

THERAPEUTIC CELLS AND METHODS RELATING TO THE SAME
RELATED APPLICATION

[0001] This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application 63/443,261 filed February 3, 2023, the entire contents of which are incorporated herein by reference.

FIELD

[0002] The present disclosure relates generally to the field of cell biology, stem cells, cellular differentiation, and cell-based therapies.

BACKGROUND

[0003] The following discussion is provided to aid the reader in understanding the disclosure and is not admitted to describe or constitute prior art thereto.

[0004] Diabetes mellitus (*i.e.*, diabetes) is a disease in which the body's ability to produce or respond to the hormone insulin is impaired, resulting in abnormal metabolism of carbohydrates and elevated levels of glucose in the blood and urine. The disease is subdivided into several subtypes, described alternatively as Type 1 diabetes mellitus, insulin-dependent diabetes mellitus (IDDM), mature onset diabetes of the young (MODY), latent adult diabetes (LADA), brittle diabetes, lean diabetes, Type 1.5, Type 2, Type 3, obesity-related diabetes, gestational diabetes, and other nomenclature accepted by the field.

[0005] Insulin-dependent diabetes patients can potentially be cured by transplant of new hormone-secreting islet cells, but this approach has been limited to date because these cells are hard to obtain in sufficient quantity, quality, and purity. *See e.g.*, Rickels MR, Roberts, RP. *Endocrine Reviews*, 40(2): 631–668 (2019). It has, therefore, long been a goal of biomedical research to generate hormone-secreting islet cells from human stem cells in an efficient and consistent manner. *See e.g.*, Pagliuca FW, *et al.* *Cell*, 154(2): 428-439 (2014). To achieve the goal of a diabetes cure, a cell product must be generated that meets the criteria of: (1) being able to establish a functional graft of therapeutically relevant hormone-secreting islet cells in a patient, and; (2) not harming the

patient through unregulated hormone secretion, neoplasms, or other adverse effects. To date, cells that meet these criteria have not been demonstrated clinically.

[0006] The vast majority of established protocols for generating hormone-secreting islet cells from human stem cells focus on generating the largest population of cells that express markers of mature insulin-secreting beta cells: Pdx1, Nkx6.1, and C-peptide (a surrogate marker of insulin secretion) *See e.g.* Veres A, *et al.* *Nature*, 569(7756): 368-373 (2019), Sharon N, *et al.* *Cell* 176, 1–15 (2019), Hogrebe NJ, *et al.* *Nature Protocols*, 16(9): 4109-4143 (2021). These protocols yield highly variable cell populations that include non-pancreatic cells, actively dividing cells and cells with pancreatic hallmarks but no known native equivalents. This impurity detracts from the potential efficacy of cell-based therapy to treat diabetes by having decreased potency compared to native islets, and also introduces the risk of tumorigenicity. Additionally, low reproducibility between cell batches directly affects the cost of producing the cells and limits the translation of this protocol to the clinic.

[0007] Thus, there remains a need for improved and predictable methods of generating therapeutic, hormone-producing cells for the treatment of diabetes. This disclosure fulfills the needs for a more pure, potent, and safe islet replacement therapy for diabetes.

SUMMARY

[0008] Described herein are cells and cellular compositions that produce pancreatic hormones and may be used to treat diabetes, as well as methods of making and identifying the same.

[0009] In one aspect, the present disclosure provides an isolated synthetic replacement endocrine (SRE) cell, that expresses transcription factors Pdx1 and ISL1, but does not express the transcription factor Nkx6.1. In another aspect, the present disclosure provides an isolated synthetic replacement endocrine (SRE) cell, that expresses transcription factors MAFA and ISL1, but does not express the transcription factor Nkx6.1.

[0010] In some embodiments of the two aspects, the isolated SRE cell is differentiated from a stem cell. In some embodiments, the stem cell is selected from an embryonic stem cell, an induced pluripotent stem cell, and a multipotent reprogrammed stem cell. In some embodiments of the two

aspects, the stem cell is derived from a cell line. In some embodiments of these two aspects, the stem cell is a multipotent reprogrammed stem cell. In some embodiments of the two aspects, the multipotent reprogrammed stem cell was obtained by reprogramming a pancreatic cell. In some embodiments of these two aspects, the multipotent reprogrammed stem cell was reprogrammed with an expression plasmid encoding (i) Oct4, Sox2, Klf4, and L-Myc; (ii) Oct4, Sox2, Klf4, and C-Myc; (iii) LIN28, Oct4, Sox2, and Nanog; or (iv) Gils1, Oct3/4, Sox2, and Klf4. In some embodiments of these aspects, the stem cell is genetically modified to avoid targeting by lymphocytes. In some embodiments of the two aspects, the stem cell is genetically modified to lack expression of MHC I and MHC II. In some embodiments of these aspects, the stem cell is genetically modified to avoid targeting by natural killer cells. In some embodiments of these two aspects, the stem cell genetically modified to express CD47. In some embodiments of the two aspects, the multipotent reprogrammed stem cell can differentiate into endoderm or ectoderm cell types, but not mesoderm cell types. In some embodiments of these aspects, the multipotent reprogrammed stem cell does not comprise any reprogramming genes incorporated into its genome.

[0011] In some embodiments of these aspects, the isolated SRE cell expresses C-peptide. In some embodiments of these aspects, C-peptide is expressed at levels equivalent to mature, native pancreatic islet cells. In some embodiments of these two aspects, C-peptide is expressed at levels higher than an SRE cell that expresses Nkx6.1. In some embodiments of these aspects, C-peptide expression is responsive to fluctuations in glucose concentration.

[0012] In some embodiments of the two aspects, the isolated SRE cell secretes insulin. In some embodiments, the isolated SRE cell secretes glucagon.

[0013] In some embodiments of these aspects, the isolated SRE cell is human.

[0014] In another aspect, the present disclosure provides pharmaceutical compositions comprising at least one isolated SRE cell disclosed herein and a therapeutically acceptable carrier.

[0015] In another aspect, the present disclosure provides a method of treating diabetes, comprising administering to a subject with diabetes at least one isolated SRE cell disclosed herein (e.g., a cell

of any one of the foregoing aspects or embodiments) or the pharmaceutical composition comprising the same.

[0016] In some embodiments, the diabetes is type 1.

[0017] In some embodiments, Nkx6.1 is expressed after the cell or pharmaceutical composition is administered to the subject.

[0018] In some embodiments, administration comprises implanting the isolated cell or pharmaceutical composition in the subject.

[0019] In some embodiments, the subject is human.

[0020] In another aspect, the present disclosure provides a method of preparing an isolated SRE cell, comprising: contacting an undifferentiated stem cell with a combination of growth factors to drive differentiation of the stem cell to an endocrine lineage, wherein the combination of growth factors comprises retinoic acid, a Hedgehog antagonist, and a bone morphogenetic protein (BMP) signaling inhibitor.

[0021] In some embodiments, the Hedgehog antagonist is selected from SANT1 and cyclopamine.

[0022] In some embodiments, the BMP signaling inhibitor is LDN193189.

[0023] In some embodiments, the stem cell is not exposed to tri-iodothyronine (T3) or analog thereof during the differentiation process.

[0024] In some embodiments, the stem cell is not exposed to a protein kinase C activator during the differentiation process.

[0025] In some embodiments, the stem cell is not exposed to nicotinamide, a form of vitamin B3 during the differentiation process.

[0026] In some embodiments, the stem cell is not exposed to transforming growth factor beta 1 (TGFbeta1) protein.

[0027] In some embodiments, the stem cell is not exposed to insulin-like growth factor 1 (IGF-1).

[0028] In some embodiments, the stem cell is derived from a cell line.

[0029] In some embodiments, the stem cell is selected from an embryonic stem cell, an induced pluripotent stem cell, and a multipotent reprogrammed stem cell.

[0030] In some embodiments, the stem cell is a multipotent reprogrammed stem cell. In some embodiments, the multipotent reprogrammed stem cell was obtained by reprogramming a pancreatic cell. In some embodiments, the multipotent reprogrammed stem cell was reprogrammed with an expression plasmid encoding (i) Oct4, Sox2, Klf4, and L-Myc; (ii) Oct4, Sox2, Klf4, and C-Myc; (iii) LIN28, Oct4, Sox2, and Nanog; or (iv) Gils1, Oct3/4, Sox2, and Klf4. In some embodiments, the multipotent reprogrammed stem cell can differentiate into endoderm or ectoderm cell types, but not mesoderm cell types. In some embodiments, the multipotent reprogrammed stem cell does not comprise any reprogramming genes incorporated into its genome. In some embodiments, the stem cell is genetically modified to avoid targeting by lymphocytes. In some embodiments, the stem cell is genetically modified to lack expression of MHC I and MHC II. In some embodiments, the stem cell is genetically modified to avoid targeting by natural killer cells. In some embodiments, the stem cell is genetically modified to express CD47.

[0031] In another aspect, the present disclosure provides an isolated SRE cell obtained by the methods of differentiation disclosed here (e.g., the immediately preceding aspect and embodiments). In some embodiments, the cell expresses transcription factors Pdx1 and ISL1, and does not express the transcription factor Nkx6.1.

[0032] In another aspect, the present disclosure provides a population of the foregoing SRE cells. In some embodiments, the population may be substantially pure, meaning it includes at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 99%, or 100% SRE cells. In some embodiments, the SRE cells may be formulated in with one or more therapeutically acceptable carriers.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] FIG. 1 shows flow cytometry analysis of SRE clusters. SRE clusters are comprised of cells that express predominantly Pdx1, ISL1, and insulin. A smaller proportion of cells express glucagon. In contrast Nkx6.1 expressing cells are almost undetectable.

[0034] FIG. 2 shows immunofluorescent images of SRE clusters. Fluorescent staining revealed that ISL1 is expressed in the nuclei of cells that express C-peptide (A). Nkx6.1-expressing cells are nearly absent (B), but cells that do express Nkx6.1 do not co-express ISL-1 (C). ISL1 is expressed in the nuclei of cells that express the endocrine pancreas marker chromogranin A (D). C-peptide-expressing cells are more abundant than glucagon-expressing cells (E). C-peptide-expressing cells do not express Nkx6.1 (F).

[0035] FIG. 3 shows that SRE clusters secrete C-peptide in response to glucose. Static incubation of 50 SRE clusters for 4X 20 min intervals in 2.2 mM glucose demonstrated a gradually decreasing C-peptide secretion. Exposure to 16.7 mM glucose caused increased C-peptide secretion by 40 min. Artificially stimulating C-peptide secretion in the presence of 30 mM KCl shows a high reserve of C-peptide available for secretion.

[0036] FIGS. 4A and 4B show SR1423 RNA analysis by hPSC Scorecard qPCR Assay for the expression of a panel of 94 genes associated with pluripotency, endoderm, mesoderm, and ectoderm following the Scorecard manual. FIG. 4A depicts a box and whisker plot representing the reference gene datasets. Results reveal a transcriptome with a differentiation expression profile outside of the significant range of the mean of the reference dataset. FIG. 4B depicts the relative gene expression heatmap, which shows that genes related to mesodermal differentiation were the most downregulated, correlating with inability for mesodermal differentiation *in vitro*.

[0037] FIGS. 5A, 5B, and 5C show the characterization of the SR1423 stem cell line. FIG. 5A shows UMAP embedding of SR1423U, H1 hESC, and iPSC cells analyzed by snRNA-Seq. FIG. 5B depicts the ridge plot of the gene signature for transcriptional regulation of pluripotent stem cells. FIG. 5C depicts the violin plot, showing expression of key stem cell transcription factors in SR1423, hESC, and iPSC cells.

[0038] FIGS. 6A and 6B show characterization of NKX6-1-high and -low clusters. FIG. 6A depicts the flow cytometry results of NKX6-1-high and -low clusters. FIG. 6B depicts the immunofluorescent images of NKX6-1-high and -low clusters. Day 30-35 clusters from each protocol were characterized for expression of key pancreatic islet transcription factors (Pdx1, Nkx6.1, ISL-1, MAFA) and hormones (C-peptide, glucagon).

[0039] FIGS. 7A and 7B show characterization of the secretion of islet-like clusters. FIG. 7A depicts the secretion index (C-peptide secreted during static exposure to 16.7 mM glucose divided by C-peptide secreted during exposure to 2.2 mM glucose) of islet-like clusters. The secretion index is above 1 for islet-like clusters derived from NKX6-1-low cultures (26 batches), NKX6-1-high cultures (16 batches), and primary human islets (6 preparations), indicating glucose sensitivity. FIG. 7B depicts total C-peptide release that includes exposure to KCl. Release was consistently higher in primary human islets, though not statistically significantly higher.

[0040] FIGS. 8A and 8B show the effect of implanted NKX6-1-low islet equivalents. FIG. 8A shows the blood glucose of immune-compromised, STZ-induced diabetic mice, which was restored to levels similar to non-diabetic controls 5 weeks after implant of 2,500 (low) or 5,000 (high) islet equivalents to the renal subcapsular space or the fat pad. FIG. 8B shows the quantification of the total area under the curve measurements of blood glucose following oral glucose challenge, showing that treated animals, but not diabetic control animals, are capable of lowering blood glucose similarly to non-diabetic controls.

[0041] FIG. 9 shows the nonfasting blood glucose levels by implant site. 10,000 to 40,000 NKX6-1-rich islet-like clusters did not control blood glucose after implant to STZ-induced, diabetic immune-compromised rats. Implant site did not influence glucose control.

[0042] FIGS. 10A, 10B, 10C, 10D, 10E, 10F, 10G, and 10H show tissue section immunofluorescent staining of explanted grafts at various time points. The staining images demonstrate a remodeled tissue with elements of functional endocrine pancreas. NKX6-1-expressing cells from NKX6-1-high grafts demonstrated gradual loss of C-peptide expression over the first 8 weeks post-implant (FIGS. 10A-10D). NKX6-1-low grafts maintained persistent expression of C-peptide or insulin (FIGS. 10E and 10G), CHGA (FIG. 10E), PDX1 (FIG. 10G), and activated expression of NKX6-1 (FIG. 10F). Pancreatic endocrine cells co-expressed human mitochondria antigen (hMito) (FIG. 10H).

[0043] FIGS. 11A, 11B, 11C, 11D, 11E, 11F, 11G, and 11H show transcriptome analysis of NKX6-1-low clusters and differentiation stages. FIG. 11A depicts the UMAP embedding of SR1423 stem cells, stages 1-9 of differentiation and human islets. FIG. 11B depicts the grouping

of SR1423 differentiation stages by pancreatic differentiation phases. FIG. 11C depicts the dot plot of key genes associated with pancreatic differentiations from FIG. 11B. FIG. 11D depicts the differentiation trajectory and pseudotime determined by Monocle. FIG. 11E depicts the differentiation trajectory split by differentiation stage. FIG. 11F shows the projection of pancreatic beta cells. FIG. 11G shows the regulation of gene expression in beta cells and gene signatures in pseudotime. FIG. 11H depicts expression of CHGA, INSM1, FEV, and YBX1 in stages 7-9 and human islet cells.

