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STEM CELLS OF THE ISLETS OF LANGERHANS AND THEIR USE IN TREATING DIABETES MELLITUS

PRIORITY

The present application claims priority to U.S. Application Serial No. 09/963,875, filed September 26, 2001, U.S. Application Serial No. 10/120,687, filed April 11, 2002 and U.S. Application Serial No. 10/136,891, filed May 2, 2002.

TECHNICAL FIELD OF THE INVENTION

The invention is related to the field of stem cells and their differentiation. In particular, it is related to the field of beta cells of the islets of Langerhans in the pancreas and nestin positive liver stem cells and their differentiation from stem cells or progenitor cells, and the use of pancreatic stem cells, progenitor cells, and differentiated beta cells or nestin positive liver stem cells or progenitor cells in transplantation.

BACKGROUND OF THE INVENTION

The invention was made at least in part using U.S. government funds, grants DK30457, DK30834, DK55365, DK60125, awarded by the National Institutes of Health, and therefore the U.S. government may retain certain rights in the invention.

The origin of pancreatic islet cells, both during embryonic development and in a mature mammal, has remained uncertain despite intensive study. Certain ductal epithelial cells are capable of either differentiation or transdifferentiation to form beta cells and other cell types found in mature islets (Bouwens, 1998). Ductal cells from isolated islets can proliferate in culture and, if transplanted into an animal, can differentiate into functional beta cells (Cornelius et al., 1997).

It has been demonstrated that exendin-4, a long acting GLP-1 agonist, stimulates both the differentiation of β-cells from ductal progenitor cells (neogenesis) and proliferation of β -cells when administered to rats. In a partial pancreatectomy rat model of type 2 diabetes, the daily administration of exendin-4 for 10 days post pancreatectomy attenuated the development of diabetes. It has also been demonstrated that exendin-4 stimulates the regeneration of the

pancreas and expansion of β–cell mass by neogenesis and proliferation of β–cells (Xu et al., 1999, Diabetes, 48:2270-2276).

Ramiya et al. have demonstrated that islets generated in vitro from pluripotent stem cells isolated from the pancreatic ducts of adult prediabetic non-obese diabetic (NOD) mice differentiate to form glucose responsive islets that can reverse insulin-dependent diabetes after being implanted, with or without encapsulation, into diabetic NOD mice (Ramiya et al., 2000, Nature Med., 6:278-282).

The insulinotropic hormone glucagon-like peptide (GLP)-1 which is produced by the intestine, enhances the pancreatic expression of the homeodomain transcription factor IDX-1 that is critical for pancreas development and the transcriptional regulation of the insulin gene. Concomitantly, GLP-1 administered to diabetic mice stimulates insulin secretion and effectively lowers their blood sugar levels. GLP-1 also enhances β–cell neogenesis and islet size (Stoffers et al., 2000, Diabetes, 49:741-748).

Ferber et al. have demonstrated that adenovirus-mediated *in vivo* transfer of the PDX-1 (also known as IDX-1) transgene to mouse liver results in the transconversion of a hepatocyte subpopulation towards a β–cell phenotype. It has been demonstrated that after intravenous infusion of mice with the PDX-1 adenoviral vector, up to 60% of hepatocytes synthesized PDX-1. Although 60% of liver cells became infected with the adenovirus and expressed PDX-1, only a subset of 5-8% of these cells turned into insulin expressing beta cells. The concentration of immunoreactive insulin was increased in the liver and serum of treated mice. Mice treated with PDX-1 survive streptozotocin-induced diabetes, and can even normalize glycemia (Ferber et al., 2000, Nature Med., 6:568-572).

While ductal cell cultures obtained from isolated islets apparently contain cells that can give rise to insulin-secreting cells, it has remained unclear whether those cells represent true stem cells or merely ductal epithelial cells undergoing transdifferentiation. Even if such preparations contain genuine stem cells, it is unknown what fraction represent stem cells and what contaminating cell types may be present. There is a need in the art for the isolation of specific cell types from pancreatic tissue, the cell types being characterized as stem cells using molecular markers and demonstrated to be pluripotent and to proliferate long-term.

Pluripotent stem cells that are capable of differentiating into neuronal and glial tissues have been identified in brain. Neural stem cells specifically express nestin, an intermediate filament protein (Lendahl et al., 1990; Dahlstrand et al., 1992). Nestin is expressed in the neural tube of the developing rat embryo at day E11, reaches maximum levels of expression in the cerebral

cortex at day E16, and decreases in the adult cortex, becoming restricted to a population of ependymal cells (Lendahl et al., 1990). Developing neural and pancreatic islet cells exhibit phenotypic similarities characterized by common cellular markers.

The invention relates to a population of pancreatic islet stem/progenitor cells (IPCs) that are similar to neural and hepatic stem cells and differentiate into islet α-cells (glucagon) and β--cells (insulin). The invention also relates to nestin-positive liver cells. IPCs according to the invention are immunologically silent/immunoprivileged and are recognized by a transplant recipient as self. The IPCs according to the invention can be used for engraftment across allogeneic and xenogeneic barriers.

There is a need in the art for a method of engrafting stem cells across allogeneic and xenogeneic barriers.

There is also a need in the art for a method of treating type I diabetes mellitus wherein islets, nestin-positive pancreatic stem cells or nestin-positive liver stem cells are transferred into a recipient across allogeneic or xenogeneic barriers and graft rejection does not occur.

There is also a need in the art for a method of transplantation into a mammal wherein islets, nestin-positive pancreatic stem cells or nestin-positive liver stem cells are transferred into a recipient across allogeneic or xenogeneic barriers and graft rejection does not occur.

SUMMARY OF THE INVENTION

It is an object of the invention to provide mammalian pancreatic or liver stem cells for use in treating diabetes mellitus and other disorders. It is also an object of the invention to provide methods for identifying, localizing, and isolating pancreatic stem cells. It is a further object of the invention to provide methods for differentiating pancreatic stem cells to obtain cells that produce insulin and other hormones. It is also an object of the invention to provide methods for transplantation into a mammal that utilize mammalian pancreatic or liver stem cells. These and other objects of the invention are provided by one or more of the embodiments described below.

One embodiment of the invention provides a method of treating a patient with diabetes mellitus. A nestin-positive pancreatic stem cell is isolated from a pancreatic islet of a donor. In another embodiment, a pancreatic stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II is isolated from a pancreatic islet of a donor. In another embodiment, a pancreatic

stem cell that is positive for SST-R2, SST-R3 and/or SST-R4 is isolated from a pancreatic islet of a donor. The stem cell is transferred into the patient, where it differentiates into an insulin-producing cell.

Another embodiment provides another method of treating a patient with diabetes. A nestin-positive pancreatic stem cell is isolated from a pancreatic islet of a donor and expanded *ex vivo* to produce a progenitor cell. In another embodiment, a pancreatic stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II is isolated from a pancreatic islet of a donor and expanded ex vivo to produce a progenitor cell. The progenitor cell is transferred into the patient, where it differentiates into an insulin-producing beta cell. Another embodiment provides still another method of treating a diabetes patient. A nestin-positive pancreatic stem cell is isolated from a pancreatic islet of a donor and expanded to produce a progenitor cell. In another embodiment, a pancreatic stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II is isolated from a pancreatic islet of a donor and expanded to produce a progenitor cell. The progenitor cell is differentiated in culture to form pseudo-islet like aggregates that are transferred into the patient.

Another embodiment provides another method of treating a patient with diabetes mellitus. A nestin-positive pancreatic stem cell is isolated from a pancreatic islet of a donor and cultured ex vivo to produce a progenitor cells. In another embodiment, a pancreatic stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II is isolated from a pancreatic islet of a donor and cultured ex vivo to produce a progenitor cell. The progenitor cell is transferred into the patient, where it differentiates into an insulin-producing beta cell.

In these embodiments, the patient can also serve as the donor of the pancreatic islet tissue, providing an isograft of cells or differentiated tissue.

In another preferred embodiment, prior to the step of transferring, the stem cell is treated *ex vivo* with an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, GLP-1, exendin-4, IDX-1, a nucleic acid molecule encoding IDX-1, betacellulin,

activin A, TGF-β, and combinations thereof.

In another preferred embodiment, the step of transferring is performed via endoscopic retrograde injection or injection into the pancreatic artery.

In another preferred embodiment, the method of treating a patient with diabetes mellitus additionally comprises the step of treating the patient with an immunosuppressive agent.

In another preferred embodiment, the immunosuppressive agent is selected from the group consisting of FK-506, cyclosporin, and GAD65 antibodies.

Another embodiment provides a method of isolating a stem cell from a pancreatic islet of Langerhans. A pancreatic islet is removed from a donor, and cells are cultured from it. A nestin-positive stem cell clone is selected from the culture. In another embodiment, a clone of pancreatic stem cells that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or do not express one or all of CD34, CD45, CD133, MHC class I and MHC class II is selected from the culture. Optionally, the islet is first purged of non-islet cells by culturing in a vessel coated with concanavalin A, which binds the non-islet cells.

In a preferred embodiment, the method of isolating a stem cell further comprises the additional step of expanding the clone by treatment with an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, GLP-1, exendin-4, IDX-1, a nucleic acid molecule encoding IDX-1, betacellulin, activin A, TGF-β, and combinations thereof.

A further embodiment provides a method of inducing the differentiation of a nestin-positive pancreatic stem cell into a pancreatic progenitor cell. Another embodiment provides a method of inducing the differentiation of a pancreatic stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II . As used herein, "differentiation" refers to the process by which a cell undergoes a change to a particular cell type, e.g. to a specialized cell type. The stem cell is treated with an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, IDX-1, a nucleic acid molecule encoding IDX-1, GLP-1, exendin-4, betacellulin, activin A, TGF-β, and combinations thereof. The stem cell subsequently differentiates into a pancreatic progenitor cell.

In a preferred embodiment, the pancreatic progenitor subsequently forms pseudo-islet like aggregates.

Yet another embodiment provides an isolated, nestin-positive human pancreatic or liver stem cell. Yet another embodiment provides an isolated pancreatic stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II. In versions of this embodiment, the stem cell differentiates into either a beta cell, an alpha cell, a pseudo-islet like aggregate, or a hepatocyte. In versions of this embodiment, the stem cell is immunoprivileged. In versions of this embodiment, the stem cell does not express class I MHC antigens. In versions of this embodiment, the stem cell does not express class II MHC antigens. In versions of this embodiment, the stem cell does not express class I or class II MHC antigens.

Still another embodiment provides a method of identifying a pancreatic cell as a stem cell. A cell is contacted with a labeled nestin-specific antibody. If the cell becomes labeled with the antibody, then the cell is identified as a stem cell. In another embodiment, a pancreatic stem cell is identified as a stem cell by contacting the cell with an antibody specific for any of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1 or Kir 6.2 wherein a cell that becomes labeled with an antibody is identified as a stem cell. Optional additional steps include contacting the cell with an antibody to cytokeratin 19 and an antibody to collagen IV; the cell is identified as a stem cell if it does not become labeled with either the cytokeratin 19 or the collagen IV antibody.

In another embodiment, a pancreatic stem cell is identified as a stem cell by contacting the cell with an antibody specific for any of CD34, CD35, CD133, MHC Class I and MHC Class II, wherein the cell is identified as a stem cell if it does not become labeled with any of the antibodies.

Another embodiment provides a method of inducing a nestin-positive pancreatic stem cell to differentiate into hepatocytes. The nestin-positive pancreatic stem cell is treated with an effective amount of an agent that induces the stem cell to differentiate into hepatocytes or into progenitor cells that differentiate into hepatocytes. In a preferred embodiment, the agent is

cyclopamine. Another embodiment provides a method of inducing a pancreatic stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II to differentiate into hepatocytes by treatment with an effective amount of an agent that induces the

stem cell to differentiate into hepatocytes or into progenitor cells that differentiate into hepatocytes.

Yet another embodiment provides a method of treating a patient with liver disease. A nestin-positive pancreatic stem cell is isolated from a pancreatic islet of a donor and transferred into the patient, where the stem cell differentiates into a hepatocyte. In another embodiment, a pancreatic stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II is isolated from a pancreatic islet of a donor and transferred into the patient where the stem cell differentiates into a hepatocyte.

In a related embodiment, the stem cell is expanded *ex vivo* to a progenitor cell, which is transferred into the patient and further differentiates into a hepatocyte.

In another related embodiment, the stem cell is differentiated *ex vivo* to a progenitor cell, which is transferred into the patient and further differentiates into a hepatocyte. In another related embodiment, the stem cell is differentiated *ex vivo* into hepatocytes, which are transplanted into the patient.

In these embodiments, the patient can also serve as the donor of the pancreatic islet tissue, providing an isograft of cells or differentiated tissue.

