(19) DANMARK

(10) **DK/EP 3234110 T3**

Patent- og
Varemærkestyrelsen

(12) Oversættelse af
europæisk patentskrift

(51) Int.Cl.: ***C 12 N 5/071 (2010.01)*** ***A 61 K 35/39 (2015.01)*** ***C 12 N 5/0735 (2010.01)***

(45) Oversættelsen bekendtgjort den: **2024-05-13**

(80) Dato for Den Europæiske Patentmyndigheds
bekendtgørelse om meddelelse af patentet: **2024-02-28**

(86) Europæisk ansøgning nr.: **15871229.9**

(86) Europæisk indleveringsdag: **2015-12-18**

(87) Den europæiske ansøgnings publiceringsdag: **2017-10-25**

(86) International ansøgning nr.: **US2015066858**

(87) Internationalt publikationsnr.: **WO2016100909**

(30) Prioritet: **2014-12-18 US 201462093999 P**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **President and Fellows of Harvard College, 17 Quincy Street, Cambridge, MA 02138, USA**

(72) Opfinder: **MELTON, Douglas, A., 22 Slocum Road, Lexington, MA 02421, USA**
MILLMAN, Jeffrey, R., 9533 Park Lane, St. Louis, MO 63124, USA

(74) Fuldmægtig i Danmark: **Plougmann Vingtoft A/S, Strandvejen 70, 2900 Hellerup, Danmark**

(54) Benævnelse: **FREMGANGSMÅDER TIL GENERERING AF STAMCELLE-AFLEDTE ß-CELLER OG ANVENDELSER DERAF**

(56) Fremdragne publikationer:
WO-A1-2014/033322
WO-A1-2014/105546
WO-A1-2015/028614
US-A1- 2012 135 015
US-A1- 2014 242 693
H. M. SHAHJALAL ET AL: "Generation of insulin-producing ?-like cells from human iPS cells in a defined and completely xeno-free culture system", JOURNAL OF MOLECULAR CELL BIOLOGY, vol. 6, no. 5, 26 June 2014 (2014-06-26), pages 394-408, XP055278811, ISSN: 1674-2788, DOI: 10.1093/jmcb/mju029
Annalisa Natalicchio ET AL: "Exendin-4 protects pancreatic beta cells from palmitate-induced apoptosis by interfering with GPR40 and the MKK4/7 stress kinase signalling pathway", DIABETOLOGIA, vol. 56, no. 11, 31 August 2013 (2013-08-31), pages 2456-2466, XP055608346, BERLIN, DE ISSN: 0012-186X, DOI: 10.1007/s00125-013-3028-4

DESCRIPTION

Description

BACKGROUND OF THE INVENTION

[0001] Diabetes affects more than 300 million people worldwide according to the International Diabetes Federation. Type 1 diabetes and type 2 diabetes involve β cell destruction and/or β cell dysfunction. Diabetic patients, particularly those suffering from type 1 diabetes, could potentially be cured through transplantation of β cells. While cadaveric human islet transplantation can render patients insulin independent for 5 years or longer, such approach is limited due to the scarcity and quality of donor islets (Bellin et al., 2012). Generating an unlimited supply of human β cells from stem cells could provide therapy to millions of patients as only a single cell type, the β cell, likely needs to be produced, and the mode of delivery is well understood: transplantation to a vascularized location within the body with immunoprotection. In addition, screening to identify novel drugs that improve β cell function, survival, or proliferation is also delayed due to limited islet supply and variability resulting from different causes of death, donor genetics, and other aspects in their isolation. As such, a steady, uniform supply of stem-cell-derived β cells would offer a useful drug discovery platform for diabetes. Moreover, genetically diverse stem-cell-derived β cells could be used for disease modeling in vitro or in vivo.

[0002] Shahjalal et al., J. Mol. Cell Biol. (2014) 6: 394-408, discloses a method of differentiating endocrine progenitor cells into insulin-producing beta cells involving use of exendin-4 at a concentration of 50 ng/ml.

[0003] Natalicchio et al., Diabetologia (2013) 56: 2456-2466, discloses experimentation on pancreatic islet cells or INS-1E cells with palmitate or exendin-4 treatment.

SUMMARY OF THE INVENTION

[0004] There is a need for methods of generating stem cell-derived β (SC-β) cells. The present invention is directed toward solutions to address this need, in addition to having other desirable characteristics.

[0005] In accordance with the present invention, there is provided a method for generating stem cell-derived β (SC-β) cells. The method includes contacting a cell population comprising endocrine progenitor cells under conditions suitable to direct differentiation of said endocrine

progenitor cells into said SC-β cells with an effective amount of an agent that decreases activity of c-Jun N-terminal kinase (JNK) in said endocrine progenitor cells, thereby generating said SC-β cells, wherein the endocrine progenitor cells comprise PDX1+/NKX6.1+/NEUROD1+/insulin+/glucagon-/somatostatin- cells, wherein the effective amount comprises a concentration between about 0.1 μm and about 110 μm, wherein the agent is SP600125. SC- β cells produced by methods described herein may exhibit one or more improved characteristics (e.g., stimulation index) in comparison with cells produced by the same method in the absence of the JNK inhibitor.

[0006] Further aspects and embodiments of the present invention are defined in the appended claims.