DETAILED DESCRIPTION

[0044] Described herein are Synthetic Replacement Endocrine (SRE) cells that can be used to treat diabetes, improved methods of generating such cells, populations of such cells, and methods of using the disclosed cells for treating diabetes. More specifically, the present disclosure provides differentiation and isolation of SRE cells that express transcription factors comprising a combination of either (a) pancreatic duodenal homeobox-1 (Pdx1) and ISL LIN homeobox-1 (ISL1) or (b) MAF BZIP Transcription Factor A (MAFA) and ISL1, wherein the SRE cells do not express homeobox protein NKX6.1 (Nkx6.1). Additionally, the disclosure also provides methods of producing cells with the foregoing expression profile. Such protocols may include, among other steps, exposing stem cells (in particular, stem cells that exhibit a preference or predisposition toward an endoderm lineage) to retinoic acid, a hedgehog antagonist, and a bone morphogenetic protein (BMP) signaling inhibitor. The cultures may be initiated in adhesion, allowing the cells to naturally and spontaneously form three-dimensional structures; and then transferring the three-dimensional structures to suspension culture. The cells may not be exposed to Wnt3a during culture, either while grown in adhesion or in suspension. Similarly, the cells may not be exposed to tri-iodothyronine (T3) or analog thereof, a protein kinase C activator during culture, nicotinamide, transforming growth factor beta 1, or insulin-like growth factor-1, either while grown in adhesion or in suspension.

[0045] Another marker of the pancreatic endocrine cell, in addition to the insulin-secreting beta cell and the glucagon-secreting alpha cell, is the transcription factor ISL1 (Sharon 2019b). *In vivo*, ISL1-expressing cells that express insulin also express Nkx6.1. The present disclosure describes a

novel cell population derived from stem cells that express ISL1 and insulin, but not Nkx6.1. Other cells express ISL1 and glucagon. A method is disclosed that generates a highly pure population of ISL1-positive, Nkx6.1-negative cells that express insulin or glucagon, and minimal to no non-pancreatic cells present. This population is differentiated from stem cells by a differentiation protocol that employs a minimal amount of transcription factors, hormones, and chemical analogs. Eschewing the use of complex formulas of growth factors improves reproducibility and consistency. This population is highly potent at creating stable, functional pancreatic grafts after implant to a suitable host. This cell population may activate expression of Nkx6.1 after implant to the host.

I. Definitions

[0046] As used herein, the term “about” will be understood by persons of ordinary skill in the art and will vary to some extent depending upon the context in which it is used. If there are uses of the term which are not clear to persons of ordinary skill in the art given the context in which it is used, “about” will mean up to plus or minus 10% of the particular term.

[0047] As used herein, the term “substantially free of” refers to the composition to which the agent has not been added, but it does not exclude that trace amounts of the agent exist.

[0048] As used herein, the term “islet cell” refers to terminally differentiated pancreatic endocrine cells, and any precursor cell that is committed to form progeny normally classified as pancreatic endocrine. The islet cell exhibits some of the morphological features and phenotypic markers (exemplified below) typical of an islet cell lineage. Mature alpha cells secrete glucagon; mature beta cells secrete insulin; mature delta cells secrete somatostatin; PP cells secrete pancreatic polypeptide.

[0049] As used herein, “Synthetic Replacement Endocrine” or “SRE” cells are cells that are definitively identifiable as pancreatic endocrine cells based upon specific expression of ISL1, Pdx1 or MAFA, and pancreatic hormones insulin or glucagon. These cells, upon implantation into a human subject, can mature or alter their gene expression profile to more closely resemble mature endocrine cells of the mammalian pancreas. For the purposes of the present disclosure, “SRE”

cells that are differentiated according to the disclosed protocol express a combination of pancreatic transcription factors of Pdx1 and ISL1 or MAFA and ISL1, but do not express the transcription factor Nkx6.1. After implantation into a suitable host, the SRE cell may ultimately express Nkx6.1.

[0050] As used herein, “pancreatic progenitors,” “pancreatic precursors,” or “pancreatic stem cells” are pancreatic cells that may mature or change their pancreatic gene expression profile after implant to a suitable host. The matured cells may adopt a gene expression pattern that more closely resembles native pancreatic islets.

[0051] As used herein, the term “stem cells” denotes undifferentiated cells that are able to differentiate into specialized cells (*e.g.*, insulin-producing pancreatic cells). For the purposes of this application, the term “stem cell” can include pluripotent cells derived from pre-embryonic, embryonic, or fetal tissue after fertilization that are capable of producing progenitors of all of the three germinal layers (*i.e.*, endoderm, mesoderm, and ectoderm); induced pluripotent cell (*i.e.*, cells that have been transduced with reprogramming genes) that are capable of producing progenitors of all of the three germinal layers; and multipotent cells, such as reprogrammed cells (*i.e.*, cells that have been transduced with reprogramming genes) that can differentiate into only one or two germ layers or that preferentially differentiate into a certain germ layer (*e.g.*, reprogrammed cells that preferentially differentiate into ectoderm or endoderm cell types). Examples of suitable multipotent cells are disclosed in PCT/US2013/047243 (published as WO2014/004341) and PCT/US2019/017281 (published as WO2019/157329). The term includes both established lines of stem cells of various kinds (including cells obtained from primary tissue) that are pluripotent or multipotent in the manner described.

[0052] As used herein, the terms “induced pluripotent cells” or “induced pluripotent stem cells” (“iPS cells”) denote pluripotent cells derived by reprogramming of adult somatic cells, reproductive cells, multipotent cells, or other cell types, following standard art accepted methods (*e.g.*, somatic-cell nuclear transfer, transduction with reprogramming genes, chemical inducement (*see* De Los Angeles *et al.*, *Cell Research*, 23: 1337–1338 (2013); Federation *et al.*, *Trends in Cell Biology*, 24: 179–187 (2013)), *etc.*). The term includes both established induced pluripotent stem cells, and cells obtained from primary tissue that are pluripotent in the manner described.

[0053] As used herein, the terms “non-pluripotent reprogrammed cells” or “multipotent reprogrammed cells” denote cells derived by reprogramming of adult somatic cells, reproductive cells, pluripotent cells, or other cell types, with known reprogramming methods, such as transduction/expression of reprogramming genes and other methods discussed above. Unlike induced pluripotent cells, “non-pluripotent reprogrammed cells” or “multipotent reprogrammed cells” may differentiate into only one or two germ layers or possess a preference to differentiate into a certain germ layer (*e.g.*, reprogrammed cells that preferentially differentiate into ectoderm or endoderm cell types, but which cannot efficiently differentiate into mesoderm cell). The term includes both established induced multipotent cells (*e.g.*, SR1423), and cells obtained from primary tissue that are reprogrammed to be multipotent in the manner described. Examples of suitable multipotent reprogrammed cells are disclosed in PCT/US2013/047243 (published as WO2014/004341) and PCT/US2019/017281 (published as WO2019/157329).

[0054] As used herein, the term “reprogramming genes” denotes known genes and transcription factors that are commonly used in the art to induce pluripotency or multipotency in differentiated cells. Exemplary reprogramming genes include, but are not limited to, Oct4 (*i.e.*, Oct-3/4 or Pou5f1); Sox family transcription factors such as Sox1, Sox2, Sox3, Sox15, and Sox18; Klf family transcription factors such as Klf4, Klf1, Klf2, and Klf5; Myc family transcription factors such as C-myc, N-myc, and L-myc; Nanog; LIN28; and Glis1. Those of skill in the art will understand that the disclosed reprogramming genes, as well as other reprogramming genes known in the art may be combined in various ways in order to induce pluripotency or multipotency. For example, Yu *et al.*, *Science*, 318(5858): 1917–20 (2007) demonstrated that a combination of LIN28, Oct4, Sox2, and Nanog can be used to generate iPS cells, while Maekawa *et al.*, *Nature*, 474(7350): 225–29 (2011) demonstrated that a combination of Glis1, Oct-3/4, Sox2, and Klf4 can be used to generate iPS cells.

[0055] As used herein, the term “differentiate” or “differentiation” denotes a change in cell type from a less specific cell to a more specific cell. For example, any cell that has exited the pluripotent state and progressed along a developmental pathway toward a defined germ line has undergone differentiation. The term “differentiated” is a relative term, so differentiating cells can be at

different stages during their developmental path towards a mature functional cell type. A cell at a later stage of developmental progression can therefore be said to be more differentiated than a cell at an earlier stage.

[0056] As used herein, “differentiation inducing factors,” as used in this disclosure, refers to one of a collection of compounds that are used in culture systems of this invention to induce differentiation of stem cells to differentiated cells of the islet lineage (including precursor cells, progenitor cells, cells with the ability to change their gene expression profile and terminally differentiated cells). No limitation is intended as to the mode of action of the compound. For example, the agent may assist the differentiation process by inducing or assisting a change in phenotype, promoting growth of cells with a particular phenotype or retarding the growth of others. It may also act as an inhibitor to other factors that may be in the medium or synthesized by the cell population that would otherwise direct differentiation down the pathway to an unwanted cell type. Within the category of “differentiation inducing factors” a person of ordinary skill in the art will understand that certain factors are known to induce certain steps throughout the differentiation process. For instance, a person of ordinary skill in the art would understand that an “endoderm-inducing factor” can include, but is not limited to, Activin-A. Similarly, an “endocrine-inducing factor” can include, but is not limited to, retinoic acid and/or a hedgehog antagonist and/or a BMP inhibitor, either alone or in combination.

[0057] As used herein, “long-term,” when used in relation to the survival and functioning of foreign therapeutic cells used in a cell-based therapy/implant, means a period of at least six months or longer.

[0058] As used herein, the phrases “therapeutically effective amount” means an amount of cells transplanted into a subject that provides the specific pharmacological effect for which the cells are transplanted, *i.e.* to produce insulin and lower blood glucose. It is emphasized that a therapeutically effective amount of cells will not always cause normal regulation of blood glucose in a given diabetic subject, even though such concentration is deemed to be a therapeutically effective amount by those of skill in the art. For convenience only, exemplary amounts are provided below.

[0059] Those skilled in the art can adjust such amounts in accordance with standard practices as needed to treat a specific subject. The therapeutically effective amount may vary based on the site of implantation, the age and weight of the subject, and/or the subject's condition, including the severity of the subject's disease, the subject's diet, and/or the subject's overall health.

[0060] The terms "treatment" or "treating" as used herein with reference to diabetes refer to one or more of: reducing, ameliorating or eliminating one or more symptoms or co-morbidities of diabetes, such as hyper- and hypo-glycemia, heart disease, renal disease, hepatic disease, retinopathy, neuropathy, non-healing ulcers, periodontal disease; reducing the subject's reliance on exogenous insulin to regulate blood glucose, regulating the subject's blood glucose without the use of exogenous insulin; reducing the subject's percentage of glycosylated hemoglobin, or HbA1C levels; and/or reducing the subject's reliance on other pharmaceutical interventions, such as insulin sensitizers, enhancers of glucose excretion, and other treatment modalities known in the art.

[0061] The terms "individual," "subject," and "patient" are used interchangeably herein, and refer to any individual mammalian subject, *e.g.*, non-human primate, porcine, bovine, canine, feline, equine, or human. Preferably, the "individual," "subject," or "patient" is a human.

II. Identification of Cells for Cell-Based Therapy

[0062] One limitation of conventional stem cell-based therapy is that different stem cells possess different propensities to differentiate into mature cell types. For instance, it has been reported that epigenetic signatures of the starting cell population can persist in reprogrammed cells, a phenomenon called "epigenetic memory." As a result, iPS cells and other reprogrammed cells may preferentially differentiate into cells that belong to the same germ layer from which they were derived. Accordingly, in some embodiments, the stem cells used in the disclosed methods for generating insulin-producing cells may be derived from mature endodermal cells that have been reprogrammed into pluripotent or multipotent stem cells. In some embodiments, the stem cells used in the disclosed methods may be derived from human pancreatic cells that have been reprogrammed. Such donor pancreatic cells may come from the subject being treated for diabetes (*i.e.*, an autologous donor) or from a person that is not being treated for diabetes (*i.e.*, an allogeneic

donor). In some embodiments, the stem cells used in the disclosed methods may be reprogrammed primary cells from the islets of Langerhans of consented healthy adult donor pancreata.

[0063] Primary cells grown in cell culture can become homogenous and lose functional mature traits over time, possibly as a result of adaptation to artificial culture conditions or genetic drift. Accordingly, when primary cells are used as a starting cell population, it may be advantageous to reprogram the primary cells within, for example, 7 days, 6 days, 5 days, 4 days, 3 days, 2 days, or within 1 day of cell harvest or isolation. For examples, isolated primary cells may be transduced with reprogramming genes within 5 days of cell harvest.

[0064] Those of ordinary skill in the art will understand that when primary cells are reprogrammed using reprogramming genes, there are numerous combinations of reprogramming genes that can be used. In some embodiments, a primary cell can be reprogrammed via transduction with Oct4, Sox2, Klf4, and L-Myc. In some embodiments, a primary cell can be reprogrammed via transduction with Oct4, Sox2, Klf4, and C-Myc. In some embodiments, a primary cell can be reprogrammed via transduction with LIN28, Oct4, Sox2, and Nanog. In some embodiments, a primary cell can be reprogrammed via transduction with Glis1, Oct-3/4, Sox2, and Klf4. These exemplary combinations are not intended to be limiting, as other combinations of reprogramming genes are known in the art and may be used for purpose of the disclosed methods.

[0065] In some embodiments, the cells used in the disclosed differentiation and treatment methods may possess a preference for differentiating toward one germ line over another. For instance, in some embodiments, a primary cell or stem cell (*e.g.*, SR1423) may efficiently differentiate to ectoderm or endoderm lineages, but be substantially unable to differentiate into a mesodermal lineage. This could be determined by, for example, employing differentiation protocols or kits to push a stem cell toward a specific germ line, yet failing to detect a germ line marker (*e.g.*, OTX2 for ectoderm, Sox17 for endoderm, or Brachyury for mesoderm).

[0066] In some embodiments, a stem cell or primary cell that preferentially differentiates along an endodermal lineage can be identified by certain molecular markers. For example, a stem cell or primary cell that preferentially differentiates along an endodermal lineage may express markers typical of pluripotency and have a normal karyotype, yet even if markers typical of pluripotency

are expressed, the stem cells may be only multipotent, and therefore not fit the accepted criteria for pluripotency.

