivalent (IEQ) units.

51. The method of any one of claims 45-50, wherein the disease or condition associated with impaired pancreatic islet function is diabetes, such as type I diabetes (T1D), type II diabetes (T2D) Type 3c diabetes, or Maturity-onset diabetes of the young (MODY).

52. Use of a population of endoderm stem cells in the manufacture of E-islets for treating a disease or condition associated with impaired pancreatic islet function in a subject in need thereof.

53. The use of claim 52, wherein the manufacture comprises the steps defined in any one of claims 26-42.

54. A kit for producing a population of endoderm stem cells from a population of pluripotent stem cells, wherein the kit comprises a first set of factors, a second set of factors, a third set of factors and a fourth set of factors, wherein

the first set of factors comprises a Nodal signaling agonist and a WNT signaling agonist,

the second set of factors comprises a Nodal signaling agonist and a fibroblast growth factor (FGF),

the third set of factors comprises a factor belonging to the TGF-beta superfamily, FGF, a hepatocyte growth factor (HGF) and VEGF, and

the fourth set of factors comprises a WNT signaling agonist, a TGF-beta inhibitor and an epidermal growth factor (EGF).

55. The kit of claim 54, wherein the fourth set of factors does not comprise FGF2 and/or Chir99021.

56. A kit for producing a population of pancreatic endocrine cells from a population of endoderm stem cells, wherein the kit comprises a fifth set of factors, a sixth set of factors and a seventh set of factors, wherein

the fifth set of factors comprises a BMP inhibitor, Nodal signaling agonist, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and/or Nicotinamide,

the sixth set of factors comprises a BMP inhibitor, a TGF-beta inhibitor and/or a gamma-secretase inhibitor, and

the seventh set of factors comprises T3 and/or Nicotinamide.

57. A kit for producing a population of pancreatic endocrine cells from a population of pluripotent stem cells, wherein the kit comprises first to seventh sets of factors, wherein

the first set of factors comprises a Nodal signaling agonist and a WNT signaling agonist,

the second set of factors comprises a Nodal signaling agonist and a fibroblast growth factor (FGF),

the third set of factors comprises a factor belonging to the TGF-beta superfamily, FGF, a hepatocyte growth factor (HGF) and VEGF,

the fourth set of factors comprises a WNT signaling agonist, a TGF-beta inhibitor and an epidermal growth factor (EGF),

the fifth set of factors comprises a BMP inhibitor, Nodal signaling agonist, FGF10, EGF, SANT1, retinoic acid, ascorbic acid and Nicotinamide,

the sixth set of factors comprises a BMP inhibitor, a TGF-beta inhibitor and a gamma-secretase inhibitor, and

the seventh set of factors comprises T3 and Nicotinamide.

58. The kit of claim 57, wherein the fourth set of factors does not comprise FGF2 and/or Chir99021.

59. A kit for producing a population of pancreatic endocrine cells from a population of PP cells, wherein the kit comprises a set of factors comprising a BMP inhibitor, a TGF-beta inhibitor and a gamma-secretase inhibitor.