[0007] The practice of the present invention will typically employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant nucleic acid (e.g., DNA) technology, immunology, and RNA interference (RNAi) which are within the skill of the art. Non-limiting descriptions of certain of these techniques are found in the following publications: Ausubel, F., et al., (eds.), Current Protocols in Molecular Biology, Current Protocols in Immunology, Current Protocols in Protein Science, and Current Protocols in Cell Biology, all John Wiley & Sons, N.Y., edition as of December 2008; Sambrook, Russell, and Sambrook, Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2001; Harlow, E. and Lane, D., Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1988; Freshney, R.I., "Culture of Animal Cells, A Manual of Basic Technique", 5th ed., John Wiley & Sons, Hoboken, NJ, 2005. Non-limiting information regarding therapeutic agents and human diseases is found in Goodman and Gilman's The Pharmacological Basis of Therapeutics, 11th Ed., McGraw Hill, 2005, Katzung, B. (ed.) Basic and Clinical Pharmacology, McGraw-Hill/Appleton & Lange; 10th ed. (2006) or 11th edition (July 2009). Non-limiting information regarding genes and genetic disorders is found in McKusick, V.A.: Mendelian Inheritance in Man. A Catalog of Human Genes and Genetic Disorders. Baltimore: Johns Hopkins University Press, 1998 (12th edition) or the more recent online database: Online Mendelian Inheritance in Man, OMIM™. McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), as of May 1, 2010, World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim/ and in Online Mendelian Inheritance in Animals (OMIA), a database of genes, inherited disorders and traits in animal species (other than human and mouse), at http://omia.angis.org.au/contact.shtml. In case of a conflict between the specification and any of the references, the specification (including any amendments thereof shall control. Standard art-accepted meanings of terms are used herein unless indicated otherwise. Standard abbreviations for various terms are used herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] These and other characteristics of the present invention will be more fully understood by reference to the following detailed description in conjunction with the attached drawings. The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

FIG. 1 is a graph demonstrating that SC-β cells generated by contacting endocrine progenitor cells directed to differentiate into SC-β cells with an exemplary agent that decreases the level and/or activity of c-Jun terminal kinase (JNK), e.g., JNK inhibitor SP600125, exhibit a greater stimulation index relative to SC-β cells generated by contacting endocrine progenitor cells directed to differentiate into SC-β cells in the absence of treatment with the JNK inhibitor. Stimulation index = [insulin@20mM glucose]/[insulin@2mM glucose]. Relative Stim Index = Stim Index/Stim Index of Control.

FIG. 2A is a schematic illustrating six stages of differentiation of human pluripotent stem cells to SC-β cells. hPSC = human pluripotent stem cell, DE = definitive endoderm cell, GT = gut tube cell, PP1 = pancreatic progenitor cell 1, PP2 = pancreatic progenitor cell 2, EN = endocrine progenitor cell, SC-β = stem cell-derived β cells.

FIG. 2B is a schematic illustrating an exemplary six step differentiation protocol for generating SC-β cells from pluripotent stem cells, as described further in Pagliuca et al. 2014 and PCT International Application No. PCT/US2014/041992.

FIG. 2C is a schematic illustrating an exemplary method for generating SC-β cells by adding a JNK inhibitor to Step 6 of the exemplary protocol shown in FIG. 2B.

DETAILED DESCRIPTION OF THE INVENTION

[0009] The present invention is directed to generating SC-β cells, in particular SC-β cells that exhibit in vitro and in vivo function. More particularly, work described herein demonstrates that contacting endocrine progenitor cells progressing to SC- β cells in a directed differentiation protocol with an agent that decreases the level and/or activity of c-Jun terminal kinase (JNK) produces SC- β cells that exhibit a greater stimulation index relative to SC-β cells generated by the protocol in the absence of contact with the agent.

Some Definitions

[0010] "Differentiation" is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell such as, for example, a pancreatic cell. A differentiated cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. The term "committed", when applied to the process of

differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. As used herein, the lineage of a cell defines the heredity of the cell, i.e., which cells it came from and to what cells it can give rise. The lineage of a cell places the cell within a hereditary scheme of development and differentiation. A lineage-specific marker refers to a characteristic specifically associated with the phenotype of cells of a lineage of interest and can be used to assess the differentiation of an uncommitted cell to the lineage of interest.

[0011] As used herein, "markers", are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. Differential expression means an increased level for a positive marker and a decreased level for a negative marker as compared to an undifferentiated cell. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

[0012] As used herein, a cell is "positive" or "+" for a specific marker when the specific marker is sufficiently detected in the cell. Similarly, the cell is "negative" or "-" for a specific marker when the specific marker is not sufficiently detected in the cell. For example, positive by FACS is usually greater than 2%, whereas the negative threshold by FACS is usually less than 1%.

[0013] The process of differentiating pluripotent stem cells into functional pancreatic endocrine cells (i.e., SC-β cells) in vitro may be viewed in some aspects as progressing through six consecutive stages, as is shown in the exemplary protocol depicted in FIG. 2A. In this step-wise progression, "Stage 1" or "S1" refers to the first step in the differentiation process, the differentiation of pluripotent stem cells into cells expressing markers characteristic of definitive endoderm cells ("DE", "Stage 1 cells" or "S1 cells"). "Stage 2" refers to the second step, the differentiation of cells expressing markers characteristic of definitive endoderm cells into cells expressing markers characteristic of gut tube cells ("GT", "Stage 2 cells" or "S2 cells"). "Stage 3" refers to the third step, the differentiation of cells expressing markers characteristic of gut tube cells into cells expressing markers characteristic of pancreatic progenitor 1 cells ("PP1", "Stage 3 cells" or "S3 cells"). "Stage 4" refers to the fourth step, the differentiation of cells expressing markers characteristic of pancreatic progenitor 1 cells into cells expressing markers characteristic of pancreatic progenitor 2 cells ("PP2", "Stage 4 cells" or "S4 cells"). "Stage 5" refers to the fifth step, the differentiation of cells expressing markers characteristic of pancreatic progenitor 2 cells into cells expressing markers characteristic of pancreatic endoderm cells and/or pancreatic endocrine progenitor cells ("EN", "Stage 5 cells" or "S5 cells"). "Stage 6" refers to the differentiation of cells expressing markers characteristic of pancreatic endocrine progenitor cells into cells expressing markers characteristic of pancreatic endocrine β cells ("SC-β cells", "Stage 6 cells" or "S6 cells"). It should be appreciated, however, that not all cells in a particular population progress through these stages at the same rate, i.e., some cells may have progressed less, or more, down the differentiation pathway than the

majority of cells present in the population.