[0067] A stem cell or primary cell that preferentially differentiates along an endodermal lineage may also possess a unique gene expression profile. For example, in some embodiments, a stem cell or primary cell that preferentially differentiates along an endodermal lineage may down-regulate expression of BHMT2, Cox7A1, and HSPB2 relative to a control level or control cell. In some embodiments, a stem cell or primary cell that preferentially differentiates along an endodermal lineage may up-regulate expression of NAP1L1 relative to a control level or control cell. Additionally, cells that preferentially differentiate along an endodermal lineage may up-regulate expression of GLIS2, CCDC58, and MTX3 and down-regulate expression of C7orf29 relative to a control cell. Expression levels may be determined by any means known in the art, such as qRT-PCR or microarray analysis, and the control cells used as a standard of comparison may include pluripotent cells that do not exhibit preferential differentiation to the endodermal lineage or a substantial inability to differentiate to the mesodermal lineage, such as the standard embryonic stem cell lines found in the NIH registry. While not being bound by theory, it is believed that at least BHMT2 and NAP1L1 play roles in DNA modification and may contribute to epigenetic memory.

[0068] In some embodiments, the differential expression of BHMT2, Cox7A1, HSPB2, and/or NAP1L1 may be at least about 1 log, at least about 2 logs, or at least about 3 logs increased (for BHMT2, Cox7A1, and HSPB2) or decreased (for NAP1L1) relative to pluripotent cell that does not display preferential differentiation to the endodermal lineage and is not substantially unable to differentiate to the mesodermal lineage, or a stem cell that meets the standard criteria for pluripotency.

[0069] Identifying a stem cell with the disclosed expression profile indicates a preference for differentiating into an endodermal lineage and thereafter an insulin-producing cell. Direct testing of differentiation preference to specific germ layers increases efficiency of generating cell lines inclined to a particular fate and therefore are suitable for cell-based therapy.

III. Protocol for generating SRE cells

[0070] Mature endocrine cells of native human islets express the transcription factors Pdx1, ISL1 and Nkx6.1. It is generally believed that Nkx6.1 expression is limited to the insulin-secreting beta cells. For example, in rodents each of these three transcription factors are essential for endocrine pancreatic development. Isolated mature islets containing Pdx1+/Nkx6.1+/Insulin+ beta cells are capable of regulating blood glucose after transplant into insulin-dependent diabetic subjects (Marfil-Garza *et al.*, *The Lancet Diabetes & Endocrinology*, 10(7): 519-532 (2022)). The expression of ISL1 and MAFA are also expressed in early endocrine progenitors and again in maturing islets, indicating roles in both specification of endocrine-committed progenitors and in maturation or maintenance of functional endocrine cells. MAFA expression is limited to insulin expressing cells of rodents and MAFA deficient mice exhibit impaired glucose sensitivity and diabetes. Overexpression of MAFA, in combination with Pdx1 and Ngn3, can drive reprogramming of the beta cell phenotype in human pluripotent cells and transdifferentiate exocrine pancreas when combined with Pax4. It is therefore possible that MAFA and ISL1 in the absence of Nkx6.1 in stem cell derived pancreatic populations may reflect beta cell fate and have high *in vivo* potency for islet replacement therapy.

[0071] Pluripotent human stem cells are capable of maturing into pancreatic endocrine cells in the laboratory (Silva *et al.*, *Stem cell Research and Therapy*, 13:308 (2022)). To verify their authenticity, the gene and protein expression of pluripotent stem cell-derived human pancreatic endocrine cells are benchmarked against mature and developing pancreatic cells of rodents and humans (Alvarez Dominguez *et al.*, *Cell*, 185(2): 235-249 (2022); Silva *et al.*, *Stem cell Research and Therapy*, 13:308 (2022); Yoshihara, *Frontiers in Cell and Developmental Biology*, 10 (2022)). The state-of-the-art describes the main stem cell-derived, anti-diabetes cell type as the insulin-secreting beta cell that progresses along a maturation timeline in which pancreatic cells express Pdx1 and Nkx6.1, followed by gain of expression of insulin and C-peptide (a by-product of insulin synthesis) (Pagliuca FW, *et al. Cell*, 154(2): 428-439 (2014); Velazco-Cruz *et al.*, *Stem Cell Reports*, 12(2): 351-365 (2019); Hogrebe 2020, Nostro 2015). This order of expression is the same as during native development of islets in rodents and humans, although the timeline of these events,

the spatial organization of the developing cells and their cellular environments are vastly different to stem cell-derived islets grown in the laboratory.

[0072] To mimic as closely as possible the native islet, current state-of-the art literature instructs that the ideal pluripotent stem cell-derived islet contains the maximum number of cells that express the definitive beta cell transcription factor Nkx6.1, and insulin by-product C-peptide. Additionally, the state-of-the art instructs that pluripotent stem cell-derived populations of pancreatic cells that express Pdx1 gain expression of ISL1 *after* gaining expression of Nkx6.1 (Veres *et al.*, *Nature* 569: 368-373 (2019)). Thus, state-of-the art literature suggests that pancreatic cells that can be implanted to treat diabetes must express Nkx6.1 and Pdx1, and this same literature is silent with respect to ISL1 expression in stem cell-derived islets or pancreatic precursors.

[0073] Pluripotent stem cell-derived islets that express Nkx6.1 and C-peptide have been found to make and secrete insulin, but at levels that are far lower than native mature pancreatic islets (Fantuzzi *et al.*, *Frontiers in Cell and Developmental Biology*, 10 (2022); Veres *et al.*, *Nature* 569: 368-373 (2019)). Thus, it was believed that these populations may continue to mature after implantation in a subject, thereafter, leading to improved potency over time (Augsornworawat *et al.*, *Cell Reports*, 32(8): 108850 (2020)). However, nonclinical and clinical data prove that pluripotent stem cell-derived islets lack potency compared to native islets.

[0074] For the disclosed protocols, cell sources may include, but are not limited to, human embryonic stem cells, induced pluripotent stem cells, non-pluripotent or multipotent reprogrammed cells (*e.g.*, SR1423), and other conventional cell sources known in the art.

[0075] The disclosed methods of generating isolated insulin-producing SRE cells from stem cells comprise a multi-step process wherein endoderm differentiation is first initiated, followed by differentiation towards the endocrine cell lineage to generate hormone-secreting cells. The hormone-secreting cells then undergo further maturation or adoption of functional endocrine cell characteristics post-transplantation. The endoderm differentiation is typically initiated by contacting the stem cells with an endoderm-inducing agent, such as Activin-A. When a sufficient number of endoderm cells have been reached, the cells are contacted with an endocrine-inducing agent, such as retinoic acid and/or a hedgehog antagonist and/or a BMP inhibitor, to further

differentiate the cells into the SRE cells. Hedgehog antagonists include SANT1 and cyclopamine, among others. BMP signaling inhibitors include LDN193189, among others. Through exposure to further differentiation factors and/or transplantation into a host, which is discussed in more detail below, the SRE cells can be further differentiated into hormone-secreting cells that can be used for cell-based therapies to treat diabetes.

[0076] In some embodiments, stem cells are cultured in a first medium comprising an endoderm-inducing agent. In some embodiments, the endoderm-inducing agent comprises at least Activin-A. In some embodiments, the endoderm-inducing agent comprises Activin-A and Wortmannin. In some embodiments, the disclosed methods do not employ or include use of an activator of Wnt signaling, such as CHIR-99021 (a small molecule activator of Wnt signaling) and/or the growth factor Wnt3A. In some embodiments, the disclosed methods do employ or include use of an activator of Wnt signaling, such as CHIR-99021, and/or the growth factor Wnt3A. Exposure of the stem cells to an endoderm-inducing agent results in differentiation of the cells into endoderm cells.

[0077] In contrast to conventional methods, in some embodiments, the disclosed differentiation method exposes the cells long-term to retinoic acid (RA). In contrast to conventional methods, in some embodiments, the disclosed differentiation protocol exposes the cells long-term to a Hedgehog antagonist such as cyclopamine or SANT1. In some embodiments, the disclosed differentiation protocol exposes the cells to a BMP inhibitor such as LDN193189. In contrast to conventional methods, in some embodiments, the disclosed differentiation protocol exposes the cells long-term to a BMP inhibitor such as LDN193189.

[0078] In contrast to conventional methods, in some embodiments, the disclosed methods do not employ or include use of tri-iodothyronine (T3). In some embodiments, the disclosed methods do employ or include use of T3. In some embodiments, the disclosed methods do not employ a protein kinase C activator or analog thereof. In some embodiments, the disclosed methods do not employ nicotinamide, a form of vitamin B3. In some embodiments, the disclosed methods do employ nicotinamide. In some embodiments, the disclosed methods do not employ transforming growth factor beta 1 (TGFbeta1) protein. In some embodiments, the disclosed methods do employ

TGFbeta1. In some embodiments, the disclosed methods do not employ insulin-like growth factor 1 (IGF-1). In some embodiments, the disclosed methods do employ IGF-1.

[0079] In contrast to conventional methods, in some embodiments, the disclosed differentiation protocol does not employ the creation of three-dimensional suspension cultures through dissociation of adhesion cultures and re-aggregation of cells in suspension.

[0080] In some embodiments, the stem cells are differentiated into endoderm cells in the presence of Activin A at a concentration of about 1 to about 200 ng/mL, about 25 to about 175 ng/mL, about 50 to about 150 ng/mL, or about 75 to about 125 ng/mL. For example, the Activin A concentration may be about 1 ng/mL, about 10 ng/mL, about 20 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 110 ng/mL, about 120 ng/mL, about 130 ng/mL, about 140 ng/mL, about 150 ng/mL, about 160 ng/mL, about 170 ng/mL, about 180 ng/mL, about 190 ng/mL, or about 200 ng/mL.

[0081] In some embodiments, the stem cells are differentiated into endoderm cells in the presence of Wortmannin at a concentration of about 0.1 to about 2.0 μ M, about 0.25 to about 1.75 μ M, about 0.5 to about 1.5 μ M, or about 0.75 to about 1.25 μ M. For example, the Wortmannin concentration may be about 0.1 μ M, about 0.5 μ M, about 1.0 μ M, about 1.5 μ M, or about 2.0 μ M.

[0082] In some embodiments, the medium used to differentiate endoderm cells to SRE cells can comprise KGF, but in some embodiment, the medium used to differentiate endoderm cells to SRE cells can comprise retinoic acid, a Hedgehog antagonist (e.g. cyclopamine and/or SANT1), and a BMP signaling inhibitor (e.g. LDN193189) and combinations thereof without KGF.

[0083] In some embodiments, the step of differentiating endoderm cells to SRE cells may comprise culturing the cells for 1-5 days in a medium comprising retinoic acid, a Hedgehog antagonist (e.g. cyclopamine or SANT1), and a BMP signaling inhibitor (e.g. LDN193189), with or without KGF. For example, the endoderm cells may be cultured in the presence of these endocrine-inducing agents for about 1, about 2, about 3, about 4, or about 5 days, thereby differentiating the endoderm cells into SRE cells.

[0084] In some embodiments, the cells are differentiated in the presence of retinoic acid for at least twenty (20) days at a concentration of about 0.05 μM , about 0.1 μM , about 0.5 μM , about 1.0 μM , about 1.5 μM , or about 2.0 μM .

[0085] In some embodiments, the cells are differentiated in the presence of Hedgehog antagonist cyclopamine for at least twenty (20) days at a concentration of about 0.05 μM , about 0.1 μM , about 0. μM , or about 0.5 μM .

[0086] In some embodiments, the cells are differentiated in the presence of a chemical analog of cyclopamine SANT-1 ((4-Benzyl-piperazin-1-yl)-(3,5-dimethyl-1-phenyl-1H-pyrazol-4-ylmethylene)-amine) at a concentration of about 0.05 μM , about 0.1 μM , about 0.25 μM , or about 0.5 μM .

[0087] In some embodiments, the cells are differentiated in the presence of LDN193189 at a concentration of about 0.05 μM , about 0.01 μM , about 0.1 μM , or about 0.5 μM .

[0088] In some embodiments, the endoderm cells are differentiated into SRE cells in the presence of retinoic acid at a concentration of about 1.0 to about 10.0 μM , about 2.0 to about 8.0 μM , or about 3.0 to about 5.0 μM . For example, the retinoic acid concentration may be about 1.0 μM , about 1.5 μM , about 2.0 μM , about 2.5 μM , about 3.0 μM , about 3.5 μM , about 4.0 μM , about 4.5 μM , about 5.0 μM , about 5.5 μM , about 6.0 μM , about 6.5 μM , about 7.0 μM , about 7.5 μM , about 8.0 μM , about 8.5 μM , about 9.0 μM , about 9.5 μM , or about 10.0 μM .

[0089] In some embodiments, the endoderm cells are differentiated into SRE cells in the presence of cyclopamine at a concentration of about 0.1 to about 1.0 μM or about 0.25 to about 0.75 μM . For example, the cyclopamine concentration may be about 0.1 μM , about 0.2 μM , about 0.25 μM , about 0.3 μM , about 0.4 μM , about 0.45 μM , about 0.5 μM , about 0.55 μM , about 0.6 μM , about 0.7 μM , about 0.75 μM , about 0.8 μM , about 0.9 μM , or about 1.0 μM .

[0090] In some embodiments, the endoderm cells are differentiated into SRE cells in the presence of LDN193189 at a concentration of about 0.1 to about 1.0 μM or about 0.25 to about 0.75 μM . For example, the cyclopamine concentration may be about 0.1 μM , about 0.2 μM , about 0.25 μM ,

about 0.3 μ M, about 0.4 μ M, about 0.45 μ M, about 0.5 μ M, about 0.55 μ M, about 0.6 μ M, about 0.7 μ M, about 0.75 μ M, about 0.8 μ M, about 0.9 μ M, or about 1.0 μ M.

[0091] In some embodiments, the endoderm cells are differentiated into SRE cells in the presence of KGF at a concentration of 1 to about 100 ng/mL, about 25 to about 75 ng/mL, or about 60 to about 70 ng/mL. For example, the KGF concentration may be about 1 ng/mL, about 10 ng/mL, about 20 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, or about 100 ng/mL. In some embodiments, the cells are not exposed to KGF until after the cells have been differentiated from endoderm cells to endocrine cells.