Yet another embodiment provides an isolated, nestin-positive human liver stem cell. In versions of this embodiment, the stem cell is immunoprivileged. In versions of this embodiment, the stem cell does not express class I MHC antigens. In versions of this embodiment, the stem cell does not express class II MHC antigens. In versions of this embodiment, the stem cell does not express class I or class II MHC antigens.

Yet another embodiment provides an isolated, nestin-positive human stem cell that is not a neural stem cell, that is capable of transplant into an animal without causing graft versus host rejection. In versions of this embodiment, the stem cell is not major histocompatibility complex class I or class I restricted. Still another embodiment provides an isolated human stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II, that is not a neural stem cell, that is capable of transplant into an animal without causing graft versus host rejection.

A "stem cell" as used herein is a undifferentiated cell which is capable of essentially

unlimited propagation either *in vivo* or *ex vivo* and capable of differentiation to other cell types. This can be to certain differentiated, committed, immature, progenitor, or mature cell types present in the tissue from which it was isolated, or dramatically differentiated cell types, such as for example the erythrocytes and lymphocytes that derive from a common precursor cell, or even to cell types at any stage in a tissue completely different from the tissue from which the stem cell is obtained. For example, blood stem cells may become brain cells or liver cells, neural stem cells can become blood cells, such that stem cells are pluripotential, and given the appropriate signals from their environment, they can differentiate into any tissue in the body.

In one embodiment, a "stem cell" according to the invention is immunologically blinded or immunoprivileged. As used herein, "immunologically blinded" or "immunoprivileged" refers to a cell that does not elicit an immune response. As used herein, an "immune response" refers to a response made by the immune system to a foreign substance. An immune response, as used herein, includes but is not limited to transplant or graft rejection, antibody production, inflammation, and the response of antigen specific lymphocytes to antigen. An immune response is detected, for example, by determining if transplanted material has been successfully engrafted or rejected, according to methods well-known in the art, and as defined herein in the section entitled, "Analysis of Graft Rejection". In one embodiment, an "immunogically blinded stem cell" or an "immunoprivileged stem cell" according to the invention can be allografted or xenografted without transplant rejection, and is recognized as self in the transplant recipient or host.

Transplanted or grafted material can be rejected by the immune system of the transplant recipient or host unless the host is immunotolerant to the transplanted material or unless immunosupressive drugs are used to prevent rejection.

As used herein, a host that is "immunotolerant", according to the invention, fails to mount an immune response, as defined herein. In one embodiment, a host that is "immunotolerant" does not reject or destroy transplanted material. In one embodiment, a host that is "immunotolerant" does not respond to an antigen by producing antibodies capable of binding to the antigen.

As used herein, "rejection" refers to rejection of transplanted material by the immune system of the host. In one embodiment, "rejection means an occurrence of more than 90% cell or tissue necrosis of the transplanted material in response to the immune response of the host. In another embodiment, "rejection" means a decrease in the viability such that the viability of the transplanted material is decreased by 90% or more as compared to the viability of the

transplanted material prior to transplantation, in response to the immune response of the host. A decrease in viability can be determined by methods well known in the art, including but not limited to trypan blue exclusion staining. In another embodiment, "rejection" means failure of the transplanted material to proliferate. Proliferation can be measured by methods known in the art including but not limited to hematoxylin/eosin staining. The occurrence of transplant rejection and/or the speed at which rejection occurs following transplantation will vary depending on factors, including but not limited to the transplanted material (i.e., the cell type, or the cell number) or the host (i.e., whether or not the host is immunotolerant and/or has been treated with an immunosuppressive agent. As used herein, "graft versus host rejection" or "graft versus host response" refers to a cell-mediated reaction in which T-cells of the transplanted material react with antigens of the host.

As used herein, "host versus graft rejection" or "host versus graft response" refers to a cell-mediated reaction in which cells of the host's immune system attack the foreign grafted or transplanted material.

In another embodiment of the invention, an immune response has occurred if production of a specific antibody (for example an antibody that binds specifically to an antigen on the transplanted material, or an antibody that binds specifically to the foreign substance or a product of the foreign substance) is detected by immunological methods well-known in the art, including but not limited to ELISA, immunostaining, immunoprecipitation and Western Blot analysis.

Stem cells express morphogenic or growth hormone receptors on the cell surface, and can sense, for example, injury-related factors then localize to and take residence at sites of tissue injury, or sense their local microenvironment and differentiate into the appropriate cell type.

"Essentially unlimited propagation" can be determined, for example, by the ability of an isolated stem cell to be propagated through at least 50, preferably 100, and even up to 200 or more cell divisions in a cell culture system. Stem cells can be "totipotent," meaning that they can give rise to all the cells of an organism as for germ cells. "Totipotent" also means the fertilized egg that can give rise to both embryo and trophoblast. Stem cells can also be "pluripotent," meaning that they can give rise to many different cell types, but not all the cells of an organism. "Pluripotent" also means gives rise to embryo only and not to trophoblast. When a stem cell differentiates it generally gives rise to a more adult cell type, which may be a partially differentiated cell such as a progenitor cell, a differentiated cell, or a terminally differentiated cell. Stem cells can be highly motile.

"Nestin" refers to an intermediate filament protein having a sequence disclosed in Genbank Access No. X65964 (Figure 7).

"ABCG2" refers to ATP-binding cassette multidrug resistance transporter G2 having a sequence disclosed in Genbank Access No. XM_032424 (Figure 18). One of skill in the art will recognize that equivalents exist wherein the DNA sequence encoding ABCG2 may vary, but wherein the encoded amino acid sequence remains the same.

"Oct3/4" refers to a POU/Homeodomain transcription factor having a sequence disclosed in Genbank Access No. NM 013633.

"GLP-1R" refers to the glucagon-like peptide-1-receptor encoded by the nucleic acid sequence shown in Figure 21 (GenBank Accession No. U01156), and having the amino acid sequence also shown in Figure 17 (GenBank Accession No. U01156). One of skill in the art will recognize that equivalents exist wherein the DNA sequence encoding GLP-1R may vary, but wherein the encoded amino acid sequence remains the same.

"Latrophilin (type 2)" refers to a G protein-coupled receptor having a sequence disclosed in Genbank Access No. AJ131581.

"Hes-1" refers to a bHLH transcription factor having a sequence disclosed in Genbank Access No. NM_005524.

Integrins are integral cell-surface proteins composed of an alpha chain and a beta chain. A given chain may combine with multiple partners resulting in different integrins. For example, alpha 6 may combine with beta 4 in the integrin referred to as TSP180, or with beta 1 in the integrin VLA-6.

The integrin subunit "α6" refers to the human integrin α6 cDNA having a sequence disclosed in Genbank Access No. NM_000210.

The integrin subunit "β1" refers to the human β1 cDNA having a sequence disclosed in Genbank Access No.: X07979.

"c-Kit" refers to a cell surface receptor tyrosine kinase having a sequence disclosed in Genbank Access No. X06182.

"MDR-1" refers to the multidrug resistance transporter 1 having a sequence disclosed in Genbank Access No. NM_000927.

"SST-R2" SST-R3" and "SST-R4" refer to somatostatin receptors having a sequence disclosed in Genbank Access Nos. XM_085745 (SSTR-2); NM_001051 (SSTR-3); XM_009594 (SSTR-4).

"SUR-1" refers to a human sulfonylurea receptor having a sequence disclosed in Genbank Access No. XM_042740.

"Kir 6.2" refers to a human inward rectifier potassium channel having a sequence disclosed in Genbank Access No. AF021139.

"CD34" refers to a transmembrane glycoprotein having a sequence disclosed in Genbank Access No. NM_001773.

"CD45" refers to the leukocyte common antigen having a sequence disclosed in Genbank Access No. Y00638.

"CD133" refers to a five-transmembrane hematopoietic stem cell antigen having a sequence disclosed in Genbank Access No. NM_006017.

A "pancreatic" stem cell means a stem cell that has been isolated from pancreatic tissue and/or a cell that has all of the characteristics of: nestin -positive staining, nestin gene expression, cytokeratin-19 negative staining, long-term proliferation in culture, and the ability to differentiate into pseudo-islets in culture.

A "pancreatic" stem cell also refers to a stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II.

In one embodiment, a "pancreatic" stem cell also refers to a stem cell that is positive for SST-R2, SST-R3 and/or SST-R4.

A "liver" stem cell means a stem cell that has been isolated from liver tissue and/or a cell that has all of the characteristics of: nestin -positive staining, nestin gene expression, and long-term proliferation in culture.

A "progenitor cell" is a cell that is derived from a stem cell by differentiation and is capable of further differentiation to more mature cell types.

As used herein, the term "insulin-producing beta cell" refers to any cell which can produce and secrete insulin in a similar amount to that produced and secreted by a beta cell of the islets of Langerhans in the human pancreas. Preferably, the secretion of insulin by an insulin-producing beta cell is also regulated in a similar fashion to the regulation of insulin secretion by a human beta cell *in situ*; for example, insulin secretion should be stimulated by an increase in the glucose concentration in the solution surrounding the insulin-producing beta cell.

"Pseudo-islet like" aggregates are artificial aggregates of insulin-secreting cells which resemble in form and function the islets of Langerhans of the pancreas. Pseudo-islet like

aggregates are created *ex vivo* under cell culture conditions. They are approximately 50-150 μm in diameter (compared to an average diameter of 100 μm for *in situ* islets) and spheroid in form.

"Isolating" a stem cell refers to the process of removing a stem cell from a tissue sample and separating away other cells which are not stem cells of the tissue. An isolated stem cell will be generally free from contamination by other cell types and will generally have the capability of propagation and differentiation to produce mature cells of the tissue from which it was isolated. However, when dealing with a collection of stem cells, *e.g.*, a culture of stem cells, it is understood that it is practically impossible to obtain a collection of stem cells which is 100% pure. Therefore, an isolated stem cell can exist in the presence of a small fraction of other cell types which do not interfere with the utilization of the stem cell for analysis or production of other, differentiated cell types. Isolated stem cells will generally be at least 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 98%, or 99% pure. Preferably, isolated stem cells according to the invention will be at least 98% or at least 99% pure.

A stem cell is "expanded" when it is propagated in culture and gives rise by cell division to other stem cells and/or progenitor cells. Expansion of stem cells may occur spontaneously as stem cells proliferate in a culture or it may require certain growth conditions, such as a minimum cell density, cell confluence on the culture vessel surface, or the addition of chemical factors such as growth factors, differentiation factors, or signaling factors.

A stem cell, progenitor cell, or differentiated cell is "transplanted" or "introduced" into a mammal when it is transferred from a culture vessel into a patient. Transplantation, as used herein, can include the steps of isolating a stem cell according to the invention and transferring the stem cell into a mammal or a patient. Transplantation can involve transferring a stem cell into a mammal or a patient by injection of a cell suspension into the mammal or patient, surgical implantation of a cell mass into a tissue or organ of the mammal or patient, or perfusion of a tissue or organ with a cell suspension. The route of transferring the stem cell or transplantation, will be determined by the need for the cell to reside in a particular tissue or organ and by the ability of the cell to find and be retained by the desired target tissue or organ. In the case where a transplanted cell is to reside in a particular location, it can be surgically placed into a tissue or organ or simply injected into the bloodstream if the cell has the capability to migrate to the desired target organ.

Transplantation, as used herein, can include the steps of isolating a stem cell according to the invention, and culturing and transferring the stem cell into a mammal or a patient. Transplantation, as used herein, can include the steps of isolating a stem cell according to the

invention, differentiating the stem cell, and transferring the stem cell into a mammal or a patient. Transplantation, as used herein, can include the steps of isolating a stem cell according to the invention, differentiating and expanding the stem cell and transferring the stem cell into a mammal or a patient.

Treatment with an immunosuppressive agent can be accomplished by administering to a patient in need thereof any agent which prevents, delays the occurrence of or decreases the intensity of the desired immune response, *e.g.*, rejection of a transplanted cell, tissue, or organ.

As used herein, "immunosuppression" refers to prevention of the immune response (for example by the administration of an "immunosuppresive agent", as defined herein) such that an "immune response", as defined herein, is not detectable. As used herein, "prevention" of an immune response means an immune response is not detectable. An immune response (for example, transplant rejection or antibody production) is detected according to methods well-known in the art and defined herein.

"Immunosuppression" according to the invention also means a delay in the occurrence of the immune response as compared to any one of a transplant recipient that has not received an immunosuppresive agent, or a transplant recipient that has been transplanted with material that is not "immunologically blinded" or "immunoprivileged", as defined herein. A delay in the occurrence of an immune response can be a short delay, for example 1hr-10 days, i.e., 1 hr, 2, 5 or 10 days. A delay in the occurrence of an immune response can also be a long delay, for example, 10 days-10 years (i.e., 30 days, 60 days, 90 days, 180 days, 1, 2, 5 or 10 years).