[0014] Characteristics of the various cell types associated with the stages shown in FIG. 2A are now described. "Definitive endoderm cells," as used herein, refers to cells which bear the characteristics of cells arising from the epiblast during gastrulation and which form the gastrointestinal tract and its derivatives. Definitive endoderm cells express at least one of the following markers: FOXA2 (also known as hepatocyte nuclear factor 3β ("HNF3β")), GATA4, SOX17, CXCR4, Brachyury, Cerberus, OTX2, goosecoid, C-Kit, CD99, and MIXL1. Markers characteristic of the definitive endoderm cells include CXCR4, FOXA2 and SOX17. Thus, definitive endoderm cells may be characterized by their expression of CXCR4, FOXA2 and SOX17. In addition, depending on the length of time cells are allowed to remain in Stage 1, an increase in HNF4α may be observed.

[0015] "Gut tube cells," as used herein, refers to cells derived from definitive endoderm that can give rise to all endodermal organs, such as lungs, liver, pancreas, stomach, and intestine. Gut tube cells may be characterized by their substantially increased expression of HNF4α over that expressed by definitive endoderm cells. For example, a ten to forty fold increase in mRNA expression of HNF4α may be observed during Stage 2.

[0016] "Pancreatic progenitor 1 cells," as used herein, refers to endoderm cells that give rise to the esophagus, lungs, stomach, liver, pancreas, gall bladder, and a portion of the duodenum. Pancreatic progenitor 1 cells express at least one of the following markers: PDX1, FOXA2, CDX2, SOX2, and HNF4α. Pancreatic progenitor 1 cells may be characterized by an increase in expression of PDX1, compared to gut tube cells. For example, greater than fifty percent of the cells in Stage 3 cultures typically express PDX1.

[0017] "Pancreatic progenitor 2 cells," as used herein, refers to cells that express at least one of the following markers: PDX1, NKX6.1, HNF6, NGN3, SOX9, PAX4, PAX6, ISL1, gastrin, FOXA2, PTF1a, PROXI and HNF4α. Pancreatic progenitor 2 cells may be characterized as positive for the expression of PDX1, NKX6.1, and SOX9.

[0018] "Pancreatic endocrine progenitor cells" or "endocrine progenitor cells" are used interchangeably herein to refer to pancreatic endoderm cells capable of becoming a pancreatic hormone expressing cell. Pancreatic endocrine progenitor cells express at least one of the following markers: NGN3; NKX2.2; NeuroD1; ISL1; PAX4; PAX6; or ARX. Pancreatic endocrine progenitor cells may be characterized by their expression of NKX2.2 and NeuroD1.

[0019] A "precursor thereof" as the term relates to a pancreatic endocrine progenitor cell refers to any cell that is capable of differentiating into a pancreatic endocrine progenitor cell, including for example, a pluripotent stem cell, a definitive endoderm cell, a gut tube cell, or a pancreatic progenitor cell, when cultured under conditions suitable for differentiating the precursor cell into the pancreatic pro endocrine cell.

[0020] "Pancreatic endocrine cells," as used herein, refer to cells capable of expressing at

least one of the following hormones: insulin, glucagon, somatostatin, ghrelin, and pancreatic polypeptide. In addition to these hormones, markers characteristic of pancreatic endocrine cells include one or more of NGN3, NeuroDl, ISL1, PDX1, NKX6.1, PAX4, ARX, NKX2.2, and PAX6. Pancreatic endocrine cells expressing markers characteristic of β cells can be characterized by their expression of insulin and at least one of the following transcription factors: PDX1, NKX2.2, NKX6.1, NeuroDl, ISL1, HNF30, MAFA and PAX6.

[0021] The terms "stem cell-derived β cell" and "SC-β cell" are used interchangeably herein to refer to cells differentiated in vitro (e.g., from pluripotent stem cells) that display at least one marker indicative of a pancreatic β cell (e.g., PDX-1 or NKX6-1), expresses insulin, and display a GSIS response characteristic of an endogenous mature β cell both in vitro and in vivo. The GSIS response of the SC-β cells can be observed within two weeks of transplantation of the SC-β cell into a host (e.g., a human or animal). It is to be understood that the SC-β cells need not be derived (e.g., directly) from stem cells, as the methods of the disclosure are capable of deriving SC-β cells from any endocrine progenitor cell that expresses insulin or precursor thereof using any cell as a starting point (e.g., one can use embryonic stem cells, induced-pluripotent stem cells, progenitor cells, partially reprogrammed somatic cells (e.g., a somatic cell which has been partially reprogrammed to an intermediate state between an induced pluripotent stem cell and the somatic cell from which it was derived), multipotent cells, totipotent cells, a transdifferentiated version of any of the foregoing cells, etc, as the invention is not intended to be limited in this manner). In some aspects, human cells are excluded that are derived from human embryonic stem cells obtained exclusively by a method necessitating the destruction of an embryo. The skilled artisan is well aware of such methods and how to avoid them for the purposes of generating SC-β cells according to the methods of the present invention.