[0092] In some embodiments, the SRE cells can be further cultured in the presence of additional growth factors and/or hormones. In some embodiments, the SRE cells may be cultured in a medium comprising Noggin, EGF, γ -secretase inhibitor XXI (GSXXi), Alk5i II, fibroblast growth factor 7 (FGF7), and/or combinations thereof. In some embodiments, the SRE cells may be cultured in a medium comprising Noggin, EGF, γ -secretase inhibitor XXI, Alk5i II, FGF7, and combinations thereof and further comprising retinoic acid and/or cyclopamine (*e.g.*, cyclopamine KAAD). For example, the pancreatic progenitor cells may be cultured in the presence of these agents for about 1, about 2, about 3, about 4, or about 5 days, thereby differentiating the pancreatic progenitor cells into a pancreatic lineage.

[0093] In some embodiments, the endoderm cells are differentiated into SRE cells in the presence of Noggin at a concentration of about 1 to about 100 ng/mL, about 25 to about 75 ng/mL, or about 60 to about 70 ng/mL. For example, the Noggin concentration may be about 1 ng/mL, about 5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 45 ng/mL, about 50 ng/mL, about 55 ng/mL, about 60 ng/mL, about 65 ng/mL, about 70 ng/mL, about 75 ng/mL, about 80 ng/mL, about 85 ng/mL, about 90 ng/mL, about 95 ng/mL, or about 100 ng/mL. In some embodiments, the SRE cells are further cultured in the presence of EGF at a concentration of about 1 to about 100 ng/mL, about 25 to about 75 ng/mL, or about 60 to about 70 ng/mL. For example, the EGF concentration may be about 1 ng/mL, about 5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 45 ng/mL, about 50 ng/mL, about 55

ng/mL, about 60 ng/mL, about 65 ng/mL, about 70 ng/mL, about 75 ng/mL, about 80 ng/mL, about 85 ng/mL, about 90 ng/mL, about 95 ng/mL, or about 100 ng/mL.

[0094] In some embodiments, the SRE cells are further cultured in the presence of IGF-I at a concentration of about 1 to about 100 ng/mL, about 25 to about 75 ng/mL, or about 60 to about 70 ng/mL. For example, the IGF-I concentration may be about 1 ng/mL, about 5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 45 ng/mL, about 50 ng/mL, about 55 ng/mL, about 60 ng/mL, about 65 ng/mL, about 70 ng/mL, about 75 ng/mL, about 80 ng/mL, about 85 ng/mL, about 90 ng/mL, about 95 ng/mL, or about 100 ng/mL. In some embodiments, the SRE cells are further cultured in the presence of FGF7 at a concentration of about 1 to about 100 ng/mL, about 25 to about 75 ng/mL, or about 60 to about 70 ng/mL. For example, the FGF7 concentration may be about 1 ng/mL, about 5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 45 ng/mL, about 50 ng/mL, about 55 ng/mL, about 60 ng/mL, about 65 ng/mL, about 70 ng/mL, about 75 ng/mL, about 80 ng/mL, about 85 ng/mL, about 90 ng/mL, about 95 ng/mL, or about 100 ng/mL.

[0095] In some embodiments, the stem cells or endoderm cells are differentiated on an adhesive substrate comprised of vitronectin and/or laminin and/or collagen. In some embodiments, the stem cells or endoderm cells can spontaneously and naturally form three-dimensional structures are collected and transferred to suspension culture.

[0096] In some embodiments, the stem cells used in the disclosed differentiation method are derived from pancreatic primary tissue. In some embodiments, the stem cells used in the disclosed differentiation method are embryonic stem cells. In some embodiments, the stem cells used in the disclosed differentiation method are induced pluripotent stem cells. In some embodiments, the stem cells used in the disclosed differentiation method are non-pluripotent reprogrammed cells. In some embodiments, the stem cells are human stem cells.

[0097] For the purposes of the present disclosure, it may be desirable to reprogram cells by expressing reprogramming genes in the cell without incorporating the reprogramming genes into the genome of the cell. Those of ordinary skill in the art will recognize that transduced genes can

be expressed in a cell without incorporating those genes into the genome using, for example, episomal expression plasmids. The reprogramming genes may be expressed on at least 1, at least 2, at least 3, or at least 4 or more episomal expression plasmids. As discussed above, multiple reprogramming genes are known in the art and may be used for the purposes of the disclosed methods, but in some embodiments, the reprogramming genes comprise Oct4, Sox2, Klf4, and L-Myc.

[0098] In some embodiments, the total culturing time required for differentiating cells from a stem cell into an insulin-producing cell may be about 30 days or less. For instance, the cells may be cultured for about 30 days, about 29 days, about 28 days, about 27 days, about 26 days, about 25 days, or less.

[0099] Those of skill in the art will also understand that the overall culturing time in each differentiation step may vary. Accordingly, in some embodiments, the present disclosure provides a method of producing insulin-secreting pancreatic cells, comprising (a) culturing human stem cells in a first medium comprising Activin-A and Wortmannin, and optionally are not exposed to keratinocyte growth factors (KGF) prior to differentiation into endoderm cells, thereby differentiating the human stem cells into endoderm cells; and (b) culturing the endoderm cells from (a) in a second medium comprising retinoic acid, a Hedgehog antagonist, and a BMP signaling inhibitor and optionally comprising KGF, thereby differentiating the endoderm cells into endocrine cells.

[0100] In some embodiments, the present disclosure provides a method of producing insulin-secreting SRE cells, comprising (a) culturing human stem cells in a first medium comprising Activin-A and Wortmannin, and optionally are not exposed to keratinocyte growth factors (KGF) prior to differentiation into endoderm cells, thereby differentiating the human stem cells into endoderm cells; and (b) culturing the endoderm cells from (a) in a second medium comprising retinoic acid, a Hedgehog antagonist, and a BMP signaling inhibitor and optionally comprising KGF, thereby differentiating the endoderm cells into SRE cells.

[0101] In some embodiments, the SRE cells are further matured in a medium wherein the human cells are not exposed to T3.

[0102] In some embodiments, the SRE cells are further matured in a medium wherein the human cells are not exposed to a protein kinase C activator.

[0103] In some embodiments, the SRE cells are further matured in a medium wherein the human cells are not exposed to nicotinamide, a form of vitamin B3.

[0104] In some embodiments, the SRE cells are further matured in a medium wherein the human cells are not exposed to transforming growth factor beta 1 (TGFbeta1) protein.

[0105] In some embodiments, the SRE cells are further matured in a medium wherein the human cells are not exposed to insulin-like growth factor 1 (IGF-1).

[0106] In some embodiments, the total culturing time for steps (a) and (b) may be 30 days or less. For instance, the cells may be cultured for about 30 days, about 29 days, about 28 days, about 27 days, about 26 days, about 25 days, or less. In some embodiments, step (a) may comprise days 1-3 of culture and step (b) may comprise days 4-27 of culture.

[0107] In some embodiments, the present disclosure provides a method of producing insulin-secreting SRE cells, comprising (a) culturing human stem cells in a first medium comprising Activin-A and Wortmannin, thereby differentiating the human stem cells into endoderm cells; (b) exposing the cells during subsequent culture steps to retinoic acid for at least twenty (20) days; (c) exposing the cells during subsequent culture steps to a Hedgehog antagonist such as cyclopamine or a chemical analog for at least twenty (20) days; (d) exposing the cells during subsequent culture steps to a BMP signaling inhibitor such as LDN193189 for no more than six (6) days; (e) not exposing the cells to Tri-iodothyronine (T3), (f) not exposing the cells to a protein kinase C activator, (g) not exposing the cells to IGF, (h) not exposing the cells to TGFβ, (i) not exposing the cells to nicotinamide, (j) initiating cell culture on an adhesive substrate; and (k) transferring cells that naturally and spontaneously form three-dimensional structures to suspension culture.

[0108] In some embodiments, the present disclosure provides a method of producing insulin-secreting SRE cells, comprising (a) culturing human stem cells in a first medium comprising Activin-A and Wortmannin or Activin-A, Wortmannin, and an activator of Wnt signaling such as CHIR-99021, thereby differentiating the human stem cells into endoderm cells; (b) exposing the

cells during subsequent culture steps to retinoic acid for at least sixteen (16) days; (c) exposing the cells during subsequent culture steps to a Hedgehog antagonist such as cyclopamine or a chemical analog such as SANT1 for no more than six (6) days; (d) exposing the cells during subsequent culture steps to a BMP signaling inhibitor such as LDN193189 for no more than six (6) days; (e) not exposing the cells to Tri-iodothyronine (T3), (f) not exposing the cells to a protein kinase C activator, (g) not exposing the cells to IGF, (h) not exposing the cells to TGF β , (i) not exposing the cells to nicotinamide, (j) initiating cell culture on an adhesive substrate; and (k) transferring cells that naturally and spontaneously form three-dimensional structures to suspension culture.

[0109] In some embodiments, the present disclosure provides a method of producing insulin-secreting SRE cells, comprising (a) culturing human stem cells in a first medium comprising Activin-A and Wortmannin or Activin-A, Wortmannin thereby differentiating the human stem cells into endoderm cells; (b) exposing the cells during subsequent culture steps to retinoic acid for at least twenty (20) days; (c) exposing the cells during subsequent culture steps to a Hedgehog antagonist such as cyclopamine or a chemical analog such as SANT1 for no more than twelve (12) days; (d) exposing the cells during subsequent culture steps to a BMP signaling inhibitor such as LDN193189 for no more than six (6) days; (e) exposing the cells to Tri-iodothyronine (T3), a protein kinase C activator, IGF, TGF β , and nicotinamide, (f) initiating cell culture on an adhesive substrate; and (g) transferring cells that naturally and spontaneously form three-dimensional structures to suspension culture.

[0110] In some embodiments, the present disclosure provides methods of producing mammalian insulin-secreting cells, comprising: culturing mammalian stem cells in adhesion, thereby allowing the mammalian stem cells to spontaneously form three-dimensional structures; and culturing of the three-dimensional structures in suspension; wherein the culturing steps comprise at least a 20-day exposure to retinoic acid, a Hedgehog antagonist, and do not comprise exposing the stem cells of three-dimensional structures to T3, an activator of protein kinase C, IGF, TGF β , or Nicotinamide.

[0111] In some embodiments, the present disclosure provides methods of producing insulin-secreting cells, comprising: culturing mammalian stem cells on an adhesive substrate in a first

medium comprising Activin-A and Wortmannin, further culturing the cells in at least one additional medium comprising retinoic acid, a Hedgehog antagonist, and a BMP signaling inhibitor; and transferring the cells to a suspension culture when the cells form three-dimensional cell structures; wherein the cells are exposed to retinoic acid, a Hedgehog antagonist, for at least 20 days.

[0112] In some embodiments, the present disclosure provides methods of producing insulin-secreting cells, comprising: culturing mammalian stem cells on an adhesive substrate in a first medium comprising Activin-A and Wortmannin, further culturing the cells in at least one additional medium comprising retinoic acid, a Hedgehog antagonist, and a BMP signaling inhibitor; and transferring the cells to a suspension culture when the cells form three-dimensional cell structures; wherein the cells are exposed to retinoic acid and a Hedgehog antagonist for around 16 days.

[0113] In some embodiments, it may be preferable to select a starting stem cell or non-pluripotent progenitor cell that preferentially differentiates or is predisposed to differentiation to the endoderm lineage. This may allow for simpler differentiation and may achieve a purer and more mature culture of insulin-secreting cells compared to traditional methods.

[0114] For the purposes of the presently disclosed method, it was determined that the production of insulin-secreting cells is optimized when the cells are initially grown in adhesion or the culture is initiated on an adhesive substrate (*e.g.*, a positively charged surface or a surface coated with vitronectin or Matrigel), thus allowing the cells to naturally and spontaneously form three-dimensional structures (*e.g.*, aggregates of cells). These three-dimensional structures made up of adhered cells can then be cultured in suspension for the duration of the disclosed methods.

[0115] Thus, in some embodiments, the starting stem cell population is grown in adhesion, while the later stages of culture take place in suspension. For the purposes of this disclosure, the phrases “grown in adhesion” or “cultured in adhesion” refer to standard cell culture wherein cells adhere to the surface of the culture dish. In some cases, the culture dish may be coated with a substrate to promote adhesion, and in some cases the dish may be given a net positive charge to promote adhesion. In general, culturing of stem cells, such as iPS cells, requires an adhesive substrate, and

various adhesion-promoting substrates are known in the art. For example, vitronectin or Matrigel can be applied to a cell culture vessel to promote adhesion, but Matrigel is harvested from mouse sarcoma cells and is therefore not preferred for clinical use. In some embodiments, differentiation is commenced by culturing the starting stem cell population with an endoderm-inducing media, and grown in adhesion. Formation of 3D structures (*e.g.*, aggregates of differentiated/differentiating cells) on the substrate/plate may occur gradually as differentiation progresses. By day about 15, the 3D structures begin to detach from the plate, and these 3D structures can be transferred into vessels that are not coated with an adhesive substrate (*e.g.*, vitronectin), such that the 3D structures are cultured in a free-floating suspension. This transition, from culturing in adhesion to a suspension culture is novel and allows for a more natural differentiation into an insulin-secreting cell.

[0116] In some embodiments, the cells are preferably exposed to retinoic acid, a Hedgehog antagonist, and a BMP signaling inhibitor for at least about 16 days, at least about 17 days, at least about 18 days, at least about 19 days, at least about 20 days, at least about 21 days, at least about 22 days, at least about 23 days, at least about 24 days, at least about 25 days, at least about 26 days, at least about 27 days, or at least about 28 days. In some embodiments, this continued exposure to retinoic acid, a Hedgehog antagonist, and a BMP signaling inhibitor may commence after the starting stem cell population has been forced toward an endodermal lineage, for example, after the starting stem cell population has been cultured in the presence of Activin A and wortmannin for about 1, about 2, about 3, about 4, or about 5 days.

[0117] Those of skill in the art will understand that the disclosed methods can be applied generally to mammalian stem cells, such as human and non-human primate stem cells. However, additional mammalian cells, such as pig, cow, horse, sheep, dog, or cat stem cells may also be differentiated according to the disclosed methods. Additionally, those of skill in the art will recognize that the disclosed methods can employ various forms of cells culture including, for example, adherent cultures and/or suspension cultures.

[0118] The disclosed protocols for generating insulin-producing cells enhanced the yield of insulin-producing beta cell progenitor cells from both human embryonic stem cells and

reprogrammed pancreatic tissue. In contrast to conventional methods of preparing insulin producing cells or pancreatic precursors, the protocol disclosed herein yields near homogeneous populations of insulin-producing cells. Producing a homologous cell population is not only important for therapeutic efficacy, but also for safety, as residual stem cells or other proliferative cells can form teratomas when transplanted and have tumorigenic potential. The high degree of differentiation and homogeneity provided by the disclosed differentiation methods yields fewer cells with tumorigenic potential, which is essential in the development of a useful cell therapy.