"Immunosuppression" according to the invention also means a decrease in the intensity of an immune response. According to the invention, the intensity of an immune response can be decreased such that it is 5-100%, preferably, 25-100% and most preferably 75-100% less than the intensity of the immune response of any one of a transplant recipient that has not received an immunosuppresive agent, or a transplant recipient that has been transplanted with material that is not "immunologically blinded" or "immunoprivileged", as defined herein. The intensity of an immune response can be measured by determining the time point at which transplanted material is rejected. For example, an immune response comprising rejection of transplanted material at day 1, post-transplantation, is of a greater intensity than an immune response comprising the rejection of transplanted material at day 30, post-transplantation. The intensity of an immune response can also be measured by quantitating the amount of a particular antibody capable of binding to the transplanted material, wherein the level of antibody production correlates directly with the intensity of the immune response. Alternatively, the intensity of an immune response

can be measured by determining the time point at which a particular antibody capable of binding to the transplanted material is detected.

Various strategies and agents can be utilized for immunosuppression. For example, the proliferation and activity of lymphocytes can be inhibited generally with agents such as, for example, FK-506, or cyclosporin or other immunosuppressive agents. Another possible strategy is to administer an antibody, such as an anti-GAD65 monoclonal antibody, or another compound which masks a surface antigen on a transplanted cell and therefore renders the cell practically invisible to the immune system of the host.

An "immunosuppressive agent" is any agent that prevents, delays the occurrence of or reduces the intensity of an immune reaction against a foreign cell in a host, particularly a transplanted cell. Preferred are immunosuppressive agents which suppress cell-mediated immune responses against cells identified by the immune system as non-self. Examples of immunosuppressive agents include but are not limited to cyclosporin, cyclophosphamide, prednisone, dexamethasone, methotrexate, azathioprine, mycophenolate, thalidomide, FK-506, systemic steroids, as well as a broad range of antibodies, receptor agonists, receptor antagonists, and other such agents as known to one skilled in the art.

A "mitogen" is any agent that stimulates mitosis and cell proliferation of a cell to which the agent is applied.

A "differentiation factor" is any agent that causes a stem cell or progenitor cell to differentiate into another cell type. Differentiation is usually accomplished by altering the expression of one or more genes of the stem cell or progenitor cell and results in the cell altering its structure and function.

A "signaling factor" as used herein is an agent secreted by a cell which has an effect on the same or a different cells. For example, a signaling factor can inhibit or induce the growth, proliferation, or differentiation of itself, neighboring cells, or cells at distant locations in the organism. Signaling factors can, for example, transmit positional information in a tissue, mediate pattern formation, or affect the size, shape and function of various anatomical structures.

As used herein, a mammal refers to any mammal including but not limited to human, mouse, rat, sheep, monkey, goat, rabbit, hamster, horse, cow or pig.

A "non-human mammal", as used herein, refers to any mammal that is not a human.

As used herein, "allogeneic" refers to genetically different members of the same species.

As used herein, "isogeneic" refers to of an identical genetic constitution.

As used herein, "xenogeneic" refers to members of a different species.

As used herein, "culturing" refers to propagating or nurturing a cell, collection of cells, tissue, or organ, by incubating for a period of time in an environment and under conditions which support cell viability or propagation. Culturing can include one or more of the steps of expanding and proliferating a cell, collection of cells, tissue, or organ according to the invention.

As used herein, "pre-inducing or inducing a state of immune tolerance" refers to the treatment of a potential transplant recipient with stem cells derived from a transplant donor such that, upon transplantation of an organ, tissue or cells from the donor, the immune system of the recipient recognizes the donor tissue as self and consequently does not initiate a "graft versus host" response. In a preferred embodiment, the stem cells are nestin positive stem cells.

As used herein, "tolerance" refers to a state of the immune system in which certain foreign antigens are recognized by the cells of the immune system as self.

As used herein, a "recipient" refers to a mammal that receives an organ, tissue or cells taken from a donor.

As used herein, a "donor" is a mammal from which organs, tissues or cells are taken for transplant into a recipient.

The invention also provides for a pharmaceutical composition comprising the isolated stem cells of the invention admixed with a physiologically compatible carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show dual fluorescence immunocytochemical staining of rat pancreatic islets at embryonic day 16 (Fig. 1A) and at day 60 after birth (Fig. 1B). Immunostaining with an antibody for nestin is shown in white (red in the original, with Cy3 as fluorophore) and with an antibody for insulin is shown in grey (green in the original, with Cy2 as fluorophore).

Figure 2 shows the result of RT-PCR performed using mRNA obtained from 50 rat islets. Forward and reverse primers are indicated. The single band of 834 bp was sequenced and identified substantially as the sequence for nestin.

Figure 3 shows nestin-positive cells that have proliferated out from a cultured rat islet.

Figure 4 shows the development of islet like structures in culture.

Figure 5 shows the results of RT-PCR analysis of islet-like structures generated in culture. Expression of NCAM and cytokeratin-19 (CK19) was detected.

Figure 6 shows the stimulation of nestin mRNA expression by high glucose. APRT was examined as a control.

Figure 7 is the amino acid and nucleotide sequences of nestin.

Figure 8 depicts expression of the neural stem cell-specific marker nestin in a distinct cell population within pancreatic islets as determined by immunocytochemistry or RT-PCR.

Figure 9 depicts characterization of nestin in stem cells isolated from the pancreas by immunocytochemistry and RT-PCR.

Figure 10 depicts expression of homeodomain protein IDX-1 and proglucagon in human islet-like clusters derived from nestin-positive islet progenitor cells (NIPs).

Figure 11 demonstrates localization of nestin-positive cells to localized regions of the ducts of the rat pancreas.

Figure 12 depicts alternative models for the origin of pancreatic duct cells that are progenitors of islet endocrine cells.

Figure 13A and B depicts immunofluorescent staining of nestin positive liver stem cells.

Figure 14 depicts the sequential appearance of transcription factors during development of the murine endocrine pancreas.

Figure 15 depicts expression of neuroendocrine, exocrine pancreatic and hepatic markers in human NIP cultures containing stem cells.

Figure 16 depicts expression of proglucagon and insulin mRNA as determined by RT-PCR and insulin secretion.

Figure 17 depicts NIP markers.

Figure 18 depicts the nucleic acid(a) and amino acid(b) sequence of human ABCG2.

Figure 19a depicts expression of the ATP-binding cassette transporter ABCG2 by RT-PCR and Southern blot hybridization.

Figure 19b demonstrate that nestin-positive islet derived progenitor cells (NIPs). include a significant number of SP cells as demonstrated by Hoechst 33342 staining.

Figure 19c demonstrates that dye efflux from SP cells is inhibited in the presence of verapamil. SP-gated cells are 2.1% of the total number of analyzed cells in B versus 0.1% in C.

Figure 20 demonstrates that SP cells isolated by FACS co-express high levels of ABCG2, MDR1 and nestin. Figure 20A depicts that SP cells and non-SP control cells were isolated by FACS after Hoechst 33342 staining (R1 and R2, respectively). Figure 20B demonstrates that the

cells were analyzed for the expression of ABCG2, MDR1, nestin, and GAPDH RNA by RT-PCR. The identity of the PCR products for ABCG2 and MDR1 was confirmed by Southern blot hybridization.

Figure 21 shows the nucleic acid (21A) and amino acid (21B) sequence of the human GLP-1R.

Figure 22 shows the expression of GLP-1R on pancreatic islet-derived Stem/progenitor cells. Panel A shows the immunocytochemical detection of GLP-1R (Cy-3) and nuclei stained with DAPI. Panel B shows RT-PCR of RNA prepared from NIPS with primers specific for GLP-1R.

Figure 23 shows GLP-1 (7-36) amide and tolbutamide stimulation of Ca^{2+} influx in nestin-positive NIPs.

Figure 24 shows immunohistochemical staining of NIPs demonstrating GLP-1 induced differentiation. Panel A shows immunohistochemical identification of nestin and insulin. Panel B shows nestin and insulin immunohistochemical staining after cells were challenged with GLP-1.

Figure 25 shows the levels of insulin secretion from NIP cultures challenged with GLP-1.

Figure 26 shows the immunohistochemical analysis of insulin expression in NIP cultures transfected with human Idx-1.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have identified and isolated a special subclass of ductal cells from the islets of Langerhans of mammalian pancreas that have the functional and molecular characteristics of stem cells. In particular, these newly discovered pancreatic stem cells are characterized *inter alia* by one or more (and preferably all of): nestin-positive staining, nestin gene expression, GLP-1R-positive staining, GLP-1R gene expression, ABCG2 positive staining, ABCG2 gene expression,Oct3/4 positive staining, Oct3/4 gene expression, latrophilin (type 2) positive staining, latrophilin (type 2) gene expression, Hes-1 positive staining, Hes-1 gene expression, Integrin subunits α6 and β1 positive staining, Integrin subunits α6 and β1 gene expression, C-kit positive staining, C-kit gene expression, MDR-1 positive staining, MDR-1 gene expression, SST-R, 2, 3, 4 positive staining, SST-R, 2, 3, 4 gene expression, SUR-1 positive staining, SUR-1 gene expression, Kir 6.2 positive staining, Kir 6.2 gene expression CD34 negative staining, CD45 negative staining, CD133 negative staining, MHC class I negative

staining, MHC class II negative staining,cytokeratin-19 negative staining, long-term proliferation in culture, and the ability to differentiate into pseudo-islets in culture. The present inventors have also identified liver cells that exhibit nestin-positive staining.

In one embodiment, the invention provides stem cells for a variety of applications, including but not limited to cellular replacement therapy for type I insulin-dependent diabetes and other forms of diabetes as well as the development of research tools to study the onset and progression of various diabetic conditions, hormonal abnormalities, and genetic diseases or conditions, such as the association of polymorphisms with particular physiologic or pathologic states. The stem cells of the invention can also be used to carry out gene therapy of endocrine pancreatic or other tissues in isograft, allograft or xenograft transplantations. Further, the stem cells described herein can be used to produce recombinant cells, artificial tissues, and replacement organs in culture. They can also be used for the *ex vivo* production of insulin and other hormones. Molecular characteristics of pancreatic stem cells discovered by the inventors, such as nestin-positive, GLP-1R-positive, ABCG2 positive staining, Oct3/4 positive, latrophilin (type 2) positive, Hes-1 positive, Integrin subunits $\alpha 6$ and $\beta 1$ positive, C-kit positive, MDR-1 positive, SST-R, 2, 3, 4 positive, SUR-1 positive, Kir 6.2 positive and cytokeratin-19 negative, CD34 negative, CD45 negative, CD133 negative, MHC class I negative and MHC class II negative staining, or liver stem cells, such as nestin-positive staining, can be used in various diagnostic, pathological, or investigative procedures to identify, localize, and quantitate stem cells in tissues from a patient or experimental animal.

Identification of Stem Cells in Pancreatic Islets

Previous investigators have focused on ductal epithelial cells of pancreatic islets or exocrine tissue as a possible source of stem cells for the neogenesis of islet endocrine cells. Nestin is an intermediate filament protein that was cloned by screening a cDNA library from E15 rat embryos with a monoclonal antibody named R.401 (Hockfield & McKay, 1985; Lendahl et al., 1990). Nestin was primarily found in neuroepithelial stem cells and is expressed in the developing central nervous system. After maximum levels are reached in the rat embryo at E16, nestin expression declines to almost undetectable levels in adult cerebral cortex, coinciding with terminal differentiation of early nestin-expressing progenitor cells (Lendahl et al., 1990). Nestin was initially found exclusively in stem cells of the embryonic developing brain and skeletal muscle (Lendahl et al., 1990). Later studies identified nestin-positive neural stem cells in the

subependymal layer of the adult mammalian forebrain (Morshead et al., 1994). Nestin-positive stem cells have been shown to be pluripotential even when isolated from adult mice or rat brain. For example, nestin-positive stem cells can generate all three major classes of neural cells in culture: neurons, astrocytes, and oligodendrocytes (Reynolds & Weiss, 1996). Nestin-positive neural stem cells respond to spinal cord injury by proliferation and degeneration of migratory cells that differentiate into astrocytes, participate in scar formation (Johansson et al., 1999) and restore hematopoietic cells of the bone marrow after infusion into irradiated mice (Bjornson et al., 1999).

Characterization of Stem Cells

Stem cells according to the invention can be identified by their expression of nestin and/or GLP-1R, ABCG2, Oct3/4, latrophilin (type 2), Hes-1, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R, 2, 3, 4, SUR-1 and Kir 6.2, by, for example, FACS, immunocytochemical staining, RT-PCR, Southern Northern and Western blot analysis, and other such techniques of cellular identification as known to one skilled in the art.