[0022] Used interchangeably herein are "d1", "1d", and "day 1"; "d2", "2d", and "day 2", etc.. These number letter combinations refer to a specific day of incubation in the different stages during the stepwise differentiation protocol of the instant application.

Methods for generating SC-B cells

[0023] In accordance with one aspect of the present invention, a method for generating stem cell-derived β (SC-β) cells comprises contacting a cell population comprising endocrine progenitor cells under conditions suitable to direct differentiation of said endocrine progenitor cells into said SC-β cells with an effective amount of an agent that decreases activity of c-Jun N-terminal kinase (JNK) in said endocrine progenitor cells, thereby generating said SC-β cells, wherein the endocrine progenitor cells comprises PDX1+/NKX6.1+/NEUROD1+/insulin+/glucagon-/somatostatin- cells, wherein the effective amount comprises a concentration between about 0.1 μm and about 110 μm, wherein the agent is SP600125.

[0024] "Contacting", "contacting the cell" and any derivations thereof as used herein, refers to

any means of introducing an agent (e.g., nucleic acids, peptides, ribozymes, antibodies, small molecules, etc.) into a target cell or an environment in which the cell is present (e.g., cell culture), including chemical and physical means, whether directly or indirectly. Contacting also is intended to encompass methods of exposing a cell, delivering to a cell, or "loading" a cell with an agent by viral or non-viral vectors, and wherein such agent is bioactive upon delivery. The method of delivery will be chosen for the particular agent and use. Parameters that affect delivery, as is known in the medical art, can include, inter alia, the cell type affected, and cellular location. In some aspects, contacting refers to exposing a cell or an environment in which the cell is located (e.g., cell culture medium) to at least one agent that decreases the level and/or activity of JNK.

[0025] It is believed that SC-β cells generated by contacting endocrine progenitor cells (or their precursors) directed to differentiate into SC-β cells according to any protocol will exhibit improved in vitro and in vivo function when contacted with an agent that decreases the activity of c-Jun N-terminal kinase (JNK). As used herein, "directed to differentiate" refers to the process of causing a cell of a first cell type to differentiate into a cell of a second cell type.

[0026] Recently, two protocols for directing the differentiation of pluripotent stem cells into insulin-producing endocrine cells that express key markers of mature pancreatic β cells (e.g., SC-β cells) have been reported, each of which includes differentiating cells into endocrine progenitor cells that can be directed to differentiate into SC-β cells, as well as protocols for directing the pancreatic endocrine progenitor cells into SC-β cells, which can be used in the method disclosed herein for generating SC-β cells. First, a six-stage protocol for the large-scale production of functional human β cells using human pluripotent stem cells (hPSC) by sequential modulation of multiple signaling pathways in a three-dimensional cell culture system, without using any transgenes or genetic modification, was used to generate glucose-responsive, monohormonal insulin-producing cells that exhibited key β cell markers and β cell ultrastructure (see Pagliuca et al., 2014 and PCT International Publication No. WO/2014/201167. Pagliuca and colleagues reported that such cells mimicked the function of human islets in vitro and in vivo, and demonstrated the potential utility of such cells for in vivo transplantation to treat diabetes. Secondly, a seven-stage protocol that converts human embryonic stem cells (hESCs) into insulin-producing cells that expressed key markers of mature pancreatic β cells, such as MAFA, and displayed glucose-stimulated insulin secretion like that of human islets using static incubations in vitro was described (Rezania et al., 2014). Cells produced by such protocol, referred to as S7 cells, were found to rapidly reverse diabetics in mice within a little over a month.

[0027] Described herein, the endocrine progenitor cells are directed to differentiate into SC-β cells by contacting the endocrine progenitor cells under conditions that promote cell clustering with i) a transforming growth factor β (TGF-β) signaling pathway inhibitor and ii) a thyroid hormone signaling pathway activator to induce the *in vitro* maturation of at least some of the endocrine progenitor cells into SC-β cells. The endocrine progenitor cells may be optionally contacted with a protein kinase inhibitor (e.g., staurosporine).

[0028] An "effective amount" of an agent (or composition containing such agent) refers to the amount sufficient to achieve a desired effect, e.g., when delivered to a cell or subject according to a selected administration form, route, and/or schedule. As will be appreciated by those of ordinary skill in this art, the absolute amount of a particular agent or composition that is effective may vary depending on such factors as the desired biological or pharmacological endpoint, the agent to be delivered, the target tissue, etc. Those of ordinary skill in the art will further understand that an "effective amount" may be contacted with cells or administered in a single dose, or the desired effect may be achieved by use of multiple doses. An effective amount of a composition may be an amount sufficient to reduce the severity of or prevent one or more symptoms or signs of a disorder (e.g., diabetes). According to the method of the present invention, the effective amount of the agent that decreases the activity of JNK comprises a concentration of between about 0.1 μM and about 110 μM. In one embodiment, the effective amount of the agent comprises 1 μM. In some aspects, the endocrine progenitor cells (S5 cells) are contacted with 1 μM of SP600125 to generate SC-β cells exhibiting an improved in vitro or in vivo function.

c-Jun N-terminal Kinase (JNK) inhibitors

[0029] The present invention uses SP600125 in the method for generating SC-β cells. Agents that decrease the activity of JNK (also referred to herein as a "JNK inhibitor") can be small organic or inorganic molecules; saccharides; oligosaccharides; polysaccharides; biological macromolecules, e.g., peptides, proteins, and peptide analogs and derivatives; peptidomimetics; nucleic acids and nucleic acid analogs and derivatives (including but not limited to microRNAs, siRNAs, shRNAs, antisense RNAs, a ribozymes, and aptamers); an extract made from biological materials such as bacteria, plants, fungi, or animal cells; animal tissues; naturally occurring or synthetic compositions; and any combinations thereof.