[0119] Prior to employing the disclosed differentiation methods, stem cells that preferentially differentiate into endoderm cell may be identified according to the methods disclosed in Section II of this application. This can increase the overall efficiency of the differentiation process as well as increase the yield of insulin-producing cells.

IV. Cell-Based Compositions and Methods of Treatment

[0120] The SRE cells disclosed herein can be used to treat diabetes in a subject in need thereof. In some embodiments, the subject in need of treatment is a mammal, for example, a human subject with insulin-dependent diabetes.

[0121] The present disclosure provides methods for producing a population of substantially homologous SRE cells, which can be incorporated into a cell-based composition for treating diabetes. Accordingly, provided herein are cell-based compositions for treating diabetes, comprising a population of SRE cells and a suitable carrier for implantation into a human subject in need thereof, wherein at least 66% of the cells are insulin-producing beta pancreatic cells. In some embodiments, the cell-based composition may comprise at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% insulin-producing pancreatic cells that express the transcription factors Pdx1 and ISL1, and do not express the transcription factor Nkx6.1. In some embodiments, the cell-based composition may comprise at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at

least about 99%, or at least about 100% insulin-producing pancreatic cells that express the transcription factors MAFA and ISL1, and do not express the transcription factor Nkx6.1

[0122] Suitable carriers for implanting therapeutic cells are known in the art and may include but are not limited to hydrogels, natural and synthetic polymer scaffolds, extracellular matrix (which may comprise, *e.g.*, collagen, laminin, fibronectin, etc.), hyaluronic acid, biomimetic scaffolds, polylactide (PLA) scaffolds, polyglycolide (PGA) scaffolds, PLA-PGA copolymer (PLGA) scaffolds, as well as hydroxyapatite scaffolds, and macro-porous cryogels. In some embodiments, the carrier suitable for transplantation may comprise encapsulating the SRE cells in macro-capsules, such as macro-capsules comprising alginate, cellulose sulfate, glucomannan, or a combination thereof.

[0123] In some embodiments, at least 66% of the SRE cells express Pdx1. In some embodiments, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% of the SRE cells express Pdx1.

[0124] In some embodiments, at least 66% of the SRE cells express MAFA. In some embodiments, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% of the SRE cells express MAFA.

[0125] In some embodiments, at least 66% of the SRE cells express ISL1. In some embodiments, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% of the SRE cells express ISL1.

[0126] In some embodiments, at least about 68% of the SRE cells do not express Nkx6.1. In some embodiments, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at

least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% of the SRE cells do not express Nkx6.1.

[0127] In some embodiments, at least 66% of the SRE cells express C-peptide. In some embodiments, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 100% of the SRE cells express C-peptide.

[0128] Also disclosed herein are a population of the SRE cells disclosed herein. Such a population of SRE cells may be incorporated into a cell-based composition as described above. In some embodiments, the population may be substantially pure, meaning it includes at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 99%, or 100% SRE cells. In some embodiments, the SRE cells may be formulated in with one or more therapeutically acceptable carriers.

[0129] The cell-based compositions for treating diabetes may be prepared according to the methods disclosed herein. For example, the disclosed SRE cells of a cell-based composition may, for example, be derived according to a method comprising contacting a stem cell with a combination of growth factors to drive differentiation of the stem cell to an endocrine lineage, wherein the combination of growth factors comprises retinoic acid, a Hedgehog antagonist, and a bone morphogenetic protein (BMP) signaling inhibitor. In some embodiments, the Hedgehog antagonist is selected from SANT1 and cyclopamine. In some embodiments, the BMP signaling inhibitor is LDN193189. In some embodiments, the stem cell is not exposed to tri-iodothyronine (T3) or analog thereof during the differentiation process. In some embodiments, the stem cell is exposed to T3 or an analog thereof during the differentiation process. In some embodiments, the stem cell is not exposed to a protein kinase C activator during the differentiation process. In some embodiments, the stem cell is exposed to a protein kinase C activator during the differentiation process.

[0130] Various endoderm-inducing factors are known in the art, including, but not limited to, Activin-A and Wortmannin. Likewise, various endocrine-inducing factor are known in the art, including, but not limited to, retinoic acid, Hedgehog antagonists such as cyclopamine and SANT1, and BMP signaling inhibitors such as LDN193189.

[0131] The source of the stem cells used for preparing the disclosed cell-based composition is not particularly limited; however, choosing a cell/cell line that preferentially differentiates into an endodermal lineage, as disclosed herein, may increase the yield of insulin-producing cells and increase differentiation efficiency. Thus, in some embodiments, the stem cells used in the disclosed differentiation methods for preparing a cell-based composition are derived from pancreatic primary tissue. In some embodiments, the stem cells used in the disclosed differentiation method are embryonic stem cells. In some embodiments, the stem cells used in the disclosed differentiation method are induced pluripotent stem cells. In some embodiments, the stem cells used in the disclosed differentiation method are non-pluripotent reprogrammed cells. In some embodiments, the stem cells are human stem cells.

[0132] In some embodiments, the stem cells used in the disclosed differentiation methods are genetically modified to avoid activation of host lymphocytes. In some embodiments, the stem cells used in the disclosed differentiation methods are genetically modified to lack expression of MHC I and MHCII.

[0133] In some embodiments, the stem cells used in the disclosed differentiation methods are genetically modified to avoid targeted destruction by natural killer cells. In some embodiments, the stem cells used in the disclosed differentiation methods are genetically modified to express CD47.

[0134] For the purposes of the present disclosure, when preparing insulin-producing cells for incorporation into a cell-based composition for treating diabetes, it may be desirable to reprogram cells by expressing reprogramming genes in the cell without incorporating the reprogramming genes into the genome of the cell. Those of ordinary skill in the art will recognize that transduced genes can be expressed in a cell without incorporating those genes into the genome using, for example, episomal expression plasmids. The reprogramming genes may be expressed on at least

1, at least 2, at least 3, or at least 4 or more episomal expression plasmids. As discussed above, multiple reprogramming genes are known in the art and may be used for the purposes of the disclosed methods, but in some embodiments, the reprogramming genes comprise Oct4, Sox2, Klf4, and L-Myc.

[0135] In some embodiments, the cell-based composition is encapsulated in, for example, micro-capsules or macro-capsules.

[0136] The present disclosure also provides methods of treating diabetes using the disclosed cell-based compositions. The methods of treating diabetes generally comprise implanting a therapeutically effective amount of the disclosed SRE cells into a subject in need thereof. The therapeutically effective amount of SRE cells may be in the form of a cell-based composition, for instance, a population of SRE cells that are micro-encapsulated or macro-encapsulated.

[0137] Thus, in some embodiments, the methods comprise implanting into an individual in need thereof a therapeutically effective amount of insulin-producing cells encapsulated in macro-capsules. The composition of the macro-capsules is not particularly limited and those of skill in the art will understand that various materials can be used to encapsulate insulin-producing cells. For example, the capsules may comprise alginate, cellulose sulfate, glucomannan, or a combination thereof. In some embodiments, the macro-capsules may comprise at least one barrier in which the outer barrier is comprised of cellulose sulfate and glucomannan. In some embodiments, the macro-capsules may be formed in the shape of a cylindrical tube comprised of an inner capsule of alginate and an outer capsule of cellulose sulfate and glucomannan.

[0138] In some embodiments, the methods comprise implanting into an individual in need thereof a therapeutically effective amount of insulin-producing cells encapsulated in the disclosed macro-capsules about once a year, once every two years, once every three years, once every four years, once every five years, or more. In some embodiments, the implanted cells will survive for at least six months after implantation. Accordingly, in some embodiments, the subject may require only one implant. In some embodiments, the cell-based composition may need to be replaced once every 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 months, once every 1, 2, 3, 4, or 5 or more years or until the subject has recurring hyperglycemia, or a return to the diabetic state.

[0139] In some embodiments, the cell-based composition can be implanted into the greater omentum of the subject. The greater omentum (also known as the great omentum, omentum majus, gastrocolic omentum, epiploon, or caul) is a large apron-like fold of visceral peritoneum that hangs down from the stomach and extends from the greater curvature of the stomach back to ascend to the transverse colon before reaching to the posterior abdominal wall. Thus, the cell-based composition may be implanted into a pouch formed surgically from the omentum.

[0140] In some embodiments, the cell-based composition is implanted into the peritoneal cavity. In some embodiments, cell-based composition is implanted into the peritoneal cavity and anchored to the omentum. In some embodiments, the cell-based composition is implanted into an omentum pouch.

[0141] Exemplary doses of insulin-producing cells can vary according to the size and health of the individual being treated. For example, in some embodiments, an exemplary implant of cells encapsulated in the disclosed cell-based composition may comprise 5 million cells to 30 million cells per Kg of body weight.

[0142] Furthermore, the disclosed methods of treatment can additionally comprise the administration of a second therapeutic in addition to the encapsulated therapeutic cells. For example, in some embodiments, the additional therapeutic compound can include, but is not limited to, insulin injections, metformin, sulfonylureas, meglitinides, thiazolidinediones, DPP-4 inhibitors, GLP-1 receptor agonists, and SGLT2 inhibitors.

[0143] Particular treatment regimens comprising implanting the cell-based composition comprising insulin-producing cells may be evaluated according to whether they will improve a given patient's outcome, meaning it will help stabilize or normalize the subject's blood glucose levels or reduce the risk or occurrence of symptoms or co-morbidities associated with diabetes, including but not limited to, episodes of hypoglycemia, elevated levels of glycosylated hemoglobin (HbA1C levels), heart disease, retinopathy, neuropathy, renal disease, hepatic disease, periodontal disease, and non-healing ulcers. In some embodiments, the cell-based composition will be encapsulated, for example, in a capsule comprising alginate, cellulose sulfate, glucomannan, or a combination thereof.

[0144] Thus, for the purposes of this disclosure, a subject is treated if one or more beneficial or desired results, including desirable clinical results, are obtained. For example, beneficial or desired clinical results include, but are not limited to, one or more of the following: decreasing one or more symptoms resulting from diabetes, increasing the quality of life of those suffering from diabetes, decreasing the dose of other medications required to treat diabetes, delaying or preventing complications associated with diabetes, and/or prolonging survival of individuals.

[0145] Furthermore, while the subject of the methods is generally a subject with diabetes, the age of the patient is not limited. The disclosed methods are useful for treating diabetes across all age groups and cohorts. Thus, in some embodiments, the subject may be a pediatric subject, while in other embodiments, the subject may be an adult subject.

[0146] One skilled in the art will readily appreciate that the present disclosure is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the disclosure. The following examples are given to illustrate the present invention. It should be understood, however, that the invention is not limited to the specific conditions or details of these examples.

Examples

Example 1 – SRE Cells Expressing Pdx1 and ISL1

Materials and Methods

[0147] *Islet harvest:* Properly consented and anonymized whole human pancreata were obtained from registered organ donation. The lobes were injected with collagenase P (Roche #1129 002 001) re-suspended to 1.4 mg/ml in islet isolation solution (Hanks Balanced Salt Solution (Invitrogen #14065-056) containing 0.35 g NaHCO₃ per L and 1% Human Serum Albumin (Roche A9731)). The inflated lobes were incubated at 37°C for 15-25 min with mild agitation. The digest was diluted with cold islet isolation solution and centrifuged at 1500 RPM for 5 min. The supernatant was discarded and the pellet was washed in cold islet isolation solution with vigorous trituration. The solution was filtered through a 420 µm sieve (Bellco Glass, Inc, Cat# 1985-00040)

and centrifuged. The pellet was re-suspended in 1.100 g/ml Histopaque (Sigma #10771, Sigma #11191) and centrifuged for 30 min at 1200 RPM. The supernatant was collected, diluted 2X in islet isolation solution and centrifuged at 1500 RPM for 5 min. The pellet was rinsed in islet isolation solution, centrifuged, and cultured in E8 medium (Gibco #A1517001) in a humidified incubator at 37°C and 5% CO₂. The following day, the islets were centrifuged at 1500 RPM for 5 min and re-suspended in undiluted TrypLE Select 10X (Life Technologies #A12177) and incubated for 10 min at 37°C. The dissociated islets were diluted in E8 media, centrifuged, re-suspended in E8 supplemented with 100 ng/ml hydrocortisone (Sigma #H0135), 1 U/ml thrombin (Sigma #T9326), and 100 ng/ml EGF (Sigma E5036). Cells were cultured on dishes coated with vitronectin (Life Technologies # A14700) following manufacturer's instructions.

[0148] *Reprogramming:* Cells were rinsed with PBS (Gibco #14190144) and incubated in TrypLE select 1X for 5 min at 37°C. Digestion was arrested with E8 medium and cells centrifuged at 1000 RPM for 5 min. Cells were re-suspended in BTX electroporation solution (VWR #89130-542) at 2×10^6 cells per 200 μ l and added to electroporation cuvette with 20 μ g of reprogramming plasmids. Two reprogramming plasmids comprising EBNA episomal expression sequences, ampicillin resistance, and the reprogramming genes Oct4, Sox2, Klf4, and L-Myc under the control of the CMV promoter were constructed. Electroporation cuvette was pulsed using a gene pulser XL (Bio-Rad). Cells were transferred to vitronectin-coated dishes in E6 medium (Life Technologies # a1516401) supplemented with 100 ng/ml bFGF (Life Technologies # PHG6015) and 1 μ M hydrocortisone. Cells were cultured at 37°C in a humidified incubator with 5% CO₂. After 24 h, media was changed with E6 supplemented with 100 ng/ml bFGF, and 1 μ M hydrocortisone, and 100 μ M sodium butyrate (Sigma # P1269), and changed every other day. Stem cell colonies were manually detached and transferred to vitronectin-coated dishes in E8 medium. 73 lines generated from the primary tissue of two donors were initially screened for the ability to express endodermal markers after 4 days exposure to endoderm-inducing agents Activin-A and Wortmannin. Cultures with the highest proportion of cells expressing endodermal markers were selected. Twenty-four cell lines having passed the first screen were subsequently screened for the ability to express pancreatic markers after exposure to a 12-day pancreatic differentiation protocol. The cell line that

consistently generated the highest proportion of pancreatic cells was named SR1423, and was banked and used for all subsequent experiments.