Immunocytochemical staining, for example, is carried out according to the following method. Cryosections (6 μM) prepared from pancreata or liver, as well as cells, are fixed with 4% paraformaldehyde in phosphate. Cells are first blocked with 3% normal donkey serum for 30 min at room temperature and incubated with a primary antisera to the protein of interest overnight at 4°C. The antisera is rinsed off with PBS and incubated with the appropriate fluorescently labeled secondary antisera for 1 hour at room temperature. Slides are then washed with PBS and coverslipped with fluorescent mounting medium (Kirkegaard and Perry Labs, Gaithersburg, MD). Fluorescence images are obtained using a Zeiss Epifluorescence microscope equipped with an Optronics TEC-470 CCD camera (Optronics Engineering, Goleta, CA) interfaced with a PowerMac 7100 installed with IP Lab Spectrum analysis software (Signal Analytics Corp, Vienna, VA).

Antisera useful according to the invention include the following: mouse monoclonal antibodies to human cytokeratin 19 (clone K4.62, Sigma, St. Louis, MO), rabbit polyclonal antisera to rat nestin and to IDX-1 (prepared by immunizations of rabbits with a purified GST-nestin fusion protein or the last twelve amino acids of rat IDX-1, respectively) (McManus et al., 1999, J. Neurosci., 19:9004-9015), rabbit polyclonal antisera to GLP-1R (Heller et al., 1997, Diabetes 46: 7851) antisera to ABCG2 (MAB4146, Chemicon (Temecula, CA)) antisera to

integrin αG (SC-6597, Santa Cruz), antisera to integrin β1, (SC-6622, Santa Cruz), antisera to HES 1 (AB5702, Chemicon), antisera to CD 45 (31252X, BD Pharmingen (San Diego, CA)), antisera to CD 34 (MS-363-PO, NeoMarkers (Freemont, CA)), antisera to MHC I (MS-557-PO, NeoMarkers), antisera to MHC II (MS-162-PO, NeoMarkers) antisera to MDR-1 (p170) (MS-660-PO, NeoMarkers), antisera to Oct 3/4 (SC-5279, Santa Cruz (SC, CA)), antisera to SUR-1(SC-5789, Santa Cruz) antisera to KIR 6.2 (SC-11227, Santa Cruz), antisera to ABC G2 (SC-18841, Santa Cruz) antisera to c-kit (SC-1493, Santa Cruz), antisera to SSTR 2 (SC-11606, Santa Cruz), antisera to SSTR 3 (SC-11610, Santa Cruz) antisera to SSTR 4 (SC-11619, Santa Cruz), guinea pig anti-insulin and anti-pancreatic polypeptide antisera, obtained from Linco, St. Charles, MO, and mouse antiglucagon and rabbit antisomatostatin antisera, purchased from Sigma (St. Louis, MO) and DAKO (Carpinteria, CA), respectively, mouse anti-human galanin (Peninsula Laboratories, Belmont, CA), collagen IV antisera (Caltag Laboratories, San Francisco, CA), mouse anti-rat MHC class I serum (Seroteck), and antirat MHC class II serum. The invention contemplates that other antisera directed to such markers is available, or will be developed. Such other antisera is considered to be within the scope of the invention.

RT-PCR and Southern blot analysis are performed according to the following methods. Total cellular RNA prepared from rat or human islets is reverse transcribed and amplified by PCR for about 35 cycles depending on the desired degree of amplification, as described previously (Daniel, et al., 1998, Endocrinology, 139:3721-3729). Oligonucleotides used as primers or amplimers for the PCR and as probes for subsequent Southern blot hybridization are:

Rat nestin:	Forward, 5'gcggggcggtgcgtgactac3';
	Reverse, 5'aggcaaggggaagagaaggatgt3';
	Hybridization, 5'aagctgaagccgaatttccttgggataccagagga3'.
Rat keratin 19:	Forward, 5'acagccagtacttcaagacc3';
	Reverse, 5'ctgtgtcagcacgcacgtta3';
	Hybridization, 5'tggattccacaccaggcattgaccatgcca3'.
Rat NCAM:	Forward, 5'cagcgttggagagtccaaat3';
	Reverse, 5'ttaaactcctgtggggttgg3';
	Hybridization, 5'aaaccagcagcggatctcagtggtgtggaacgatgat3'.

Rat IDX-1 Forward, 5'atcactggagcagggaagt3'

Reverse, 5'gctactacgtttcttatct3'

Hybridization, 5'gcgtggaaaagccagtggg3'

Human nestin: Forward, 5'agaggggaattcctggag3';

Reverse, 5'ctgaggaccaggactctcta3';

Hybridization, 5'tatgaacgggctggagcagtctgaggaaagt3'.

Human keratin: Forward, 5'cttttcgcgcgcccagcatt3';

Reverse, 5'gatcttcctgtccctcgagc3';

Hybridization, 5'aaccatgaggaggaaatcagtacgctgagg3'.

Human glucagon: Forward, 5'atctggactccaggcgtgcc3';

Reverse, 5'agcaatgaattccttggcag3';

Hybridization, 5'cacgatgaatttgagagacatgctgaaggg3';

Human E-Cadherin Forward, 5' agaacagcacgtacacagcc 3'

Reverse, 5'cctccgaagaaacagcaaga 3'

Hybridization, 5' tctcccttcacagcagaactaacacacggg 3'

Human transthyretin Forward, 5' gcagtcctgccatcaatgtg 3'

Reverse, 5' gttggctgtgaataccacct 3'

Hybridization, 5' ctggagagctgcatgggctcacaactgagg 3'

Human Pancreatic Amylase Forward, 5'gactttccagcagtcccata 3'

Reverse, 5' gtttacttcctgcagggaac 3'

Hybridization, 5' ttgcactggagaaggattacgtggcgttcta 3'

Human procarboxypeptidase

Forward, 5' tgaaggcgagaaggtgttcc 3'

Reverse, 5' ttcgagatacaggcagatat 3'

Hybridization, 5' agttagacttttatgtcctgcctgtgctca 3'

Human Synaptophysin Forward, 5' cttcaggctgcaccaagtgt 3'

Reverse, 5' gttgaccatagtcaggctgg 3'

Hybridization, 5' gtcagatgtgaagatggccacagacccaga 3'

Human Hepatocyte Growth Factor (HGF)

Forward, 5' gcatcaaatgtcagccctgg 3'

Reverse, 5' caacgctgacatggaattcc 3'

Hybridization, 5' tcgaggtctcatggatcatacagaatcagg 3'

Human cMET (HGF-receptor)Forward, 5' caatgtgagatgtctccagc 3'

Reverse, 5' ccttgtagattgcaggcaga 3'

Hybridization, 5' ggactcccatccagtgtctccagaagtgat 3'

Human XBP-1 Forward, 5'gagtagcagctcagactgcc 3'

Reverse, 5' gtagacctctgggagctcct 3'

Hybridization, 5' cgcagcactcagactacgtgcacctctgca 3'

Human Glut-2 Forward, 5' gcagctgctcaactaatcac 3'

Reverse, 5' tcagcagcacaagtcccact 3'

Hybridization, 5' acgggcattcttattagtcagattattggt 3'

Human Insulin Forward, 5' aggcttcttctacaca3'

Reverse, 5' caggctgcctgcacca 3'

Hybridization, 5' aggcagaggacctgca 3'

Other such sequences are possible and such sequences are considered to be within the scope of the art. The invention includes oligonucleotides used as primers or amplimers for the PCR and as probes for Southern analysis for any of the markers selected from the group consisting of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits $\alpha 6$ and $\beta 1$, C-kit, MDR-1, SST-R, 2, 3, 4, SUR-1, Kir 6.2, CD34, CD45, CD133, MHC class I and MHC class II. As a general guide, primers are selected from two different exons and encompass at least one intronic sequence. In addition, an RT minus control is run for most samples. PCR amplification is effectuated at 94°C for 1 min followed by 94°C for 10 secs, 58/56°C for 10 secs, 72°C for 1 min, 35 cycles, and 72°C for 2 min. The annealing temperature is 58°C for rat nestin and 56°C for the remaining primer pairs.

For RT-PCR of mRNA isolated from a mammal that is not rat or human, oligonucleotides that are specific for the amplified nucleic acid from the mammalian species being analyzed are prepared. The selection and use of such primers is known to one skilled in the art.

For Southern hybridization oligonucleotide probes are labeled with an appropriate radionuclide, such as γ^{32}P ATP, using conventional techniques. Radiolabeled probes are hybridized to PCR products transferred to nylon membranes at 37°C for one hour, then washed in 1 x SSC + 0.5% SDS at 55°C for 10-20 min or 0.5 x SCC + 0.5% SDS at 42° for the human PCR products.

Nestin as a Marker of Pancreatic Stem Cells

The inventors have now unexpectedly discovered that the pancreas of adult mammals, including humans, contains cells that express nestin. Importantly, the distribution of nestin-positive cells in the pancreas does not correspond to the distribution of hormone-producing cells. For example, fluorescently labeled antibodies specifically reactive to insulin or glucagon label the beta and alpha cells of the islets, respectively, whereas the inventors have observed that in mice, rats, and humans, fluorescently labeled nestin antibodies localize only to certain cells of the ductular epithelium and not to alpha, beta, delta, and pancreatic peptide producing cells in pancreatic islets (Fig. 1). The inventors also observed that antibodies specific for collagen IV, a marker for vascular endothelial cells, galanin, a marker of nerve endings, and cytokeratin 19, a marker for ductal cells, do not colocalize with nestin antibodies. Furthermore, the inventors have found that nestin-positive islet cells do not co-label with antibodies for insulin, glucagon, somatostatin, or pancreatic polypeptide (Fig. 1). This suggests that these nestin-containing cells are not endocrine cells, ductal cells, neural cells, or vascular endothelial cells, but may represent a truly distinct cell type within the islet that has not previously been described. The inventors have found nestin-positive cells in the islets, as well as localized regions of the pancreatic ducts, and within centroacinar regions of the exocrine pancreas.

The expression of nestin mRNA in isolated islets was detected using RT-PCR with RNA from isolated rat islets (Fig. 2). The functional properties of nestin-positive pancreatic cells were investigated using cell culture techniques and by the isolation of nestin-positive cells from islets, both of which are described below.

The inventors have also discovered that the liver of rats contains cells that express nestin (Fig. 13).

ATP-Binding Cassette (ABC) Multi-Drug Rresistance Transporters (ABCG2 (Brcp1) and MDR-) as Markers ofPancreatic Stem Cells.

Human islet-derived NIPs contain a substantial subpopulation of SP cells that co-express ABCG2, MDR1 and nestin: ABCG2 expression defines the side population (SP) phenotype of pluripotential stem cells in the bone marrow, by virtue of excluding the dye Hoechst 33342. MDR-1 renders SP cells the capacity to replicate. SP cells (CD34 low/negative) do not proliferate and therefore cannot be expanded *in vitro*. The forced expression of MDR-1 in CD34 low negative SP cells causes them to proliferate (Bunting *et al.*, 2000, *Blood* 96:902). NIPs are

also CD34 low/negative. Therefore NIPs are now defined as SP cells by virtue of their expression of ABCG2 and unlike marrow-derived SP cells, NIPs express MDR-1 and therefore can proliferate making it feasible to expand NIP SP cells *ex vivo* for purposes of transplantation.

Oct3/4 as a Marker of Pancreatic Stem Cells

NIPs express Oct3/4. Oct3/4 is a transcription factor belonging to the family of Pou homeodomain proteins (Niwa et al., 2002, *Mol. Cell. Biol.* 22:1526; Niwa et al., 2000, *Nat. Genet.* 24:328; Niwa, 2001, *Cell Struct Funct* 26:137; Shimazaki et al., 1993, *EMBO* 12:4489; Wang et al., 1996, *Biochem Cell Biol* 74:579; Nichols et al., 1998, *Cell* 95:379; . This is an important finding because the expression of Oct3/4 is strictly restricted to stem/progenitor cells (Niwa *et al.* 2002, supra; Niwa et al., 2000, supra; Niwa, 2001, supra). NIPs robustly express Oct3/4, thereby defining them *per se* to be stem/progenitor cells. That is if Oct3/4 expression is absolutely restricted to stem/progenitor cells, and Oct3/4 is expressed in NIPs, then NIPs are stem/progenitor cells.

Integrin subunits α6 and B1 as a Marker of Pancreatic Stem Cells

NIPs express the integrin subunits α and *B1*. *α6/B1* integrin is the laminin receptor (Giancotti and Ruoslahti, 1999, *Science* 285:1028). It has recently been shown that ES cells can be propagated in culture dishes coated with laminin and do not need to be grown on feeder cell layers (Xu *et al.*, *Nature Biotechnology*, October 2001, 19:971). The cell surface expressed integrins are essential for the maintenance of cell growth and development (see Giancotti et al., supra). The *α6/B1* receptor is specific for recognition of laminin, which is an essential protein expressed in the blastocyst. Therefore *α6/B1* is a marker of ES cells (stem/progenitor cells). NIPs express the integrin subunits *α6* and *B1*. Therefore NIPs are similar to ES cells in this regard.