[0030] Exemplary JNK inhibitors include, but are not limited to, the JNK inhibitors of formula I as described in U.S. Patent No. 8,183,254; the benzothiazole derivatives according to formula I and tautomers, geometrical isomers, enantiomers, diastereomers, racemates and pharmaceutically acceptable salts thereof described in U.S. Patent Application Publication No. 2009/0176762; a JNK inhibitor sequence, chimeric peptide, or nucleic acid described in U.S. Patent No. 6,610,820 and U.S. Patent Application Publication No. 2009/0305968, for example cell-permeable peptides that bind to JNK and inhibit JNK activity or c-Jun activation, including peptides having amino acid sequences of DTYRPKRPTT LNLFPQVPRS QDT (SEQ ID NO:1); EEPHKHRPTT LRLTTLGAQD S (SEQ ID NO:2); TDQSRPVQPF LNLTTPRKPR YTD (SEQ ID NO:3); or SDQAGLTTLR LTTPRHKHPE E (SEQ ID NO:4); c-Jun N-terminal kinase inhibiting compounds of formula I described in U.S. Patent No. 7,612,086; a JNK inhibitor of formula I described in PCT International Application Publication No. WO/2011/018417; an agent that inhibits MKK4 as described herein (e.g., MKK4 inhibitors); an agent that inhibits JNK interacting protein (JIP) (see for example, Chen T, et al. Biochem J. 2009 May 13; 420(2):283-94, discloses small-molecules that disrupt the JIP-JNK interaction to provide an alternative approach for JNK inhibition); SP600125 (Anthra[1,9-cd]pyrazol-6(2H)-on; 1,9-

Pyrazoloanthrone) (Calbiochem., La Jolla, Calif.); a compound based on the 6,7-dihydro-5H-pyrrolo[1,2-a]imidazole scaffold (e.g., ER-181304); SB203580; a selective inhibitor of JNK3 described in PCT International Application Publication WO 2010/039647; 7-(5-7V-Phenylaminopentyl)-2H-anthra[1,9-cd]pyrazol-6-one; 7-(7-7V-Benzoylaminoheptyl)amino-2H-anthra[1,9-cd]pyrazol-6-one; and 7-(5-(p-Tolyloxy)pentyl)amino-2H-anthra[1,9-cd]pyrazol-6-one; a dominant negative form of JNK, e.g., a catalytically inactive JNK-1 molecule constructed by replacing the sites of activating Thr183 and Tyr185 phosphorylation with Ala and Phe respectively, which acts as a dominant inhibitor of the wild-type JNK-1 molecule as described in PCT International Application Publication No. WO 1996/036642; a JIP-1 polypeptide that binds JNK as described in U.S. Patent Publications 2007/0003517 and 2002/0119135, including a peptide having the amino acid sequence SGDTYRPKRPTTLNLFPQVPRSQDTLN (SEQ ID NO:5).

[0031] Described herein, a JNK inhibitor decreases the level and/or activity of JNK in cells contacted by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% relative to the level or activity of JNK in the cells in the absence of contact with the JNK inhibitor. While not required, a JNK inhibitor can completely inhibit the level and/or activity of JNK in the cells. It should be appreciated that the JNK inhibitors may decrease the level and/or activity of JNK in any cell in the population in which endocrine progenitor cells are differentiating into SC-β cells, including in the SC-β cells generated in the population, endocrine progenitor cells or any precursors thereof.

[0032] Described herein, an agent decreases the level and/or activity of JNK by inhibiting phosphorylation of JNK. It should be appreciated, however, that the agent may act via any mechanism which results in decreased level and/or activity of JNK. For example, the level and/or activity of JNK may be decreased by decreasing the level and/or activity of mitogen-activated protein kinase kinase 4 (MKK4). MKK4, a member of the MAP kinase kinase family, directly phosphorylates and activates the c-Jun NH2-terminal kinases (JNK), in response to cellular stresses and proinflammatory cytokines. JNK is a member of the MAP kinase family and a key component of a stress activated protein kinase signaling pathway. Such agents may be referred to herein as MKK4 inhibitors. A MKK4 inhibitor may decrease the level and/or activity of JNK in cells contacted by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% relative to the level or activity of JNK in the cells in the absence of contact with the JNK inhibitor.

[0033] It should be appreciated, as noted above, that the population of cells comprising the endocrine progenitor cells contacted in accordance with the method may comprise different cells types as the cells are differentiating into SC-β cells. Preferably, a maximum amount of endocrine cells in the population contacted with a JNK inhibitor or MKK4 inhibitor differentiate into SC-β cells. In some embodiments of the method, between at least 5% and 65% of the endocrine cells in the population differentiate into SC-β cells.

SC-B cells obtained by the method of generating SC-B cells

[0034] An isolated non-native SC-β cell or population thereof generated according to a method described herein that exhibits a GSIS response both *in vitro* and *in vivo* is provided, wherein the isolated SC-β cell or population thereof exhibits a stimulation index of between about 1.4 and about 2.4. The isolated SC-β cell or population also exhibits at least one characteristic feature of a mature endogenous β cell (e.g., monohormonality). In some embodiments, the isolated SC-β cell or population thereof produces between approximately 300 uIU to about 4000 uIU per 30 minute per 10^6 total cells incubation at a high glucose concentration.