[0149] *Cell line characterization:* SR1423 expressed markers typical of pluripotent cells and had a normal karyotype. The DNA STR profile of SR1423 confirmed that it is a single cell line that matches the donor tissue. Additionally, SR1423 grows at a rate typical of pluripotent cell lines. It was observed that other induced pluripotent stem cell (hereinafter called “iPSC”) lines from the same donor and reprogramming experiment demonstrated preferential differentiation as well. The iPSC line “B” also differentiated well to endoderm while lines “C” and “D” showed no preference for differentiation to the endodermal lineage (data not shown). In order to determine whether there was a correlation between gene expression profiles and the inability of iPSC to differentiate into specific lineages, whole-genome microarray profiling of expressed genes of SR1423 as well as lines B, C, and D were performed. *See e.g.,* Southard SM. *et al* PLoS ONE 13(9) (2018), Koyanagi-Aoi M. *et al. Proc. Natl. Acad. Sci.* 110 (2013). Unsupervised hierarchical clustering analysis based on fold change expression of at least Log2, revealed that SR1423 clustered together with cell line B, but not with from C and D. This identified a gene expression pattern that correlates with robust and preferential differentiation to the endodermal lineage. Of the 10 most differentially expressed genes, BHMT2, Cox7A1, HSPB2, and NAP1L1 correlated significantly with ability to form endoderm using a qRT-PCR measure.

[0150] *Stem cell culture:* Undifferentiated iPSC cells were maintained in 6-well tissue culture plates (Greiner Bio-One #657160) coated with vitronectin XF (Stem Cell Technologies #07180) or 17 $\mu\text{g}/\text{cm}^2$ Geltrex (Life Technologies #A1413301) following manufacturer’s instructions and fed daily with E8 medium. Cultures were passaged at 75-85% confluence every 3-5 days with 0.5 mM EDTA (Life Technologies #15575) and seeded at 7×10^3 cells/ cm^2 .

[0151] *Differentiation:* Undifferentiated adherent cells were exposed to a multi-stage differentiation protocol as follows: Stage 1 (3 days) comprised of a 50:50 mixture of RPMI:CMRL supplemented with B27, Activin A and Wortmannin; Stage 2 (2-8 days) comprised of low glucose DMEM supplemented with B27, SANT1, RA, LDN, and KGF; Stage 3 (4 days) comprised of low glucose DMEM supplemented with B27, SANT1, RA, KGF and EGF. During stage 3, the three-

dimensional cell clusters are transferred to suspension culture; Stage 4 (4 days) comprised of low glucose DMEM supplemented with B27, SANT1, RA, Gsxxi, Alk5i, zinc sulfate, and staurosporine; Stage 5 (4-35 days) comprised of low glucose DMEM supplemented with zinc sulfate.

Characterization of SRE Clusters

[0152] *Flow Cytometry*: Between days 28 and 40, the SRE clusters were analyzed by flow cytometry for the proportion of cells expressing endocrine pancreas-specific markers. At least 500 clusters were dispersed to single cells by incubation in Trypsin EDTA solution for 5 min at 37°C with shaking at 1000 RPM. The reaction was stopped by addition of 20% of the volume of fetal bovine serum. The cell suspension was passed through a 70 µM cell strainer to collect a suspension of single cells. Cells were quantified using a BioRad TC20 automated cell counter. 100,000 to 200,000 cells were used for flow cytometry of each marker combination and unstained controls. Cells were pelleted and re-suspended in BD Cytofix/Cytoperm and incubated at ambient temperature for 20-30 min. The cells were pelleted in a refrigerated centrifuge at 5°C for 2 min at 2000 RPM. The cell pellet was re-suspended in BD was buffer. The cells were pelleted again and re-suspended in PhosPerm buffer III for 20-30 min on ice. Cell were pelleted again and re-suspended in BD wash buffer. Cells were pelleted again and re-suspended in wash buffer supplemented with fluorophore conjugated primary antibodies or isotype controls. Antibodies used were: anti-PDX1-Alexa-647 (1:100 dilution), anti-insulin-Alexa-647 (1:2500 dilution), anti-Nkx6.1-Alexa-488 (1:50 dilution), anti-Glucagon-PE (1:500 dilution), anti-ISL1-PE (1:100 dilution), Alexa-647 isotype control(1:100 dilution), Alexa-488 isotype control (1:100 dilution), or PE isotype control (1:500 dilution). Samples were incubated for 1 h, at ambient temperature, protected from light. The cells were pelleted again and re-suspended in wash buffer. Cells were pelleted again and re-suspended in 1% FBS in PBS. Samples were analyzed using a Guava flow cytometer running InCyte software. FIG. 1 shows that SRE clusters are comprised of cells that express predominantly Pdx1, ISL1, and insulin. A smaller proportion of cells express glucagon. In contrast Nkx6.1 expressing cells are almost undetectable.

[0153] *Immunofluorescence*: Between days 28 and 40, the SRE clusters were visualized by immunofluorescence for endocrine pancreas-specific markers. At least 100 clusters were fixed in 4% paraformaldehyde for 20 min at room temperature. Fixed clusters were allowed to gravity settle and were re-suspended in 20% sucrose in PBS. The solution was incubated at ambient temperature until all clusters had settled to the bottom of the tube. Clusters were re-suspended in OCT freezing medium, transferred to a cryo-mold and snap frozen in liquid nitrogen. Frozen blocks were sectioned to a thickness of 10 μ m using a Shandon cryotome and transferred to silane-coated microscope slides. Sections on microscope slides were permeabilized in 0.5% Triton-X-100 in PBS for 20 min and rinsed in PBS. Sections were incubated in blocking buffer comprised of 10% horse serum in 0.1% TX-100 in PBS for 1 h. Sections were rinsed in PBS and incubated in primary antibodies diluted in 1% horse serum in 0.1% TX-100 in PBS. All primary antibodies were diluted 1:100. Sections were incubated at 5°C overnight. Sections were rinsed in PBS and incubated for 2 h, at ambient temperature in secondary antibodies in 1% horse serum in 0.1% TX-100 in PBS. Secondary antibodies used were horse anti-rabbit-Alexa-488, horse anti-mouse-Alexa-595, and horse anti-goat-Alexa-595. Sections were rinsed in PBS and counterstained for 2 min in 1:1000 Hoescht in PBS. Sections were rinsed in PBS covered in Fluoromount solution, covered with a coverslip and imaged using an EVOS fluorescence microscope with DAPI, Rhodamine, and fluorescein filters. All images were photographed at a total magnification of 200X. FIG. 2 confirms that SRE clusters are comprised of cells that express predominantly Pdx1 and ISL1, and that Nkx6.1 expressing cells are almost undetectable. ISL1 is expressed in the nuclei of cells that express C-peptide and in the nuclei of cells that express the endocrine pancreas marker chromogranin A. Although Nkx6.1-expressing cells are nearly absent, the few cells that do express Nkx6.1 do not co-express ISL-1.

[0154] *Performance of SRE clusters*: Between days 28 and 40, the SRE clusters were exposed to varying levels of glucose, and the supernatant was analyzed for the presence of secreted C-peptide. 50 SRE clusters were rinsed in bicarbonate buffer supplemented with sodium chloride, calcium chloride, magnesium chloride and hydrochloric acid (GSIS buffer). SRE clusters were allowed to gravity settle and re-suspended in 300 μ L GSIS buffer supplemented with 2.2 mM glucose for 20 min at 37°C and 5% CO₂. Clusters were allowed to gravity settle, supernatant was collected and

stored on ice. This procedure was repeated 3 additional times. Clusters were then re-suspended in 16.7 mM glucose for 20 min. This was repeated a second time. Clusters were then re-suspended in GSIS buffer supplemented with 16.7 mM glucose and 30 mM potassium chloride for 20 min. Supernatants were analyzed for C-peptide content using a Mercodia C-peptide ELISA kit and quantified using an absorbance plate reader. FIG. 3 shows that incubation of 50 SRE clusters for 4, 20 min intervals in 2.2 mM glucose demonstrate a gradually decreasing C-peptide secretion. Exposure to 16.7 mM glucose causes increased C-peptide secretion by 40 minutes. Artificially stimulating C-peptide secretion in the presence of 30 mM KCl shows a high reserve of C-peptide available for secretion.

Example 2 – SRE Cells Expressing MAFA and ISL-1

Introduction

[0155] The islet replacement cure for Type 1 diabetes is unavailable to most patients due to lack of donor islet supply and the requirement for potent immune suppression. Islet-like clusters grown from immortal human pluripotent stem cells are a viable replacement for cadaveric islets and can be manufactured at large scale. Pluripotent stem cells are defined by an inherent ability to follow maturation pathways that specify every tissue of the developing embryo and, eventually, all adult tissues. Protocols for guiding pancreatic maturation of pluripotent stem cells aim to trigger pancreatic fate choice and restrict non-pancreatic lineages. These protocols create populations comprised of cells with functional pancreatic features and off-target cells. It is hypothesized that the presence of cells without appropriate endocrine specification can diminish the clinical potency and safety of the population.

[0156] Induced pluripotent stem cells are epigenetically distinct from embryonic stem cells and can retain epigenetic imprinting of the source tissue, leading to differential gene expression and differentiation capacity between cell lines. The aim was to capitalize on this phenomenon by reprogramming primary pancreatic islet cells from a consenting human donor to create a stem cell line with enhanced pancreatic differentiation potential. To achieve this, a protocol was developed for episomal reprogramming of these primary pancreatic islet cells, and the immortalized clones were searched to identify populations that efficiently responded to maturation cues to re-form

populations of cells with pancreatic hallmarks. The best performing cell line (clone SR1423) is characterized by a restricted fate potential and therefore failed to meet criteria of pluripotency. Further characterization reported here emphasizes the distinction between SR1423, induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs).

[0157] Stem-cell-derived pancreatic clusters are less functionally mature than primary islets and contain cell populations with transcriptomic and epigenomic profiles that share some features with fetal, neonatal, and infant cell populations. To date, the fates of differentiating cell populations that express subsets of pancreatic markers are not well defined. In particular, distinct progenitor populations are identified based on differential expression of the transcription factor NKX6-1 among cells expressing the transcription factor PDX-1 *in vitro* and *in vivo*. PDX-1 is a key determinant of pancreatic activity that is activated early in pancreatic commitment and remains expressed through development and in the mature endocrine and exocrine pancreatic tissue. NKX6-1 is activated in early pancreatic endocrine cell commitment and is again expressed in insulin-secreting beta cells. Among populations of differentiating pluripotent stem cells, it has been suggested that PDX-1+/NKX6-1+ are fated to form functional insulin-secreting beta cells, while PDX-1+/NKX6-1- cells form polyhormonal cells that either resolve *in vivo* to glucagon-secreting alpha cells or non-functional off-target cells.

[0158] A population of beta cells lacking NKX6-1 expression, however, was recently identified in rodent islets. Additionally, Pavluch et al. demonstrated that NKX6-1 expression was found to be dispensable for glucose sensitive insulin secretion in rat insulinoma cells. Furthermore, NKX6-1 also has an important role in the specification of the enterochromaffin population of serotonin-producing enteroendocrine cells of the gut. Pancreatic and enterochromaffin populations expressing NKX6-1 emerge from a common NGN3-expressing progenitor population and share similar functional features. Because serotonin-expressing cells are common among populations of stem cell-derived pancreatic cells, it is speculated that stem cell-derived pancreatic populations expressing NKX6-1 may contain off-target cells with enterochromaffin identity. Therefore, NKX6-1 is not a clear determinant of beta cell identity or fate commitment.

[0159] The expression of transcription factors ISL1 and MAFA are also expressed in early endocrine progenitors and again in maturing islets, indicating roles in both specification of endocrine-committed progenitors and in maturation or maintenance of functional endocrine cells. MAFA expression is limited to insulin expressing cells of rodents and MAFA-deficient mice exhibit impaired glucose sensitivity and diabetes. Overexpression of MAFA, in combination with PDX1 and NGN3 can drive programming of the beta cell phenotype in human pluripotent cells and transdifferentiate exocrine pancreas when combined with Pax4. It is therefore possible that MAFA and ISL1 in the absence of NKX6-1 in stem cell-derived pancreatic populations may reflect beta cell fate and have high *in vivo* potency for islet replacement therapy. *In vivo* potency of a population of stem cell-derived pancreatic cells that predominantly expressed NKX6-1 and a population that lacked NKX6-1 expression but highly expressed MAFA and ISL1 were compared.

Materials and Methods

[0160] hPSC Scorecard. Undifferentiated SE1423u cells were thawed and routinely cultured until passage 23. Near-confluent 10 cm dishes were lysed in TriZOL and stored at -80 °C. RNA was harvested from the TriZOL lysate according to the purification protocol described in the ThermoFisher TaqMan hPSC Scorecard manual. The nucleic acid pellets were dissolved in 50 µL of nuclease-free water, and then aliquots were stored at -80 °C. Once thawed, nucleic acid concentration was determined by NanoDrop. Reverse transcription was performed in duplicate following the ThermoFisher TaqMan hPSC Scorecard Assay protocol. Briefly, 2 µg of nucleic acid was mixed with ThermoFisher High Capacity cDNA synthesis reagents (random hexamer primers, RNase inhibitor, buffer, reverse transcriptase, dNTPs, and water). The mix was incubated at 37 °C for 2 hours, and then enzymes were heat-inactivated at 85 °C for 5 minutes. cDNA was stored at -80 °C until use. Quantitative qPCR was performed according to the ThermoFisher hPSC Scorecard Assay manual. Pre-made ThermoFisher hPSC Scorecard panel 96 well plates were loaded with cDNA template and TaqMan Fast Advanced PCR Master Mix. Cycling parameters were programmed according to the Scorecard Assay manual. The procedure was repeated. The results files contained Ct values for each well, and these values were then copied into a template provided by ThermoFisher, which was formatted for use with the Scorecard online software. The

formatted values were saved as .txt files for uploading into the Scorecard online software. Results from each replicate were uploaded into the software and pooled.

[0161] Cell Culture and Differentiation. Undifferentiated stem cells were maintained in mTeSR-E8 (Stem Cell Technologies) on plastic dishes precoated with full-length vitronectin (Peprotech) with daily media changes. Guidance cues for differentiation and their durations are outlined in Table 1. Stages 1 and 2 used basal media comprised of 1:1 mixture RPMI and DMEM F-12 supplemented with BSA and SM1 (Stem Cell Technology) or B27 (ThermoFisher). Stages 7-9 used a 1:3 mixture of CMRL and RMPI basal media supplemented with BSA. Stages 6-9 were carried out in suspension culture using a vertical wheel bioreactor culture system.