Hes-1 as a Marker of Pancreatic Stem Cells

The delta/Notch signaling pathway of lateral inhibition is critical for embryonic development. Much as been written about the importance of Notch signaling. Notch signaling is also important in NIPs. Notch signaling is absolutely required for the development of the pancreas (Apelquist *et al.*, 1999, *Nature* 400:6747; Lammert *et al.*, 2000, *Mech Dev.* 94:; Jensen *et al.*, 2000, *Nat. Genet.*, 24:36; Jansen *et al. Diabetes* 2000, 49:163). Ngn-3 is the key bHLH transcription factor determinant required for the development of the endocrine pancreas lineage.

Hes-1 is a powerful suppressor of Ngn-3 expression. NIPs robustly express Hes-1. Therefore NIPs are progenitor cells prevented from differentiating into endocrine cells. Therefore a method to block the expression of Hes-1 in the NIPs will lead to their differentiation into endocrine cells (β-cells). Such a method for blocking Hes-1 expression could be the use of transfected antisense RNA, either by using small interfering RNA oligonucleotides or an expression plasmid expressing antisense Hes-1 RNA.

GLP-1R as a Marker of Pancreatic Stem Cells

The inventors have further discovered that the pancreas of adult mammals, including humans, contains cell that express the glucagon like peptide-1 receptor (GLP-1R). The invention is further based on the discovery that GLP-1R-positive cells can co-localize with nestin-positive cells. The invention is also based on the converse discovery; that nestin-positive pancreatic stem cells can co-localize with GLP-1R-positive cells. The glucagon gene encodes a multifunctional proglucagon, that is differentially process by pro-hormone convertases in the pancreas and intestine to yield glucagon and the glucagon-like peptides (GLP). GLP-1 has been shown to bind specifically to a G-protein coupled receptor found in pancreatic β-cells to stimulate insulin secretion via a cAMP-dependent pathway (Kiefer and Habener, 1999, *Endocr. Rev.* 20: 876; Drucker, 1998 *Diabetes*, 47: 159). The present invention is based, in part, on the discovery that the nestin-positive stem cells found in the pancreatic islets and ducts, as described above, also are GLP-1R-positive.

Nestin-positive-Islet-Progenitor cells (NIPs) were isolated from human islet tissue and reacted with rabbit polyclonal antisera to the GLP-1R (Heller et al., *supra)*, following the general immunocytochemical procedures outlined above. Receptor immunoreactivity was detected in greater than 60% of NIPs examined (Figure 22A). To further confirm the immunocytochemical identification of GLP-1R in NIPs, RT-PCR was performed on mRNA prepared from NIP cells. Amplification of NIP mRNA with the amplification primers 5' gtgtggcggccaattactac 3' (Forward); 5' cttggcaagtctgcatttga 3' (Reverse) produced the expected 346 bp product (Figure 22B) indicating that, in addition to expressing the GLP-1R protein, NIPs have the biosynthetic ability to produce GLP-1R. GLP-1R, therefore, in addition to nestin, is useful in the present invention as a marker for pancreatic stem cells.

Cytokeratin-19 as a Marker for a Distinct Population of Duct Epithelial Cells

Cytokeratin-19 (CK-19) is another intermediate filament protein. CK-19 and related cytokeratins have previously been found to be expressed in pancreatic ductal cells (Bouwens et al., 1994). The inventors have discovered, however, that while CK-19 expression is indeed confined to the ductules, fluorescent antibodies specific for CK-19 label distinct ductal cells from those labeled with nestin-specific antibodies. This suggests that nestin-positive cells in islets may be a distinct cell type of ductal cell from CK-19 positive cells.

NIPs are CD34 low/negative:

The SP fraction of NIPs (left lower gate of Figure 19B) express low to null levels of CD34. This finding is important because CD34 low negative marrow-derived SP cells, as contrasted to CD34-positive SP cells, are highly pluripotential (see Goodell, 1999, *Blood*, 94:12545). Marrow-derived CD34 low/negative SP cells have a limited, if any, capacity to proliferate (Goodell *et al.*, 1996, *J. Exp. Med.*, 183:1797; Goodell *et al.*, 1997 *Nature Medicine*, 3:1337). CD34 low/negative NIPs do proliferate *in vitro*, because they express Mdr-1.

Additional Markers of Pancreatic Stem Cells

The invention also provides for pancreatic stem cells that are positive for at least one of the markers selected from the group consisting of latrophilin (type 2) (Sudhof, 2001, *Annu Rev Neurosci* 24:933), C-kit (CD117) (Gibson et al., 2002, *Adv Anat Pathol* 9:65), SSTR-2, 3 and 4 (somatostatin receptors) (Schulz et al., 2000, *J Physiol Paris* 94:259), SUR-1 (sulonylurea receptor) (Winarto et al., 2001, *Arch Histol Cytol* 64:59; Landgraf, 2000, *Drugs Aging*, 17:411), and Kir 6.2 (inward rectifying K+ channel subunit with SUR-1 (Winarto et al., supra; Landgraf, supra).

The invention also provides for pancreatic stem cells that are negative for at least one of CD45 (Sasaki et al., 2001, *Int J Biochem Cell Biol* 33:1041), CD133 (Kobari et al., 2001, *J Hematother Stem Cell Res* 10:273), MHC class II and MHC class II.

Isolated Stem Cells from Pancreatic Islets and Their Use

Stem cells can be isolated from a preparation of pancreatic tissue, for example, islets obtained from a biopsy sample of tissue from a diabetic patient. The stem cells can then be

expanded *ex vivo* and the resulting cells transplanted back into the donor as an isograft. Inside the donor, they may differentiate to provide insulin-secreting cells such as beta cells to replace beta cells lost to the autoimmune attack which caused the diabetes. This approach can overcome the problems of immune rejection resulting from transplantation of tissue, for example, islets from another individual who might serve as the donor. In one embodiment of the invention, the use of isografted stem cells allows another technique to be performed in an effort to avoid the immune rejection, namely genetic therapy of the transplanted cells to render them resistant to immune attack, such as the autoimmunity present in individuals with type 1 diabetes. A further advantage of using stem cells over whole islets is that transplanted stem cells can differentiate *in situ* and better adapt to the host environment, for example, providing appropriate microcirculation and a complement of different islet cell types which responds to the physiological needs of the host. Another embodiment of the invention contemplates the use of partially differentiated stem cells *ex vivo*, for example, to form progenitor cells, which are subsequently transplanted into the host, with further differentiation optionally taking place within the host. Although the use of an isograft of stem cells, progenitor cells, or pseudo-islets is preferred, another embodiment contemplates the use of an allograft of stem cells, progenitor cells, or pseudo-islets obtained from another individual or from a mammal of another species.

In yet another embodiment of the invention, the stem cells are immunologically blind or immunoprivileged. In one embodiment of this aspect of the invention, immunoprivileged stem cells do not express sufficient amounts of class I and/or class II major histocompatibility antigens (a.k.a. HLA or human leukocyte antigen) to elicit an immune response from the host. For example, these stem cells, obtained from allogeneic or xenogeneic sources do not initiate a host versus graft response in immunocompetent transplant recipients.

In another embodiment of this aspect of the invention, immunoprivileged stem cells do not express class I MHC antigens and/or class II MHC antigens. These stem cells, obtained from allogeneic or xenogeneic sources do not initiate a host versus graft response in immunocompetent transplant recipients.

In another embodiment of the invention, human tissue grafts comprising stem cells express both human specific class I and class II MHC antigens, but are recognized by immunocompetent mice as self, and do not undergo host versus graft rejection. These stem cells, obtained from allogeneic or xenogeneic sources do not initiate a host versus graft response in immunocompetent transplant recipients.

The invention also provides for methods of isolating stem cells from a xenogenic donor, and transplanting the resulting cells into a mammal of another species (e.g. murine stem cells are transplanted into a human, for example, a diabetic human patient) as a xenograft.

The invention provides for methods of performing isogeneic, allogeneic or xenogeneic transplants of nestin-positive stem cells wherein the stem cells are cultured for a period of time, for example, 2-4 hours, 4-5 hours, 5-10 hours or 1-3 days prior to transplantation. The invention also provides for methods of performing isogeneic, allogeneic or xenogeneic transplants of a stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II wherein the stem cells are cultured for a period of time, for example, 2-4 hours, 4-5 hours, 5-10 hours or 1-3 days prior to transplantation..

The invention also provides for methods of performing isogeneic, allogeneic or xenogeneic transplants of nestin-positive stem cells wherein the stem cell is expanded for a period of time, for example, 2-4 hours, 4-5 hours, 5-10 hours or 1-3 days prior to transplantation to give rise by cell division to other stem cells or progenitor cells.

The invention also provides for methods of performing isogeneic, allogeneic or xenogeneic transplants of a stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II wherein the stem cell is expanded for a period of time, for example, 2-4 hours, 4-5 hours, 5-10 hours or 1-3 days prior to transplantation..

The invention also provides for methods of performing isogeneic, allogeneic or xenogeneic transplants of stem cells wherein the nestin-positive stem cells are induced to differentiate into a progenitor cell by treatment with an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, IDX-1, a nucleic acid molecule encoding IDX-1, GLP-1, exendin-4, betacellulin, activin A, TGF-β, and combinations thereof for a period of time, for example, 2-4 hours, 4-5 hours, 5-10 hours or 1-3 days prior to transplantation.

In the case of a pancreatic stem cell, the stem cell subsequently differentiates into a pancreatic progenitor cell.

The invention also provides for methods of performing isogeneic, allogeneic or xenogeneic transplants of a stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3,

SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II wherein the stem cells are induced to differentiate into a progenitor cell by treatment with an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, IDX-1, a nucleic acid molecule encoding IDX-1, GLP-1, exendin-4, betacellulin, activin A, TGF-β, and combinations thereof for a period of time, for example, 2-4 hours, 4-5 hours, 5-10 hours or 1-3 days prior to transplantation. In the case of a pancreatic stem cell, the stem cell subsequently differentiates into a pancreatic progenitor cell.

The invention provides for methods of performing isogeneic, allogeneic or xenogeneic transplants wherein nestin-positive stem cells are not cultured, expanded or differentiated prior to transplantation or wherein nestin-positive stem cells are cultured and/or expanded and/or differentiated prior to transplantation.

The invention also provides for methods of performing isogeneic, allogeneic or xenogeneic transplants wherein a stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II are not cultured, expanded or differentiated prior to transplantation or wherein a stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II is cultured and/or expanded and/or differentiated prior to transplantation.

Nestin-positive cells can be proliferated in culture from isolated pancreatic islets and subsequently isolated to form a stem cell line capable of essentially unlimited propagation.

In another embodiment, a stem cell that is positive for at least one of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SST-R2, SST-R3, SST-R4, SUR-1, Kir 6.2 and/or does not express one or all of CD34, CD45, CD133, MHC class I and MHC class II can be proliferated in culture from isolated pancreatic islets and subsequently isolated to form a stem cell line capable of essentially unlimited propagation.

The inventors discovered that nestin expressing cells grow out of cultured islets and can be observed growing around the islets as early as about four days in culture. These cells have a neuron-like morphology (Fig. 3), show nestin-positive staining, and express nestin mRNA. Islets containing nestin-positive cells can be separated from other cells, *e.g.*, fibroblasts, that proliferate

from the cultured islets by exposing a suspension of the islets to concanavalin A. The islets containing nestin-positive stem cells will not adhere to a concanavalin A coated culture vessel, for example, allowing the islets to be simply decanted while other cell types remain attached to the vessel. The islets are then plated on wells that do not have a concanavalin A coating, where they adhere. The details of the culture and isolation procedure are described for rat cells in Example 1 below. Similar results have been obtained with human cells.

Formation of Pseudo-Islets and Ductal Structures in Culture

One embodiment of the invention provides an alternative to transplantation of stem cells or progenitor cells by causing them to form pseudo-islet like aggregates that can be transplanted into a patient with insufficient islet cell mass to maintain physiological control without hormone therapy. Islet-derived stem cells can be prepared from cultured islets as indicated above or obtained from a propagating stem cell line. The stem cells can then be induced to differentiate by exposing them to various growth factors. This process is illustrated in Examples 2 and 3.

Differentiation of Stem Cells or Progenitor Cells to Islet Cells

Growth factors that may induce differentiation of pancreatic stem cells include but are not limited to EGF-2, basic FGF, high glucose, KGF, HGF/SF, GLP-1, exendin-4, betacellulin, activin A, TGF-β, and combinations thereof. GLP-1 refers to glucagon-like peptide-1. High glucose refers to a higher glucose concentration than the concentration normally used in culturing the stem cells. For example, the stem cells can be normally cultured and propagated in about 5.6 mM glucose, and the high glucose concentration refers to another concentration above 5.6 mM. In the preferred embodiment, a concentration of 16.7 mM is contemplated. In Example 2, one possible growth factor treatment is described using basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF).