[0035] The SC-β cells disclosed herein share many distinguishing features of native β cells, but are different in certain aspects (e.g., gene expression profiles). In some embodiments, the SC-β cell is non-native. As used herein, "non-native" means that the SC-β cells are markedly different in certain aspects from β cells which exist in nature, i.e., native β cells. It should be appreciated, however, that these marked differences typically pertain to structural features which may result in the SC-β cells exhibiting certain functional differences, e.g., although the gene expression patterns of SC-β cells differs from native β cells, the SC-β cells behave in a similar manner to native β cells but certain functions may be altered (e.g., improved) compared to native β cells. For example, a higher frequency of SC-β cells respond to 20 mM glucose compared to the frequency of native β cells. Other differences between SC-β cells and native β cells would be apparent to the skilled artisan based on the data disclosed herein.

[0036] The SC-β cells (e.g., human) generated according to the methods described herein may exhibit at least one of the following characteristics of an endogenous mature pancreatic β cell: i) a response to multiple glucose challenges that resembles the response of endogenous islets (e.g., at least one, at least two, or at least three or more sequential glucose challenges); ii) a morphology that resembles the morphology of an endogenous β cell; iii) packaging of insulin into secretory granules or encapsulated crystalline insulin granules; iv) a stimulation index of greater than at least 1.4; v) cytokine-induced apoptosis in response to cytokines; vi) enhanced insulin secretion in response to known antidiabetic drugs (e.g., secretagogues); vii) monohormonal, i.e., they do not abnormally co-express other hormones, such as glucagon, somatostatin or pancreatic polypeptide; viii) a low rate of replication; and ix) increased intracellular Ca^{2+} in response to glucose.

[0037] In addition, a microcapsule comprising the isolated SC-β cell or population thereof generated according to the method herein that exhibits a GSIS response both *in vitro* and *in vivo,* wherein the SC-β cell or population thereof exhibits a stimulation index of between about 1.4 and about 2.4 encapsulated therein is provided.

[0038] A macroencapsulation device comprising the isolated SC-β cell or population thereof generated according to the method herein that exhibits a GSIS response both *in vitro* and *in vivo,* wherein the SC-β cell or population thereof exhibits a stimulation index of between about 1.4 and about 2.4 is also provided.

[0039] Furthermore, a cell line comprising the isolated SC-β cell generated according to the method herein that exhibits a GSIS response both *in vitro* and *in vivo,* wherein the SC-β cell or population thereof exhibits a stimulation index of between about 1.4 and about 2.4 that stably expresses insulin is provided.

Assays

[0040] An isolated SC-β cell or population thereof generated according to the methods herein, or a cell line comprising the isolated SC-β cell generated according to the method herein that exhibits a GSIS response both *in vitro* and *in vivo,* wherein the SC-β cell or population thereof exhibits a stimulation index of between about 1.4 and about 2.4 stably expresses insulin, can be used in an assay (i) to identify one or more candidate agents which promote or inhibit a β cell fate selected from the group consisting of β cell proliferation, β cell replication, β cell death, β cell function, β cell susceptibility to immune attack, and β cell susceptibility to dedifferentiation or differentiation, or (ii) to identify one or more candidate agents which promote the differentiation of at least one insulin-positive endocrine cell or a precursor thereof into at least one SC-β cell. The assays typically involve contacting the isolated SC-β cell, population thereof, or the cell line, with one or more candidate agents to be assessed for its ability to i) promote or inhibit a β cell fate selected from the group consisting of β cell proliferation, β cell replication, β cell death, β cell function, β cell susceptibility to immune attack, and β cell susceptibility to dedifferentiation or differentiation, or ii) promoting the differentiation of at least one insulin-positive endocrine cell or a precursor thereof into at least one SC-β cell and assessing whether the candidate agent possesses the ability to i) promote or inhibit a β cell fate selected from the group consisting of β cell proliferation, β cell replication, β cell death, β cell function, β cell susceptibility to immune attack, and β cell susceptibility to dedifferentiation or differentiation, or ii) promoting the differentiation of at least one insulin-positive endocrine cell or a precursor thereof into at least one SC-β cell.

Methods for treatment

[0041] Described herein are methods for the treatment of a subject in need thereof. The methods entail administering to a subject in need thereof an isolated population of SC-β cells or a microcapsule comprising SC-β cells encapsulated therein. The subject may be in need of additional β cells. The subject may have an increased risk of developing diabetes. An isolated population of SC-β cells generated by a method of the present invention can be for use in administering to a subject for treatment of type 1 or type 2 diabetes. In some aspects, the subject has, or has an increased risk of developing, a metabolic disorder. Administering to the subject may comprise implanting SC-β cells, a microcapsule comprising SC-β cells, or a macroencapsulation device comprising SC-β cells into the subject. The subject may be a human subject or an animal subject. The cells may be implanted as dispersed cells or formed into clusters that may be infused into the hepatic portal vein. Cells may be provided in

biocompatible degradable polymeric supports, porous non-degradable devices or encapsulated to protect from host immune response. Cells may be implanted into an appropriate site in a recipient. The implantation sites include, for example, the liver, natural pancreas, renal subcapsular space, omentum, peritoneum, subserosal space, intestine, stomach, or a subcutaneous pocket.