Table 1: Comparison of guidance cues that drive differentiation of SR1423U to NKX6-1 high and low populations

Differentiation protocols comparison					
NKX6-1-high			NKX6-1-low		
Stage	Length (days)	Guidance cue	Stage	Length (days)	Guidance cue
1	3	Activin A	1	1	Activin A
		PI3K inhibition (Wortmannin)			Wortmannin
2	2	Fibroblast growth factor 7 (FGF7)			GSK3 inhibition (CHIR99021)
3	1	Retinoic acid (RA)	2	2	Activin A
		Hedgehog inhibition (SANT1)			Wortmannin
		FGF7	3	2	FGF7
		BMP inhibition (LDN193189)	4	2	RA
4	4	LDN193189			SANT1
		Activin A			FGF7
		Nicotinamide			LDN193189
		Epidermal growth factor (EGF)	5	4	RA (1/20th conc)
5	4	RA (1/20th conc)			SANT1
		FGF7			FGF7
		EGF			LDN193189
		SANT1	6	4	RA (1/20th conc)
6	6	SANT1			SANT1
		RA (1/20th conc)			FGF7
		Notch signaling inhibitor (GSXXi)			EGF
		LDN193189	7	6	RA (1/20th conc)
		TGFbeta inhibition (Alk5i)			SANT1
		EGF			GSXXi
7	4	SANT1			Alk5i
		RA (1/20th conc)	8, 9	19	No additive
		Alk5i			
		Nicotinamide			
		Insulin-like growth factor 1 (IGF1)			
		Triiodothyronine (T3)			
8	15	SANT1			
		RA (1/20th conc)			
		Alk5i			
		Nicotinamide			
		IGF1			
		T3			
		TGF beta 1			

[0162] Flow Cytometry. Single cell suspensions were prepared from clusters incubation in trypsin/EDTA (VWR). 20% of the total volume of fetal bovine serum was added, and the tube was placed on ice. The suspension was passed through a 70 µm filter. The eluate was quantified from

20 µL aliquots that were mixed with trypan blue and counted twice using the TC20 automated cell counter (BioRad). Cells were pelleted and resuspended in 1 mL of Cytofix/Cytoperm solution (Becton Dickinson) and incubated at ambient temperature for 30 minutes. 150,000 cells were pelleted in a refrigerated centrifuge and washed in BD wash buffer (Becton Dickinson) for 20 to 30 minutes on ice. Cells were pelleted and resuspended in 1:100 dilution of fluorophore conjugated secondary antibodies in BD wash buffer and incubated for 1 hour at room temperature or overnight at 4 °C. Cells were rinsed in BD wash buffer and resuspended in 1% BSA in PBS. Samples were quantified alongside unstained controls using a Guava flow cytometry system.

[0163] Immunofluorescence. Samples were fixed for 30 minutes in 4% paraformaldehyde, rinsed in PBS, and resuspended in 30% sucrose until sedimentation to the bottom of the vessel. Fixed samples were cryopreserved in OCT media cryosectioned to a thickness of 10 µm and collected on silane-coated microscope slides (VWR). Sections were permeabilized in 2.5% TX-100 (Sigma) dissolved in PBS for 20 minutes, rinsed, and incubated in a blocking solution of 1% TX-100 and 10% horse serum for 1 hour. Sections were rinsed and incubated overnight at 4 °C in primary antibody diluted 1:100 in PBS supplemented with 1% TX-100 and 2% horse serum. Slides were rinsed and incubated in 1:1,000 dilution of fluorophore conjugated secondary antibodies raised in horse for 1 hour. Sections were rinsed, incubated in 20 nM Hoescht dye for 2 minutes, rinsed, secured under a coverslip, and visualized using an Evos Fluorescent microscope (ThermoFisher).

[0164] scRNA-Seq. SR1423 differentiated cells were harvested on the final day of each differentiation stage. For adherent culture stages, one confluent 15 cm tissue culture dish was washed with PBS and then treated with 5 mM EDTA for 5 minutes at 37 °C, and single cell suspensions were generated by manual trituration. Single cells were cryopreserved prior to library preparation. For suspension phases of SR1423 differentiation, approximately 50 mg of clusters were collected and cryopreserved in culture media + 10% DMSO. Nuclei were isolated directly from cryopreserved cells and cluster samples, which were then used for single cell library preparation by Sigulomics, Inc. using 10X Genomics 3' library prep kit v3 chemistry. Nuclei were loaded into the chromium controller to target 5,000 nuclei for sequencing. Following library

preparation, sequencing was performed at Novogene USA using the NovaSeq 6000 sequencer (Illumina) with a target of 35,000 reads per cell.

[0165] scRNA-Seq Data Processing and Analysis. Following sequencing, reads were aligned to the human reference genome (GRCh38) using CellRanger v.7.1.0 (10X Genomics). Next, data were processed in R V.4.3.0 using Seurat v5. Filtering was performed to exclude cells with fewer than 500 genes expressed and less than 3000 transcripts detected. Following filtering, data were normalized, and the top 3000 variable genes were selected for integration. Principal component analysis was performed on these variable genes, and data were integrated via reciprocal principal component analysis using the first 30 principal components. The RNA expression matrix was loaded into VISION v3.0.1, and single cell pathway analysis was performed using default parameters and the full Reactome pathway database. The results of VISION pathway analysis were added to the Seurat object as a new assay. The top 200 variable pathways were identified and used for principal component analysis, nearest neighbor graph embedding, and clustering with a resolution of 0.5. Uniform manifold projection was calculated based on the first 20 principal components of the Reactome pathway analysis. Dropouts were imputed using the RunALRA function implemented through SeuratWrappers using default parameters. Drop out-imputed data were only used for visualizing gene expression. Trajectory and pseudotime analyses were performed using Monocle v2. For these analyses, the normalized RNA expression matrix was downsampled to 1000 cells from each starting sample and then loaded into Monocle. Within Monocle, dimensionality reduction was performed using the DDRTree method and pseudotime computed.

[0166] Animal Experiments. Animal husbandry and animal implants, care and monitoring were carried out using IACUC-approved protocols at the contract research facilities of Noble Life Sciences and Bioqual, Inc. NSG mice were sourced from Jackson Labs. SRG rats were sourced from Envigo.

[0167] Streptozotocin. Diabetes was induced with a single intraperitoneal injection of 150 mg/kg of streptozotocin (mice, Sigma) or 50 mg/kg (rats) to nonfasting animals. Diabetes was confirmed

by two blood glucose measurements above 350 mg/dL. Hyperglycemic animals were provided a dose of subcutaneous insulin pellets following manufacturer's instructions (Linshin Canada).

[0168] *Implants – Subcapsular Renal Implant.* 2,500 or 5,000 islet equivalents of Seraxis islet-like clusters were counted using the islet cell counter (Biorep) automated method and transferred to a 1.5 mL conical tube for mouse implants. 10,000 or 40,000 clusters were manually counted and transferred to a 1.5 mL conical tube for rat implants. Islet-like clusters were pelleted using a tabletop microfuge and aspirated into the tip of a 15-gauge flexible gavage needle. This was deposited into a renal subcapsular pouch using standard veterinary surgical techniques.

[0169] *Implants – Fat Pad/Omentum Implants.* The gonadal fat pad was exited from the lower abdomen using standard veterinary surgical technique and spread flat along the abdomen. 2,500 or 5,000 islet equivalents of Seraxis clusters were pelleted using a tabletop microfuge and resuspended in 20 μ L of purified human plasma (Tisseel, component A). The cluster/plasma mixture was deposited onto the fat pad. An equal volume of fibrin solution (Tisseel, component B) was layered on to the cluster plasma mixture, which was enclosed by folding of the remaining fat pad tissue. For rat implants, the omentum was exited from the upper abdomen, and 10,000 or 40,000 clusters were deposited in the same fashion.

[0170] *Implants – Intrasplenic Implants.* 50 islet-like clusters or primary islets were incubated sequentially in buffer supplemented with 2.2 mM and 16.7 mM glucose for 30 minutes, followed by a 30 minute incubation of KCl. Supernatant was analyzed for C-peptide content by ELISA (Mercodia). The secretion index represents the ratio of C-peptide secreted during exposure to 16.7 mM glucose over C-peptide secreted during exposure to 2.2 mM glucose. Total C-peptide release corresponds to the sum of C-peptide secreted during both exposures and KCl.

Results

[0171] Undifferentiated Stem Cell Line. The human stem cell line, SR1423, was generated by causing transient expression of the Yamanaka factors in cells of human islets harvested from a consented donor pancreas with high HLA and blood type compatibility for allogenic donor organ transplantation. Rather than screen for pluripotency, this cell line was selected for its ability to

differentiate toward the definitive endoderm and, subsequently, PDX-1-expressing pancreatic progenitor cell fate. Analysis showed that SR1423 does not meet criteria for pluripotency as it fails to express the master control gene for mesoderm specification, brachyury, in response to mesoderm-inducing agents. The hPSC Scorecard Assay was employed for a comparison of pluripotency-related genes to a reference dataset of multiple pluripotent stem cells. Undifferentiated SR1423 demonstrated a gene expression profile outside of the range of the reference dataset with mesodermal differentiation potential scoring lowest (FIG. 4A-4B). Consistent with previous directed differentiation experiments, the T gene (TBXT, brachyury) was highly down-regulated while the early endoderm specifying transcription factors SOX17 and HNF1B was found to be up-regulated without any external differentiation cues.

[0172] Transcriptome analysis by single-cell RNA sequencing (scRNA-Seq) also demonstrated a gene expression profile of the undifferentiated cells that manifests distinctions from previously published iPSC and ESC datasets (FIG. 5A-5C). Unsupervised clustering with iPSC (GSE202398) and hESC (GSE143783) single cell datasets revealed SR1423U are unique from each stem cell type but more similar to iPSC than hESC (FIG. 5A). Pathway analysis also demonstrated that transcriptional regulation of SR1423U falls between that of iPSC and hESC (FIG. 5B) with a unique profile of transcription factor expression (FIG. 5C). Consistent with hPSC Scorecard Assay data and lack of mesoderm potential, low expression of POU5F1 (OCT4), NANOG, and LIN28 suggest incomplete reprogramming of SR1423U. Together, these data demonstrate that SR1423U is a unique, partially reprogrammed islet-derived stem cell.

[0173] SR1423 Differentiation. SR1423 responds to basic differentiation protocols that drive endocrine pancreatic fate choice and generates highly pure populations of cells with pancreatic endocrine cell characteristics. As previously described, SR1423 is a multipotent, and not pluripotent, stem cell line. SR1423 was therefore characterized as a multipotent stem cell line with pancreatic endoderm bias. SR1423 was derived and maintained in a clean room following good manufacturing practices (GMP) principles. Master cell and working cell banks were created and characterized for lack of adventitious agents and other parameters relevant for clinical application. Most work reported here was performed on aliquots from the working cell bank.

[0174] Two maturation protocols were designed that both guided the differentiation of SR1423 cells to clusters that contained native proportions of C-peptide and glucagon-expressing cells, and demonstrated robust, physiologic glucose responsive C-peptide secretion. C-peptide, a by-product of insulin synthesis is secreted by beta cells in equimolar concentrations to insulin and is frequently used as a reliable surrogate marker of insulin production and secretion.

[0175] *First Differentiation Protocol.* A review of the literature that describes the differentiation of pancreatic islet cells from pluripotent stem cells suggest that an ideal target population contains a large proportion of cells that co-express the transcription factors PDX-1 and NKX6-1, and the hormone insulin, a profile found in the beta cells of the native adult islet. Therefore, the first profile was designed based on these principles that drives SR1423 to generate islet-like clusters with a high proportion of cells that expressed PDX-1, NKX6-1, and the by-product of insulin processing, C-peptide (detailed in methods, Table 1). Flow cytometry and immunofluorescent staining (FIG. 6A-6B) showed a population of cells that co-express PDX-1, NKX6-1, and C-peptide. These cell populations secreted C-peptide in response to glucose *in vitro* (FIG. 7A-7B). C-peptide expression is found in the absence of NKX6-1, and some NKX6-1 expressing cells lack C-peptide (FIG. 6A-6B). Thus, NKX6-1 expression is neither necessary nor sufficient for C-peptide expression in this cell population.

[0176] *Second Differentiation Protocol.* The cells from the second protocol more robustly expressed the pancreatic transcription factors ISL-1 and MAFA. The second protocol was designed that primarily generated cells that lacked expression of NKX6-1 but maintained expression of PDX-1 and C-peptide. Flow cytometry and immunofluorescent staining (FIG. 6A-6B) confirmed a population of cells that co-express PDX-1 and insulin, and a low proportion of cells that express NKX6-1. This population more robustly expresses the transcription factors ISL-1 and MAFA by immunofluorescence microscopy than cells from the first protocol. ISL-1 is expressed in early pancreatic endocrine progenitors and remains expressed in the mature pancreatic endocrine cells. ISL-1 is a key activator of NKX6-1 and other genes found in mature glucose-sensing beta cells. MAFA is a regulator of beta cell maturation. The clusters from this protocol secreted C-peptide at levels similar to native islets in static culture conditions (FIG. 7A-7B)

[0177] *In Vivo* Performance. Islet-like clusters from NKX6-1-high and NKX6-1-low populations were transplanted to immune-compromised mice and rats chemically rendered diabetic by application of streptozotocin. Clusters were delivered to the renal subcapsular space, intrasplenic, to a pouch formed from the omentum (rats), or a pouch formed from the gonadal fat pad (mice). Animals that received NKX6-1-low clusters reproducibly achieved glucose control within the time frames analyzed (FIG. 8A), while animals that received NKX6-1-high clusters did not (FIG. 9). Glucose control was not influenced by site of implant at these sites. Glucose control was achieved in mice that received doses of NKX6-1-low clusters equivalent to 2,500 and 5,000 human islets. Animals demonstrating glucose control at 11-13 weeks post-engraftment demonstrated the ability to control blood glucose in a manner similar to non-diabetic controls after oral gavage of dextrose (FIG. 8B).

[0178] Grafts of NKX6-1-high clusters (first protocol) revealed a loss of C-peptide expression in the NKX6-1-positive population over time (FIGS. 10A-10D). This contrasts with previous reports that PDX-1/NKX6-1 co-positive cells mature in a hyperglycemic background *in vivo* to generate insulin-secreting cells homologous to native beta cells in this time frame. Clusters from both protocols were found on explant to have been remodeled with host tissue to form structures with abundant, human endocrine tissue comingled with remodeled host tissue (FIGS. 10A-10H). Although the clusters were delivered as a bolus, explants revealed islet-like structures that were separated from each other by tissue that resembled parenchyma, and in some cases, ducts. Among explants of NKX6-1-low grafts, expression of endocrine markers within the islet-like structures were similar to native islets with robust chromogranin A (FIG. 10E) and PDX-1 (FIG. 10G) co-expressed with C-peptide or insulin. NKX6-1 was expressed with ISL-1 and C-peptide, indicating *in vivo* activation. Pancreatic endocrine cells were also recognized by anti-human mitochondria antibody, demonstrating human origin. These data demonstrate that pancreatic clusters derived from SR1423 stem cells actively cooperate or drive remodeling of rodent host tissue to form stable pancreatic grafts with anatomic features of endocrine pancreas. Clusters from NKX6-1-low populations maintain persistent expression of C-peptide, continue along islet maturation pathways and are potentially capable of regulating rodent blood glucose.