In addition to growth factors added to the medium of cultured cells, further growth factors can contribute to differentiation when stem cells are implanted into an animal or a human. In that situation, many growth factors which are either known or unknown may be secreted by endogenous cells and exposed to the stem cells *in situ*. Implanted stem cells can be induced to differentiate by any combination of endogenous and exogenously administered growth factors that are compatible with the desired outcome, *i.e.*, the final differentiated cell type or types and the anatomical structures (*e.g.*, tissues or organs) formed.

One embodiment provides an approach to stimulating differentiation, that is to administer downstream effectors of growth factors or to transfect stem cells or progenitor cells with a nucleic acid molecule encoding such effectors. One example is IDX-1, which is a transcription factor induced by GLP-1 or exendin-4. Introducing effectors such as IDX-1 can trigger differentiation to form endocrine islet cells.

Analysis of Graft Rejection

The invention provides for an in vivo procedure for evaluating the survival of transplanted material. Experimental transplant rejection is analyzed by transplanting an immunosuppressed or a non-immunosuppressed mammal, with a stem cell or a pseudo-islet like aggregate according to the invention.

For example, non-immunosuppressed C57BL/6 mice are transplanted (for example, under the renal capsules) with human stem cells according to the invention. Graft rejection is analyzed by sacrificing the transplant recipient and staining for viability, or performing immunocytochemical staining at the site of the grafted material (i.e., an organ or tissue present at the site of the grafted material) at a suitable post-transplantation time point. The time point at which staining (for example hematoxylin/eosin or immunostaining) of the site of the grafted material is made can vary, for example, according to the average survival time, or the expected survival time of a transplanted mammal. The site of the graft is analyzed, for example by staining, 1 day to 10 years (i.e., 1, 5, 10, 30, 100 or more days, 1, 2, 5, or 10 years) post-transplantation, preferably 10 days to 1 year post-transplantation and most preferably, 10-100 days post-transplantation. For example, if transplanted material is introduced under the renal capsule of a mouse, the kidney of the transplanted mouse is inspected. Transplanted material is successfully engrafted (i.e., not rejected) if, the transplanted material is still detectable and/or the transplanted material has proliferated into a tissue mass.

Detection of the transplanted material and proliferation of the transplanted material is determined, for example, by hematoxylin/eosin staining of a frozen section prepared from the transplant site (i.e., the kidney) and the detection of new growth that is not derived from the transplant recipient (i.e., not host kidney derived). In the case of xenogeneic transplantation, transplanted material is successfully engrafted if specific immunostaining with antisera specific for an antigen from the species from which the transplanted material is derived, according to

methods of immunocytochemical staining known in the art and described herein, identifies positive cells. Alternatively, in embodiments wherein a xenogeneic transplantation is performed, transplanted material is successfully engrafted if molecules (i.e., a protein or an antigen) derived from the transplant species (that is the species from which the transplanted material is derived) are detected in the blood of the transplant recipient.

As used herein, "rejection" refers to rejection of transplanted material by the immune system of the host. In one embodiment, "rejection means an occurrence of more than 90% cell or tissue necrosis of the transplanted material in response to the immune response of the host. In another embodiment, "rejection" means a decrease in the viability such that the viability of the transplanted material is decreased by 90% or more as compared to the viability of the transplanted material prior to transplantation, in response to the immune response of the host. A decrease in viability can be determined by methods well known in the art, including but not limited to trypan blue exclusion staining. In another embodiment, "rejection" means failure of the transplanted material to proliferate. Proliferation can be measured by methods known in the art including but not limited to hematoxylin/eosin staining. The occurrence of transplant rejection and/or the speed at which rejection occurs following transplantation will vary depending on factors, including but not limited to the transplanted material (i.e., the cell type, or the cell number) or the host (i.e., whether or not the host is immunotolerant and/or has been treated with an immunosuppressive agent.

Methods of Transplantation

The invention provides for methods of transplantation in to a mammal. A stem cell, progenitor cell, or differentiated cell is "transplanted" or "introduced" into a mammal when it is transferred from a culture vessel into a patient.

Transplantation, according to the invention can include the steps of isolating a stem cell according to the invention and transferring the stem cell into a mammal or a patient. Transplantation according to the invention can involve transferring a stem cell into a mammal or a patient by injection of a cell suspension into the mammal or patient, surgical implantation of a cell mass into a tissue or organ of the mammal or patient, or perfusion of a tissue or organ with a cell suspension. The route of transferring the stem cell or transplantation, will be determined by the need for the cell to reside in a particular tissue or organ and by the ability of the cell to find and be retained by the desired target tissue or organ. In the case where a transplanted cell is to

reside in a particular location, it can be surgically placed into a tissue or organ or simply injected into the bloodstream if the cell has the capability to migrate to the desired target organ. Transplantation, according to the invention, can include the steps of isolating a stem cell according to the invention, and culturing and transferring the stem cell into a mammal or a patient. In another embodiment, transplantation, as used herein, can include the steps of isolating a stem cell according to the invention, differentiating the stem cell, and transferring the stem cell into a mammal or a patient. Transplantation, as used herein, can include the steps of isolating a stem cell according to the invention, differentiating and expanding the stem cell and transferring the stem cell into a mammal or a patient.

Methods of Treating Insulin-Dependent Diabetes Using Pancreatic Stem Cells

Stem cells are useful to replace lost beta cells from Type 1 diabetes patients or to increase the overall numbers of beta cells in Type 2 diabetes patients. The diabetes patient will preferably serve as the donor of pancreatic tissue used to produce stem cells, progenitor cells, or pseudo-islet like aggregates. Stem cells exist within the adult pancreatic islets as well as the pancreatic ducts. After a diabetic patient undergoes pancreatic biopsy, islets are isolated from the biopsy tissue and prepared for culture *ex vivo* preferably within 24 hours. Stem cells can be proliferated and isolated by the methods described above within 2-3 weeks. Stem cells can be transplanted back into the patient directly following isolation or after a period of differentiation which is induced by growth factors. Islets can be produced by subculture as described in Example 2. The whole process of surgical pancreas biopsy and transplantation can be performed within a period of about 30 days.

In one embodiment of the invention, pluripotential stem cells are used. These cells are immunologically blinded or immunoprivileged, such that in allogeneic or xenogeneic transplants, they are recognized as self by the recipient, and are not MHC restricted by class I or class II antigens. In one aspect of this embodiment of the invention, these cells do not express MHC class I and/or class II antigens.

In another embodiment of the invention, the recipient of the transplant may demonstrate host vs. graft rejection of other transplanted cells, which can be combated by the administration of blocking antibodies to, for example, an autoantigen such as GAD65, by the administration of one or more immunosuppressive drugs described herein, or by any method known in the art to prevent or ameliorate autoimmune rejection.

Alternatively, stem cells isolated from a non-human mammal according to the invention, are transplanted into a human diabetes patient. Prior to the transplantation step the stem cells may be cultured, and/or expanded and/or differentiated.

Methods of Treating Patients Suffering from Liver Disease Using Pancreatic Stem Cells

The ability of pancreatic stem or progenitor cells to transdifferentiate to form hepatocytes is well known (Bisgaard & Thorgeirsson, 1991). The pancreatic stem cells of the present invention can be used to provide hepatocytes for a patient suffering from a liver disease such as cirrosis, hepatitis, or hepatic cancer in which the functional mass of hepatic tissue has been reduced. The stem cells of the invention can also be treated by gene therapy to correct a genetic defect and introduced into a patient to restore hepatic function. Nestin-positive stem cells can be differentiated either in culture or *in vivo* by applying one or more growth factors, or other treatment such as transfection with a nucleic acid molecule, that results in differentiation of the stem cells to hepatocytes. In one embodiment, the invention contemplates the use of cyclopamine to suppress, for example, sonic hedgehog, resulting in hepatocyte formation. In another embodiment of the invention, the stem cells can be transplanted without any *ex vivo* treatment and the appropriate growth factors can be provided in situ within the patient's body. In yet another embodiment, the stem calls can be treated with growth factors or other agents *ex vivo* and subsequently transplanted into the patient in a partially differentiated or terminally differentiated state. Other aspects of the invention, including methods of transfecting stem cells or progenitor cells, dosages and routes of administration, pharmaceutical compositions, donor-isograft protocols, and immunosuppression methods, can be practiced with transdifferentiation to hepatocytes just as for the differentiation to pancreatic tissues.

The invention specifically contemplates transplanting into patients isogeneic, allogeneic, or xenogeneic stem cells, or any combination thereof.

Methods of Stem Cell Transfection

A variety of methods are available for gene transfer into pancreatic stem cells. Calcium phosphate precipitated DNA has been used but provides a low efficiency of transformation, especially for nonadherent cells. In addition, calcium phosphate precipitated DNA methods often result in insertion of multiple tandem repeats, increasing the likelihood of disrupting gene function of either endogenous or exogenous DNA (Boggs, 1990). The use of cationic lipids, *e.g.*,

in the form of liposomes, is also an effective method of packaging DNA for transfecting eukaryotic cells, and several commercial preparations of cationic lipids are available. Electroporation provides improved transformation efficiency over the calcium phosphate protocol. It has the advantage of providing a single copy insert at a single site in the genome. Direct microinjection of DNA into the nucleus of cells is yet another method of gene transfer. It has been shown to provide efficiencies of nearly 100% for short-term transfection, and 20% for stable DNA integration. Microinjection bypasses the sometimes problematic cellular transport of exogenous DNA through the cytoplasm. The protocol requires small volumes of materials. It allows for the introduction of known amounts of DNA per cell. The ability to obtain a virtually pure population of stem cells would improve the feasibility of the microinjection approach to targeted gene modification of pancreatic stem cells. Microinjection is a tedious, highly specialized protocol, however. The very nature of the protocol limits the number of cells that can be injected at any given time, making its use in large scale production limited. Gene insertion into pancreatic stem cells using retroviral methods is the preferred method. Retroviruses provide a random, single-copy, single-site insert at very high transfection efficiencies. Other such transfection methods are known to one skilled in the art and are considered to be within the scope of this invention.

Retroviral Transformation Of Pancreatic Stem Cells

Gene transfer protocols for pancreatic cells can involve retroviral vectors as the "helper virus" (*i.e.*, encapsidation-defective viral genomes which carry the foreign gene of interest but is unable to form complete viral particles). Other carriers such as DNA-mediated transfer, adenovirus, SV40, adeno-associated virus, and herpes simplex virus vectors can also be employed. Several factors should be considered when selecting the appropriate vector for infection. It is sometimes preferable to use a viral long terminal repeat or a strong internal promoter to express the foreign gene rather than rely on spliced subgenomic RNA.

The two primary methods of stem cell transformation are co-culture and supernatant infection. Supernatant infection involves repeated exposure of stem cells to the viral supernatant. Co-culture involves the commingling of stem cells and an infected "package cell line" (see below) for periods of 24 to 48 hours. Co-culture is typically more efficient than supernatant infection for stem cell transformation. After co-culture, infected stem cells are often further cultured to establish a long term culture (LTC).

The cell line containing the helper virus is referred to as the package cell line. A variety of

package cell lines are currently available. An important feature of the package cell line is that it does not produce replication-competent helper virus.

In one embodiment of the invention animals or patients from whom stem cells are harvested may be treated with 5- fluorouracil (5-FU) prior to extraction. 5-FU treated stem cells are more susceptible to retroviral infection than untreated cells. 5-FU stem cells dramatically reduce the number of clonogenic progenitors, however.

In another embodiment, harvested stem cells may be exposed to various growth factors, such as those employed to promote proliferation or differentiation of pancreatic stem cells. Growth factors can be introduced in culture before, during, or after infection to improve cell replication and transduction. Studies report the use of growth factors increase transformation efficiency from 30 to 80%.

Typical Retroviral Transformation Protocol

The *ex vivo* transduction of mammalian pancreatic stem cells and subsequent transplantation into nonablated recipients sufficient to obtain significant engraftment and gene expression in various tissues containing their progeny cells has been shown in mice. The target cells are cultured for 2-4 days in the presence of a suitable vector containing the gene of interest, before being injected in to the recipient.

Specifically, bone marrow stem cells were harvested from male donor (4-8 weeks old) BALB/c AnNCr mice (National Cancer Institute, Division of Cancer Treatment Animal Program, Frederick, MD). The cells were plated at a density of 1-2 x 10^7 cells/10 cm dish and cultured for 48 hours in Dulbecco's modified Eagle's medium (DMEM) containing; 10% heat-inactivated fetal bovine serum, glutamine, Pen/Strep, 100 U/ml of interleukin-6 (IL-6) and stem cell factor (SCF; Immunex, Seattle, WA) to stimulate cell growth (Schiffmann, et. al., 1995).