[0042] To enhance further differentiation, survival or activity of the implanted cells in vivo, additional factors, such as growth factors, antioxidants or anti-inflammatory agents, can be administered before, simultaneously with, or after the administration of the cells. These factors can be secreted by endogenous cells and exposed to the administered cells in situ. Implanted cells can be induced to differentiate by any combination of endogenous and exogenously administered growth factors known in the art.

[0043] The amount of cells used in implantation depends on a number of various factors including the patient's condition and response to the therapy, and can be determined by one skilled in the art.

[0044] The method of treatment described herein may further comprise incorporating the cells into a three-dimensional support prior to implantation. The cells can be maintained in vitro on this support prior to implantation into the patient. Alternatively, the support containing the cells can be directly implanted in the patient without additional in vitro culturing. The support can optionally be incorporated with at least one pharmaceutical agent that facilitates the survival and function of the transplanted cells.

Artificial islet or pancreas

[0045] Moreover, an artificial islet or pancreas comprising the SC-β cells generated according to the methods described herein is provided.

[0046] An artificial pancreas is a device that encapsulates and nurtures islets of Langerhans to replace the islets and β cells destroyed by type 1 diabetes. An artificial pancreas may contain a million islets or more, and may be implanted in the peritoneal cavity or under the skin where it can respond to changing blood glucose levels by releasing hormones, such as insulin. An artificial pancreas may be made using living (e.g., glucose-sensing and insulin secreting islets) and nonliving components (e.g., to shield the islets from the diabetic's body and its destructive immune mechanism while permitting the islets to thrive).

[0047] β cells may be used in any artificial pancreas. The artificial pancreas may comprise microencapsulated or coated islets comprising SC-β cells generated according to the methods herein. The artificial pancreas may comprise a macroencapsulation device into which islet cells comprising SC-β cells generated according to the methods herein are grouped together and encapsulated. The macroencapsulation device may comprise a PVA hydrogel sheet for an artificial pancreas of the present invention (Qi et al., 2004). The artificial islet may comprise

SC-β cells generated according to the methods herein, along with other islet cells (α, δ, etc.) in the form of an islet sheet. The islet sheet comprises a layer of artificial human islets comprising the SC-β cells macroencapsulated within a membrane (e.g., of ultra-pure alginate). The sheet membrane is reinforced with mesh and may be coated on the surface to prevent or minimize contact between the cells encapsulated inside and the transplantation recipient's host immune response. Oxygen, glucose, and other nutrients readily diffuse into the sheet through the membrane nurturing the islets, and hormones, such as insulin readily diffuse out. Additional examples of membranes designed for macroencapsulation/implantation of an artificial islet or pancreas can be found in the literature (Isayeva et al. 2003). Another example of a macroencapsulated implant suitable for an artificial islet or pancreas can be found in the literature (Aurélien, et al. 2014).

Terminology

[0048] The articles "a", "an" and "the" as used herein, unless clearly indicated to the contrary, should be understood to include the plural referents. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. It should it be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, etc. For purposes of simplicity those embodiments have not in every case been specifically set forth in *haec verba* herein. It should also be understood that any embodiment of the invention, e.g., any embodiment found within the prior art, can be explicitly excluded from the claims, regardless of whether the specific exclusion is recited in the specification. For example, any agent may be excluded from the genus of JNK inhibitors.

[0049] Where ranges are given herein, the invention includes embodiments in which the endpoints are included, embodiments in which both endpoints are excluded, and embodiments in which one endpoint is included and the other is excluded. It should be assumed that both endpoints are included unless indicated otherwise. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. It is also understood that where a series of numerical values is stated herein, the invention includes embodiments that relate analogously to any intervening value or range defined by any two values in the series, and that the lowest value may be taken as a minimum and the greatest value may be taken as a maximum. Numerical values, as used herein, include values expressed as percentages. For any embodiment of the invention in which a numerical value is prefaced by "about" or "approximately", the invention includes an embodiment in which the exact value is recited. For any embodiment of the invention in which a numerical value is not prefaced by "about" or

"approximately", the invention includes an embodiment in which the value is prefaced by "about" or "approximately". "Approximately" or "about" generally includes numbers that fall within a range of 1% or in some embodiments 5% of a number in either direction (greater than or less than the number) unless otherwise stated or otherwise evident from the context (except where such number would impermissibly exceed 100% of a possible value).

References

[0050]

1. 1. Bellin et al., (2012). Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. Am. J. Transplant. 12, 1576-1583.
2. 2. Pagliuca et al. (2014). Generation of Functional Human Pancreatic β cells In Vitro. Cell. 159, 428-439.
3. 3. Rezania et al. (2014). Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat. Biotech. 32(11), 1121-1133.
4. 4. Isayeva, et al. (2003). Characterization and performance of membranes designed for macroencapsulation/implantation of pancreatic islet cells. Biomaterials 24(20), 3483-3491.
5. 5. Motté, et al. (2014). Composition and function of macroencapsulated human embryonic stem cell-derived implants: comparison with clinical human islet cell grafts. American Journal of Physiology-Endocrinology and Metabolism 307(9), E838-E846.
6. 6. Qi et al. (2004). PVA hydrogel sheet macroencapsulation of the bioartificial pancreas. Biomaterials 24(27), 5885-5892.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US2014041992W [0008]
- WO2014201167A [0026]
- US8183254B [0030]
- US20090176762 [0030]
- US6610820B [0030]
- US20090305968 [0030]
- US7612086B [0030]
- WO2011018417A [0030]
- WO2010039647A [0030]
- WO1996036642A [0030]
- US20070003517A [0030]
- US20020119135A [0030]