[0179] NKX6-1-low Characterization. Unsupervised transcriptome analysis of the NKX6-1-low protocol by scRNA-Seq using the Uniform Manifold Approximation and Projection (UMAP) method for dimensionality reduction reveals that stages 0 (undifferentiated), 1, and 2 cluster separately in discrete groupings in agreement with the drastic morphological changes that occur when stem cells launch differentiation (FIG. 11A-11H). These and subsequent stages manifest gene expression profiles consistent with progressive maturation to the pancreatic endocrine identity. Stages 1 and 2 exhibit endodermal gene expression profiles including CXCR4, SOX17, CER1, and LEFTY1. The guidance cues of stages 3-6 drive expression of genes characteristic of pancreatic commitment such as FOXA2, HNF4A, HNF1B, PDX-1, SOX9, and PTF1A. The guidance cues of stages 7, 8, and 9 promote the progression adoption of gene expression characteristic of the endocrine pancreas including NKX2-2, NEUROG3, NEUROD1, MAFB, MAFA, PAX4, PAX6, ISL-1, CHGA, and CHGB. Concurrent expression of MafA, Ngn3, Pdx1, and Pax4 are observed in stage 7 (FIG. 11C). The stage 9 cluster is adjacent to the cluster formed by native human islets (FIG. 11A). These findings of differentiation towards pancreatic islets are additionally supported by pseudotemporal trajectory analysis performed using Monocle, which demonstrates a progressive, off-target-free differentiation of SR1423 stem cells into islet-like clusters. Furthermore, pathway analysis using VISION demonstrates a stage-dependent acquisition of β cell-like identity and regulation of gene expression (FIGS. 11F-11G). Together, these data describe a progressive differentiation from stem cells through endoderm, gut tube, pancreatic progenitor, and finally pancreatic endocrine commitment.

[0180] Next, the late stages of SR1423 were further characterized by focusing analyses on pancreatic endocrine cells. Based on the snRNA-Seq data, SR1423 differentiation yields a pure population of endocrine cells exhibiting 100% positivity for CHGA among pancreatic endocrine and endocrine progenitors (stages 7-9, FIG. 11H). YBX1-INSM1-FEV is required for the specification and maintenance of endocrine progenitor identity. These genes are also found in stages 7-9 as well as mature human islets. Despite glucose responsive insulin production these cells retain expression of genes critical to maintenance of pancreatic progenitors affirming SR1423-derived pancreatic endocrine cells as less mature than adult islets but committed to that fate.

Discussion

[0181] The study demonstrates that two disparate populations of pancreatic cells, distinguished by expression of NKX6-1, have similar *in vitro* potency in terms of insulin expression and secretion, but different *in vivo* potency. The NKX6-1-low cells were better able to control blood glucose in diabetic rodents and explants showed a stable cell identity and induction of NKX6-1 *in vivo*. These results are contrary to the established differentiation path of native beta cells. The observations of co-expression of the transdifferentiating factors PDX1, MAFA, NGN3, and PAX4 may explain the adoption of beta-cell like identity *in vitro* in the absence of NKX6-1. Cells lacking NKX6-1 are reported to represent a multi-hormonal population in differentiating stem-cell derived islets. C-peptide and glucagon are largely separate populations in NKX6-1-low clusters described here; thus, NKX6-1-low cells may have an alternative identity. One possible fate would be that of the islet “hub” cells, that are not polyhormonal despite lack of NKX6-1 expression. Hub cells are reported to have low or absent NKX6-1, lower numbers of insulin granules than their NKX6-1+ counterparts and display features of both mature and immature beta cells. *In vivo*, NKX6-1 is activated in many cells of originally NKX6-1-low clusters, possibly allowing for a more standard islet architecture with both hub and follower cells.

[0182] Most protocols in the scientific literature aim to achieve maximal NKX6-1 expression. The population of NKX6-1 expressing cells described here are not claimed to be identical to NKX6-1 expressing cells derived from pluripotent cells and described by other groups. It is unknown whether the NKX6-1 expressing cells that lost C-peptide expression *in vivo* herein de-differentiated, trans-differentiated to another endocrine phenotype, or would eventually have completed maturation and re-established insulin expression.

[0183] Animal implants of stem cell-derived islets have been shown to take weeks longer to regulate blood glucose than native islets, despite showing glucose sensitive insulin secretion *in vitro*. This delay implies that stem cell derived islets are not functionally mature *in vitro* but can reach functional maturation *in vivo*. The hypothesis that only a small population of the progenitors are capable of *in vivo* maturation and that underdosing explains the delay to glucose regulation has been proposed. Transcriptome profiles shown here demonstrate that stem cell derived pancreatic

clusters share most homology with prenatal pancreatic endocrine populations. Insulin expression and secretion in stem cell derived populations is therefore promiscuous and may not be a reliable measure of beta cell function and potency. Others have reached a similar conclusion in the context of cells co-expressing insulin and other pancreatic hormones. It is hypothesized here that delay to glucose regulation after implant is occupied by ongoing endocrine cell maturation.

[0184] Significant tissue remodeling occurs within the first few weeks of each implant site evaluated (subcapsular renal space, intrasplenic, and intra-abdominal omentum or fat pad). In each of these sites, islet-like structures formed, and in some cases, duct-like structures that were not observed in the *in vitro* cell clusters. Tissue injury repair and regeneration processes, including survival response to hypoxia and cell stress, likely promote the formation of these structures from the implanted material. It has been reported that regenerating pancreata show improved islet growth and higher insulin content near ducts. Additionally, duct cells secrete angiogenic cytokines that would facilitate vascularization of the implanted site. Similarly, others have demonstrated the benefits of promoting vascularization in poorly vascularized sites, and of promoting a pancreatic niche by delivering clusters in a synthetic or natural scaffold. Tissue remodeling may therefore be key in the establishment of a functional niche in which beta cell maturation occurs. The present interpretation of explants is that the remodeling process is a critical determinant of graft function and therefore impacts the effective dose.

[0185] Transplantation of islet-like clusters to insulin-requiring diabetics in a clinical setting could be improved by enhancing the allogenic compatibility of the graft. That goal can be achieved by disrupting expression of major histocompatibility proteins I and II, the main antigenic drivers of allogenic tissue rejection. These and other edits on SR1423U have been performed, defining new cell lines to improve tolerability and safety for future use in non-immunosuppressed recipients.

[0186] Islet like clusters similar to those described here are the subject of characterization and non-clinical studies to support regulatory filing for clinical testing. The cell expansion and differentiation protocol have been adapted to a manufacturing process following cGMP guidelines using closed, scalable vessels within a clean room environment. This work demonstrates progress

towards the development of a potent and safe islet replacement therapy for insulin requiring diabetes.

Conclusion

[0187] Populations of stem cell-derived cells expressing pancreatic endocrine markers can secrete insulin in response to glucose without expression of NKX6-1. A population of cells expressing pancreatic islet transcription factor ISL-1 and MAFA, without NKX6-1 was more potent at controlling blood glucose in diabetic rodents than a similar population expressing NKX6-1, implying that NKX6-1 can be a misleading indicator of beta cell identity and commitment in stem cell derived cells. It was concluded that SR1423-derived pancreatic clusters expressing ISL-1 and MAFA, but not NKX6-1 in animal models and have significant therapeutic potential for the treatment of insulin-requiring diabetes.

Example 3 – Treating Diabetes in a Human Adult with the Disclosed Therapeutic Cells

[0188] This example illustrates methods of using the disclosed protocol to generate therapeutic cells to treat Type I diabetes in a human adult.

[0189] An adult human subject with insulin-dependent diabetes receives a transplant comprising a therapeutically effective amount of a composition comprising the disclosed SRE cells into the subject's omentum pouch or peritoneal cavity. The subject is evaluated for blood glucose levels. The subject is monitored following the implant of a therapeutically effective number of SRE cells to ensure that the subject's blood glucose levels have been stabilized. The subject is further screened for glycosylated hemoglobin, and co-morbidities of diabetes over time.

* * * * *

WHAT IS CLAIMED IS:

1. An isolated synthetic replacement endocrine (SRE) cell that expresses transcription factors comprising:
 - (a) Pdx1 and ISL1, or
 - (b) MAFA and ISL1;wherein the SRE cell does not express transcription factor Nkx6.1.
2. The isolated cell of claim 1, wherein the isolated SRE cell is differentiated from a stem cell.
3. The isolated cell of claim 2, wherein the stem cell is selected from an embryonic stem cell, an induced pluripotent stem cell, and a multipotent reprogrammed stem cell.
4. The isolated cell of claim 2, wherein the stem cell is derived from a cell line.
5. The isolated cell of claim 2, wherein the stem cell is a multipotent reprogrammed stem cell.
6. The isolated cell of claim 5, wherein the multipotent reprogrammed stem cell was obtained by reprogramming a pancreatic cell.
7. The isolated cell of claim 6, wherein the multipotent reprogrammed stem cell was reprogrammed with an expression plasmid encoding Oct4, Sox2, Klf4, and L-Myc.
8. The isolated cell of claim 6, wherein the multipotent reprogrammed stem cell was reprogrammed with an expression plasmid encoding Oct4, Sox2, Klf4, and C-Myc.
9. The isolated cell of claim 6, wherein the multipotent reprogrammed stem cell was reprogrammed with an expression plasmid encoding LIN28, Oct4, Sox2, and Nanog.
10. The isolated cell of claim 6, wherein the multipotent reprogrammed stem cell was reprogrammed with an expression plasmid encoding Gils1, Oct3/4, Sox2, and Klf4.

11. The isolated cell of claim 6, wherein the stem cell was genetically modified to avoid targeting by lymphocytes.
12. The isolated cell of claim 6, wherein the stem cell was genetically modified to lack expression of MHC I and MHC II.
13. The isolated cell of claim 6, wherein the stem cell was genetically modified to avoid targeting by natural killer cells.
14. The isolated cell of claim 6, wherein the stem cell was genetically modified to express CD47.
15. The isolated cell of claim 5, wherein the multipotent reprogrammed stem cell can differentiate into endoderm or ectoderm cell types, but not mesoderm cell types.
16. The isolated cell of claim 5, wherein the multipotent reprogrammed stem cell does not comprise any reprogramming genes incorporated into its genome.
17. The isolated cell of claim 1, wherein the isolated SRE cell expresses C-peptide.
18. The isolated cell of claim 17, wherein C-peptide is expressed at levels equivalent to mature, native pancreatic islet cells.
19. The isolated cell of claim 17, wherein C-peptide is expressed at levels higher than an SRE cell that expresses Nkx6.1.
20. The isolated cell of any one of claims 17, wherein C-peptide expression is responsive to fluctuations in glucose concentration.
21. The isolated cell of claim 1, wherein the isolated SRE cell secretes insulin.
22. The isolated cell of one of claim 1, wherein the isolated SRE cell secretes glucagon.
23. The isolated cell of one of claim 1, wherein the isolated SRE cell is human.

24. A pharmaceutical composition, comprising at least one isolated cell according to any one of claims 1-23 and a therapeutically acceptable carrier.
25. A method of treating diabetes, comprising administering to a subject with diabetes the isolated cell accordingly to any one of claims 1-23 or the pharmaceutical composition according to claim 24.
26. The method of claim 25, wherein the diabetes is type 1.
27. The method of claim 25, wherein Nkx6.1 is expressed after the isolated cell or pharmaceutical composition is administered to the subject.
28. The method of claim 25, wherein administration comprising implanting the isolated cell or pharmaceutical composition in the subject.
29. The method of claim 25, wherein the subject is human.
30. A method of preparation of an isolated synthetic replacement endocrine (SRE) cell, comprising:
contacting an undifferentiated stem cell with a combination of factors to drive differentiation of the stem cell to an endocrine lineage, wherein the combination of factors comprises retinoic acid, a Hedgehog antagonist, and a bone morphogenetic protein (BMP) signaling inhibitor.
31. The method of claim 30, wherein the Hedgehog antagonist is selected from SANT1 and cyclopamine.
32. The method of claim 30 or 31, wherein the BMP signaling inhibitor is LDN193189.
33. The method of claim 30, wherein the stem cell is not exposed to tri-iodothyronine (T3) or analog thereof during the differentiation process.
34. The method of claim 30, wherein the stem cell is not exposed to a protein kinase C activator during the differentiation process.

35. The method of claim 30, wherein the stem cell is derived from a cell line.
36. The method of claim 30, wherein the stem cell is selected from an embryonic stem cell, an induced pluripotent stem cell, and a multipotent reprogrammed stem cell.
37. The method of claim 30, wherein the stem cell is a multipotent reprogrammed stem cell.
38. The method of claim 37, wherein the multipotent reprogrammed stem cell was obtained by reprogramming a pancreatic cell.
39. The method of claim 38, wherein the multipotent reprogrammed stem cell was reprogrammed with an expression plasmid encoding Oct4, Sox2, Klf4, and L-Myc.
40. The method of claim 38, wherein the multipotent reprogrammed stem cell was reprogrammed with an expression plasmid encoding Oct4, Sox2, Klf4, and C-Myc.
41. The method of claim 38, wherein the multipotent reprogrammed stem cell was reprogrammed with an expression plasmid encoding LIN28, Oct4, Sox2, and Nanog.
42. The method of claim 38, wherein the multipotent reprogrammed stem cell was reprogrammed with an expression plasmid encoding Gils1, Oct3/4, Sox2, and Klf4.
43. The method of any one of claims 37-42, wherein the multipotent reprogrammed stem cell can differentiate into endoderm or ectoderm cell types, but not mesoderm cell types.
44. The method of any one of claims 37-42, wherein the multipotent reprogrammed stem cell does not comprise any reprogramming genes incorporated into its genome.
45. The method of any one of claims 37-42, wherein the stem cell was genetically modified to avoid targeting by lymphocytes.
46. The method of any one of claims 37-42, wherein the stem cell was genetically modified to lack expression of MHCI and MHCII.

47. The method of any one of claims 37-42, wherein the stem cell was genetically modified to avoid targeting by natural killer cells.
48. The method of any one of claims 37-42, wherein the stem cell was genetically modified to express CD47.
49. An isolated synthetic replacement endocrine (SRE) cell obtained by the method of claim 30.
50. The isolated cell of claim 49, wherein the cell expresses transcription factors Pdx1 and ISL1, and does not express transcription factor Nkx6.1.