Concurrently, a viral package cell line was cultured for 24 hours. The package cell line used by Schiffmann, et al. was GP + E86 and the viral vector was the LG retroviral vector based on the LN series of retroviral vectors.

After the appropriate incubation period, 1-2 x 10^7 stem cells were plated on a 10 cm dish containing the viral package cells and co-cultured for 48 hours in the presence of 8 μg/ml of polybrene and under the same growth factor stimulation conditions as the donor stem cells. The stem cells were then harvested, washed of growth media and injected into recipient mice at dosages of 2 x 10^7 cells/injection for multiple injections (total of 5 injections either daily or weekly).

Successful stem cell transduction and engraftment of stem cells can be determined through, for example, PCR analysis, immunocytochemical staining, Southern Northern or Western blotting, or by other such techniques known to one skilled in the art.

Mammals

Mammals that are useful according to the invention include any mammal (for example, human, mouse, rat, sheep, rabbit, goat, monkey, horse, hamster, pig or cow). A non-human mammal according to the invention is any mammal that is not a human, including but not limited to a mouse, rat, sheep, rabbit, goat, monkey, horse, hamster, pig or a cow.

Dosage and Mode of Administration

By way of example, a patient in need of pancreatic stem cells as described herein can be treated as follows. Cells of the invention can be administered to the patient, preferably in a biologically compatible solution or a pharmaceutically acceptable delivery vehicle, by ingestion, injection, inhalation or any number of other methods. A preferred method is endoscopic retrograde injection. Another preferred method is injection into the pancreatic artery. Another preferred method is injection or placement of the cells or pseudo-islet like aggregates into the space under the renal capsule. The dosages administered will vary from patient to patient; a "therapeutically effective dose" can be determined, for example but not limited to, by the level of enhancement of function (*e.g.*, insulin production or plasma glucose levels). Monitoring levels of stem cell introduction, the level of expression of certain genes affected by such transfer, and/or the presence or levels of the encoded product will also enable one skilled in the art to select and adjust the dosages administered. Generally, a composition including stem cells will be administered in a single dose in the range of 10^5 - 10^8 cells per kg body weight, preferably in the range of 10^6-10^7 cells per kg body weight. This dosage may be repeated daily, weekly, monthly, yearly, or as considered appropriate by the treating physician. The invention provides that cell populations can also be removed from the patient or otherwise provided, expanded *ex vivo*, transduced with a plasmid containing a therapeutic gene if desired, and then reintroduced into the patient.

Pharmaceutical Compositions

The invention provides for compositions comprising a stem cell according to the

invention admixed with a physiologically compatible carrier. As used herein, "physiologically compatible carrier" refers to a physiologically acceptable diluent such as water, phosphate buffered saline, or saline, and further may include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art.

The invention also provides for pharmaceutical compositions. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carrier preparations which can be used pharmaceutically.

Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethyl cellulose; and gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores are provided with suitable coatings such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, i.e., dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders such as lactose or starches, lubricants such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such

as fatty oils, liquid paraffin, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations for parenteral administration include aqueous solutions of active compounds. For injection, the pharmaceutical compositions of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer' solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention may be manufactured in a manner known in the art, e.g. by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping or lyophilizing processes.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc... Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder in 1mM-50 mM histidine, 0.1%-2% sucrose, 2%-7% mannitol at a Ph range of 4.5 to 5.5 that is combined with buffer prior to use.

After pharmaceutical compositions comprising a compound of the invention formulated in a acceptable carrier have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition with information including amount, frequency and method of administration.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples, which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

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EXAMPLE 1

Isolation of Nestin-Positive Stem Cells from Rat Pancreas.

Rat islets were isolated from the pancreata of 2-3 month old Sprague-Dawley rats using the collagenase digestion method described by Lacy and Kostianovsky. Human islets were provided by the Diabetes Research Institute, Miami, FL using collagenase digestion. The islets were cultured for 96 hrs at 37°C in 12-well plates (Falcon 3043 plates, Becton Dickinson, Lincoln Park, NJ) that had been coated with concanavalin A. The culture medium was RPMI 1640 supplemented with 10% fetal bovine serum, 1mM sodium pyruvate, 10mM HEPES buffer, 100 µg/ml streptomycin, 100 units/ml penicillin, 0.25 µg/ml amphotericin B (GIBCO BRL, Life Science Technology, Gaithersburg, MD), and 71.5 mM β-mercaptoethanol (Sigma, St. Louis, MO).

After 96 hrs, fibroblasts and other non-islet cells had adhered to the surface of concanavalin A coated wells and the islets remained floating (did not adhere to the surface). At this time, the media containing the islets were removed, centrifuged down, and the purged islets replated in 12-well plates without a coating of concanavalin A. The islets were then cultured in the above RPMI 1640 medium supplemented with 20 ng/ml of basic fibroblast growth factor-2 and 20 ng/ml of epidermal growth factor.

The islets adhered to the surface of the plates, and cells grew out and away from the islets in a monolayer. These cells that form a monolayer were nestin-positive by immunostaining with a rabbit anti-rat nestin antiserum developed by Dr. Mario Vallejo at the Massachusetts General Hospital. Other nestin antibodies may be used, for example the R.401 antibody described hereinabove, or the MAB533 antibody. A monoclonal antibody specific for rat embryo spinal cord nestin, MAB353, ATCC No. 1023889; is described in Journal of Neuroscience 1996; 16:1901-100; and also available from Chemicon International, Single Oak Dr., Temecula, CA 92590 USA. After two weeks of culture, several (3-5) of the nestin-positive monolayer cells were removed by picking with a capillary tube (cylinder cloning) and were replated on the 12-well plates (not coated with concanavalin A) and cultured in the RPMI 1640 medium further supplemented with bFGF-2 and EGF. The cells propagated at a rapid rate and reached confluence after six days of culture. After 12 days of culture, the cell monolayer formed waves in which they begin to pile up in a co-linear manner. On day 15 of culture, the cell waves began to condense, migrate into spheroid bodies and by day 17 the surface of the wells contained these spheroid bodies (ca. 100 µm in diameter), empty spaces, and a few areas of remaining monolayer

cells. Several of these monolayer cells were re-picked and re-cloned and the process described above occurred again in precisely the same temporal sequence.

EXAMPLE 2

Differentiation of Pancreatic Stem Cells to Form Islet

Pancreatic islets from rats were first cultured in RPMI medium containing 10% fetal bovine serum using concanavalin-A coated 12-well plates. The islets were maintained in culture for three days in the absence of added growth factors other than those supplied by fetal bovine serum. After this period, during which the islets did not attach, the islets were transferred to fresh plates without concanavalin A. The stem cells were then stimulated to proliferate out from the islets as a monolayer by exposing them to bFGF-2 (20 ng/ml) and EGF (20 ng/ml) for 24 days. After the 24 day period, the monolayer was confluent. Among them was a population of cells surrounding the islets. Cells from that population were picked and subcloned into new 12-well plates and again cultured in the medium containing bFGF and EGF. The subcloned cells proliferated rapidly into a monolayer in a clonal fashion, expanding from the center to the periphery. The cells became confluent at day 6 and then started to form a wave of overlapping cells on day 12. By day 17 the cells migrated almost entirely into spherical structures and tubular structures resembling islet-like structures (pseudo-islet like aggregates) and duct-like structures (pseudo-ducts) (Fig. 4). RT-PCR analysis revealed that the pseudo-islet like aggregates were expressing NCAM (a marker for endocrine cells, see Fig. 5), cytokeratin 19 (a marker for ductal cells, see Fig. 5), and the transcription factor brain-4 (a beta cell marker). Treatment with growth factors is required to achieve terminal differentiation to mature islet cells.

EXAMPLE 3

Isolation and culture of human or rat pancreatic islets

Human pancreatic islets were isolated and cultured. Human islet tissue was obtained from the islet distribution program of the Cell Transplant Center, Diabetes Research Institute, University of Miami School of Medicine and the Juvenile Diabetes Foundation Center for Islet Transplantation, Harvard Medical School, Boston, MA. Thoroughly washed islets were handpicked, suspended in modified RPMI 1640 media (11.1 mM glucose) supplemented with

10% fetal bovine serum, 10 mM HEPES buffer, 1 mM sodium pyruvate, 100 U per mL penicillin G sodium, 100 μg per mL streptomycin sulfate, 0.25 ng per mL amphotericin B, and 71.5 μM β-mercaptoethanol, and added to Falcon 3043 12-well tissue culture plates that had been coated with Concanavalin A (ConA). The islet preparation was incubated for 96 hrs at 37°C with 95% air and 5% CO_2. In these conditions, many islets remained in suspension (floated), whereas fibroblasts and other non-islet cells attached to the substratum. After 96 h of incubation the media containing the suspended islets was carefully removed, the islets were manually picked and resuspended in the modified RPMI 1640 media now further supplemented with 20 ng/mL each of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). The islet suspension (containing 20-30 islets per well) was added to 12-well tissue culture plates not coated with ConA. The islets immediately adhered to the surfaces of the plates. Within several days, a monolayer of cells was observed growing out and away from the islets. In certain instances, human-derived cells were cultured in modified RPMI media containing 2.5 mM glucose, and several growth factor combinations that include activin-A (2 nM), hepatocyte growth factor (100 pM), or betacellulin (500 pM). In those experiments in which cells were challenged with 10 mM nicotinamide, the media contained no serum or growth factors.

EXAMPLE 4

Effects of Glucose and GLP-1 on Differentiation of Pancreatic Stem Cells.

Elevation of plasma glucose concentration leads to increased pancreatic islet size. The effect of the glucose concentration in the culture medium was therefore investigated using isolated islets, which contain nestin-positive stem cells. Rat pancreatic islets were cultured in a medium containing high (16.7 mM) glucose or in normal (5.6 mM) glucose. After four days, RT-PCR was performed to determine the level of nestin mRNA. The results indicated a three-fold stimulation of nestin mRNA levels in the islets cultured in high glucose compared to the islets cultured in normal glucose (Fig. 6).

Similarly, injection of glucagon-like peptide-1 (GLP-1) into mice was found to increase islet mass by 2-fold in 48 hours. Knockout mice having a disrupted gene for GLP-1 receptor were examined for nestin expression in pancreatic islets. Immunostaining using a nexin antibody was found to be markedly reduced compared to normal mice with GLP-1 receptors.

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Animal Model of Diabetes Mellitus

Treatments for diabetes mellitus type that result in relief of its symptoms are tested in an animal which exhibits symptoms of diabetes. It is contemplated that the animal will serve as a model for agents and procedures useful in treating diabetes in humans. Potential treatments for diabetes can therefore be first examined in the animal model by administering the potential treatment to the animal and observing the effects, and comparing the treated animals to untreated controls.

The non-obese diabetic (NOD) mouse is an important model of type I or insulin dependent diabetes mellitus and is a particularly relevant model for human diabetes (see Kikutano and Makino, 1992, Adv. Immunol. 52:285 and references cited therein, herein incorporated by reference). The development of type I diabetes in NOD mice occurs spontaneously and suddenly without any external stimuli. As NOD mice develop diabetes, they undergo a progressive destruction of β-cells which is caused by a chronic autoimmune disease. The development of insulin-dependent diabetes mellitus in NOD mice can be divided roughly into two phases: initiation of autoimmune insulitis (lymphocytic inflammation in the pancreatic islets) and promotion of islet destruction and overt diabetes. Diabetic NOD mice begin life with euglycemia, or normal blood glucose levels, but by about 15 to 16 weeks of age the NOD mice start becoming hyperglycemic, indicating the destruction of the majority of their pancreatic β-cells and the corresponding inability of the pancreas to produce sufficient insulin. In addition to insulin deficiency and hyperglycemia, diabetic NOD mice experience severe glycosuria, polydypsia, and polyuria, accompanied by a rapid weight loss. Thus, both the cause and the progression of the disease are similar to human patients afflicted with insulin dependent diabetes mellitus. Spontaneous remission is rarely observed in NOD mice, and these diabetic animals die within 1 to 2 months after the onset of diabetes unless they receive insulin therapy.

The NOD mouse is used as an animal model to test the effectiveness of the various methods of treatment of diabetes by administering a stem cell preparation according to the invention. As such, treatment via administration of stem cells are tested in the NOD mouse for their effect on type I diabetes.

The stem cells are administered to a NOD mouse, typically intraperitoneally, according to the following dosage amounts. NOD mice are administered about $1X10^1$ to $1X10^4$ cells per mouse. Administration of the cells is started in the NOD mice at about 4 weeks of age, and is continued for 8 to 10 weeks, e.g., 3 times a week. The mice are monitored for diabetes

beginning at about 13 weeks of age, being tested twice per week according to the methods described below. The effects of treatment are determined by comparison of treated and untreated NOD mice.