Non-patent literature cited in the description

- **SHAHJALAL et al.**J. Mol. Cell Biol., 2014, vol. 6, 394-408 [0002]
- **NATALICCHIO et al.**Diabetologia, 2013, vol. 56, 2456-2466 [0003]
- Current Protocols in Molecular Biology, Current Protocols in Immunology, Current Protocols in Protein Science, and Current Protocols in Cell BiologyJohn Wiley & Sons20081200 [0007]
- **SAMBROOKRUSSELLSAMBROOK**Molecular Cloning: A Laboratory ManualCold Spring Harbor Laboratory Press20010000 [0007]
- **HARLOW, E.LANE, D.**Antibodies - A Laboratory ManualCold Spring Harbor Laboratory Press19880000 [0007]
- **FRESHNEY, R.I.**Culture of Animal Cells, A Manual of Basic TechniqueJohn Wiley & Sons20050000 [0007]
- **GOODMANGILMAN'S**The Pharmacological Basis of TherapeuticsMcGraw Hill20050000 [0007]
- Basic and Clinical PharmacologyMcGraw-Hill/Appleton & Lange20060000 [0007]
- Mendelian Inheritance in Man**MCKUSICK, V.A.**A Catalog of Human Genes and Genetic DisordersJohns Hopkins University Press19980000 [0007]
- **CHEN T et al.**Biochem J., 2009, vol. 420, 2283-94 [0030]
- **BELLIN et al.**Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetesAm. J. Transplant., 2012, vol. 12, 1576-1583 [0050]
- **PAGLIUCA et al.**Generation of Functional Human Pancreatic β cells In VitroCell, 2014, vol. 159, 428-439 [0050]
- **REZANIA et al.**Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells.Nat. Biotech., 2014, vol. 32, 111121-1133 [0050]

- **ISAYEVA et al.**Characterization and performance of membranes designed for macroencapsulation/implantation of pancreatic islet cells.Biomaterials, 2003, vol. 24, 203483-3491 [0050]
- **MOTTÉ et al.**Composition and function of macroencapsulated human embryonic stem cell-derived implants: comparison with clinical human islet cell grafts.American Journal of Physiology-Endocrinology and Metabolism, 2014, vol. 307, 9E838-E846 [0050]
- **QI et al.**PVA hydrogel sheet macroencapsulation of the bioartificial pancreas.Biomaterials, 2004, vol. 24, 275885-5892 [0050]

Patentkrav

1. Fremgangsmåde til generering af stamcelle-afledte β- (SC-β) celler, hvilken fremgangsmåde omfatter at bringe en cellepopulation omfattende endokrine progenitorceller under betingelser, som er egnede til at dirigere differentiering af nævnte endokrine progenitorceller til nævnte SC-β-celler, i kontakt med en effektiv mængde af et middel, der reducerer aktiviteten af c-Jun N-terminal kinase (JNK) i nævnte endokrine progenitorceller, for derved at generere nævnte SC-β-celler, hvor de endokrine progenitorceller omfatter PDX1+/NKX6.1+/NEUROD1+/insulin+/glucagon-/somatostatin-celler, hvor den effektive mængde omfatter en koncentration mellem ca. 0,1 µM og ca. 110 µM, hvor midlet er SP600125.

2. Fremgangsmåden ifølge krav 1, hvor mellem mindst 5% og 65% af de endokrine celler i populationen differentieres til nævnte SC-β-celler.

3. Fremgangsmåden ifølge krav 1 eller krav 2, hvor aktiviteten af JNK reduceres med 70%.

4. Fremgangsmåden ifølge krav 1 eller krav 2, hvor aktiviteten af JNK reduceres med 90%.

5. Fremgangsmåden ifølge et hvilket som helst af krav 1 eller krav 2, hvor aktiviteten af JNK hæmmes fuldstændigt af midlet.

DRAWINGS

Drawing

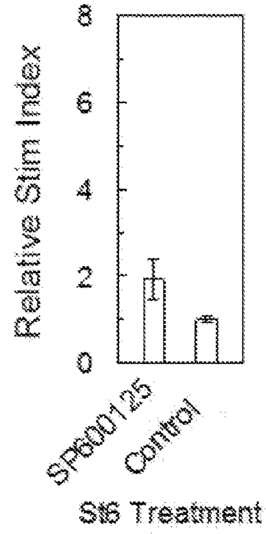

FIG. 1

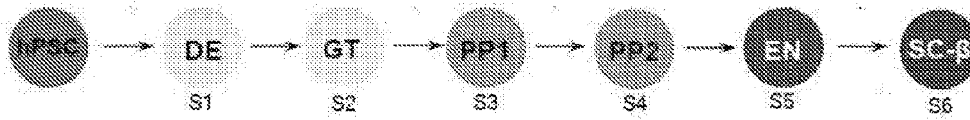

FIG. 2A

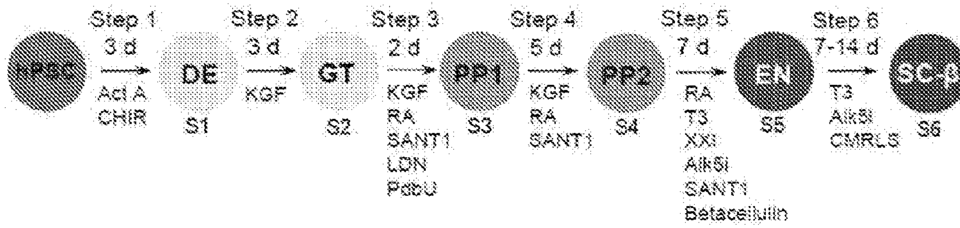

FIG. 2B

FIG. 2C