The effectiveness of the treatment methods of the invention on diabetes in the NOD mice is monitored by assaying for diabetes in the NOD mice by means known to those of skill in the art, for example, examining the NOD mice for polydipsia, polyuria, glycosuria, hyperglycemia, and insulin deficiency, or weight loss. For instance, the level of urine glucose (glycosuria) can be monitored with Testape (Eli Lilly, Indianapolis, IN.) and plasma glucose levels can be monitored with a Glucometer 3 Blood Glucose Meter (Miles, Inc., Elkhart, IN.) as described by Burkly, 1999, U.S. Patent 5,888,507, herein incorporated by reference. Monitoring urine glucose and plasma glucose levels by these methods, NOD mice are considered diabetic after two consecutive urine positive tests gave Testape values of +1 or higher or plasma glucose levels >250 mg/dL (Burkly, 1999, supra). Another means of assaying diabetes in NOD mice is to examine pancreatic insulin levels in NOD mice. For example, pancreatic insulin levels can be examined by immunoassay and compared among treated and control mice (Yoon, U.S. Patent 5,470,873, herein incorporated by reference). In this case, insulin is extracted from mouse pancreas and its concentration is determined by its immunoreactivity, such as by radioimmunoassay techniques, using mouse insulin as a standard.

In addition to monitoring NOD mice for diabetes in general, the effects of the inventive methods of treatment are also monitored for gene-specific or gene product-specific effects if the stem cells administered were transformed or transfected with a heterologous gene, thereby allowing a correlation to be drawn between expression of the heterologous gene and its effects on diabetes. For example, the presence of the heterologous gene product may be examined by immunohistochemistry of the pancreatic β-cells of NOD mice for the gene product and for insulin. The expression of the patched and smoothened genes is further examined in NOD mouse islets by detection of the RNA transcript for the patched and smoothened receptors. Reverse transcription-polymerase chain reaction (RT-PCR) amplification is performed by known means to amplify a fragment of mouse patched or smoothened cDNA, and analyzed by agarose gel electrophoresis, according to standard means. The identification of the amplified cDNA fragment is confirmed as corresponding to the patched or smoothened RNA by hybridization of the amplified fragment with a radiolabeled internal oligonucleotide probe for the patched or smoothened genes, or by other such methods as known to one skilled in the art.

EXAMPLE 5

Immunocytochemical identification of nestin positive human and rat pancreatic stem cells

Pancreatic islets were analyzed for nestin expression. Islets and stem cells were isolated as described above. Nestin expression was observed by immunocytochemical staining in a distinct population of cells within developing islet clusters of embryonic day 16 (E16) rat pancreas (Fig. 8A) and in islets of the adult pancreas (postnatal 60 days) (Fig. 8B). Immunocytochemical staining was performed as follows.

Cryosections (6 μM) prepared from embryonic day 16 and adult (60 day) rat pancreata as well as cells were fixed with 4% paraformaldehyde in phosphate. Cells were first blocked with 3% normal donkey serum for 30 min at room temperature and incubated with primary antisera overnight at 4°C. The antisera were rinsed off with PBS and incubated with the respective Cy-3 and Cy-2 labeled secondary antisera for 1 hour at room temperature. Slides were then washed with PBS and coverslipped with fluorescent mounting medium (Kirkegaard and Perry Labs, Gaithersburg, MD). Tissue sections were incubated overnight at 4°C with primary antisera. Primary antisera were then rinsed off with PBS, and slides were blocked with 3% normal donkey serum for 10 min at room temperature before incubation with donkey anti-Cy3 (indocarbocyanine) and either anti-guinea pig (insulin), anti-mouse (glucagon), or anti-sheep (somatostatin) sera DTAF (Jackson Immuno Research Laboratories, West Grove, PA) for 30 min at room temperature. Slides were then rinsed with PBS and coverslipped with fluorescent mounting medium (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Fluorescence images were obtained using a Zeiss Epifluorescence microscope equipped with an Optronics TEC-470 CCD camera (Optronics Engineering, Goleta, CA) interfaced with a PowerMac 7100 installed with IP Lab Spectrum analysis software (Signal Analytics Corp, Vienna, VA).

The nestin-positive cells are distinct from β–, α–, δ–, and PP-cells because they do not co-stain with antisera to the hormones insulin (Fig. 8A & B), glucagon, somatostatin, or pancreatic polypeptide. The nestin-positive cells also do not co-stain with antisera to collagen IV, a marker for vascular endothelial cells (Fig. 8C) nor with an antiserum to galanin, a marker for nerve cells or a monoclonal antibody to cytokeratin 19, a specific marker for ductal cells (Fig. 8). Nestin-positive staining is associated with distinct cells within the islets clearly observed by nuclear costaining (Fig. 4D).

EXAMPLE 6

Identification of nestin positive human and rat stem cells by RT-PCR

To confirm the immunocytochemical identification of nestin expression in pancreatic islets, we performed an RT-PCR of the nestin mRNA using total RNA prepared from freshly isolated rat islets and human islet tissue. RT-PCR was performed according to the following method.

Total cellular RNA prepared from rat or human islets was reverse transcribed and amplified by PCR for 35 cycles as described previously (Daniel et al., 1998, Endocrinology, 139:3721-3729). The oligonucleotides used as primers or amplimers for the PCR and as probes for subsequent Southern blot hybridization are:

Rat nestin:	Forward, 5'gcggggcggtgcgtgactac3';
	Reverse, 5'aggcaaggggaagagaaggatgt3';
	Hybridization, 5'aagctgaagccgaatttccttgggataccagagga3'.
Rat keratin 19:	Forward, 5'acagccagtacttcaagacc3';
	Reverse, 5'ctgtgtcagcacgcacgtta3';
	Hybridization, 5'tggattccacaccaggcattgaccatgcca3'.
Rat NCAM:	Forward, 5'cagcgttggagagtccaaat3';
	Reverse, 5'ttaaactcctgtggggttgg3';
	Hybridization, 5'aaaccagcagcggatctcagtggtgtggaacgatgat3'.
Rat IDX-1	Forward, 5'atcactggagcagggaagt3'
	Reverse, 5'gctactacgtttcttatct3'
	Hybridization, 5'gcgtggaaaagccagtggg3'
Human nestin:	Forward, 5'agaggggaattcctggag3';
	Reverse, 5'ctgaggaccaggactctcta3';
	Hybridization, 5'tatgaacgggctggagcagtctgaggaaagt3'.
Human keratin:	Forward, 5'cttttcgcgcgcccagcatt3';
	Reverse, 5'gatcttcctgtccctcgagc3';
	Hybridization, 5'aaccatgaggaggaaatcagtacgctgagg3'.
Human glucagon:	Forward, 5'atctggactccaggcgtgcc3';
	Reverse, 5'agcaatgaattccttggcag3';

Hybridization, 5'cacgatgaatttgagagacatgctgaaggg3'.

Primers were selected from two different exons and encompassed at least one intronic sequence. In addition, an RT minus control was run for most samples. PCR cycling was at 94°C for 1 min followed by 94°C for 10 secs, 58/56°C for 10 secs, 72°C for 1 min, 35 cycles, and 72°C for 2 min. The annealing temperature was 58°C for rat nestin and 56°C for the remaining primer pairs.

For Southern hybridization oligonucleotide probes were radiolabeled with T4 polynucleotide kinase and γ^{32}P ATP. Radiolabeled probes were hybridized to PCR products that had been transferred to nylon membranes at 37°C for one hour, then washed in 1 x SSC + 0.5% SDS at 55°C for 10-20 min or 0.5 x SCC + 0.5% SDS at 42° for the human PCR products.

The RT-PCR generated products of the correctly predicted size (Fig. 8E, upper panels) and were confirmed by Southern blotting (Fig. 8E, lower panels) and by DNA sequencing of the products. These data demonstrate the identification of a new cell type in pancreatic islets that expresses nestin and may represent an islet pluripotential stem cell similar to the nestin-positive stem cells in the central nervous system.

EXAMPLE 7

The ATP-dependent transporter ABCG2 is expressed in nestin-positive cells derived from pancreatic islets

Human islet-derived NIPs contain a substantial subpopulation of SP cells that co-express ABCG2, MDR1 and nestin. Nestin was first shown to be a marker of neural and muscle stem/progenitor cells (Lendahl, et al., 1990, *Cell* 60:585: Zimmerman et al.; 1994, *Neuron* 12:11). Neural stem cells especially exhibit a high degree of plasticity giving rise to neurons and different types of glial cells. Neural stem/progenitor cells also differentiate into hematopoietic cells, which would make them truly pluripotential stem cells (Shih et al., 2001, *Blood* 98:2412). The best examples of pluripotential adult stem cells to date are those derived from the bone marrow, so called side population (SP) cells (Goodell et al., 1996, *J. Exp Med* 183:1797). SP cells have been identified by their property to effectively exclude the fluorescent vital dye Hoechst 33342 and contain the vast majority of bone marrow repopulating cells. SP cells represent 0.05% of the whole bone marrow of adult humans and mice, and apart from giving rise to all hematopoietic lineages they can also give rise to skeletal and cardiac muscle as well as to

endothelial cells (Gussoni et al., 1999, *Nature* 401:390; Jackson et al., 2001, *J Clin Invest* 107:1395). The ATP-\binding cassette transporter ABCG2 (BCRP1) has been demonstrated to be a major component of the SP phenotype, thus offering a molecular means to specifically identify SP-cells (Kim et al., 2002, *Clin Cancer Res* 8:22; Scharenberg et al., *Blood* 99:507; Zhou et al., 2001, *Nat Med* 7:1028). Notably, marrow-derived SP cells have a limited, if any, capacity to proliferate *in vitro* (Bunting et al., 2000, *Blood* 96:902; Storms et al., 2000, *Blood* 96:2125), but enforced P-glycoprotein pump (MDR-1) function in bone marrow cells results in the expansion of SP stem cells *in vitro* and repopulating cells *in vivo* (Bunting et al., 2002, *Stem Cells* 20:11).

NIPs, linked to neural stem cells by their expression of nestin, also show characteristics of bone marrow SP stem cells, by virtue of their expression of ABCG2 in a substantial subpopulation of NIPs identified by the Hoechst 33342 exclusion assay. This side population of NIPs also expresses MDR-1, contributing to their continued expansion *in vitro*. Thus NIPs may be a potential source of adult pluripotential stem/progenitor cells useful for the production of islet tissue for transplantation into diabetic subjects.

Human pancreatic islets were obtained from the islet distribution programs of the Cell Transplant Center, Diabetes Research Institute, University of Miami School of Medicine (Miami, FL), and the Juvenile Diabetes Research Foundation Center for Islet Transplantation, Harvard Medical School (Boston, MA). NIPs were isolated as previously described (Zulewski et al., 2001, *Diabetes* 50:521). Briefly, human pancreatic islets were handpicked, incubated at 37°C at 5% CO_2 in concanavalin A (ConA) coated culture dishes in modified RPMI 1640 media supplemented with 10% fetal bovine serum, 10 mmol/l HEPES buffer, 1 mmol/l sodium pyruvate, 71.5 μmol/l β-mercaptoethanol and antibiotic-antimycotic (Gibco Life Technologies, Gaithersburg, MD). After 96 hours floating islets were transferred into fresh media further that was supplemented with 20ng/ml basic fibroblast growth factor (bFGF) and 20ng/ml epidermal growth factor (EGF) (both from Sigma, St. Louis, MO) in new plastic culture dishes without ConA coating. The islets attached to the uncoated plastic surface and within several days a monolayer of cells was observed growing out and away from the islets. Cells from the periphery of the monolayer, nestin-positive islet-derived progenitor cells (NIPs), were transferred to a new culture dish and grown to near confluence under the same conditions as above. Cells were further split into two culture dishes to perform Hoechst 33342 dye exclusion assays with and without verapamil, an inhibitor of H33342 dye transport (Goodell et al., 1996, supra).

RT-PCR demonstrated the expression of ABCG2 (Fig. 19A). The RT-PCR-generated DNA product was sequenced and shown to be identical (586 bps) with that of the human ABCG2 (GenBank Accession No. XM-032424, Figure 18)) (Data not shown). Southern blot hybridization with a cloned ABCG2 probe confirmed the amplification of the correct cDNA (Fig. 19A).

RNA was isolated from cultured NIPs as well as from sorted SP cells and non-SP controls (5000 and 10,000 cells respectively) using Trizol (Gibco) following the manufacturers protocol. Single stranded cDNA was made with the Superscript First-Strand System (Invitrogen, Carlsbad, CA). The cDNAs were amplified by polymerase chain reaction (PCR). Controls without reverse transcriptase (-RT controls) were done for all PCR reactions. PCR products were analyzed by agarose gel electrophoresis and the correct identity of products was confirmed by sequencing. Template concentrations were normalized for GAPDH (31 cycles). ABCG2, MDR1 and nestin were amplified with 34, 38, and 36 cycles respectively. Primers were: 5'tgaaggtcggagtcaacggatttggt3' and 5'catgtgggccatgaggtccaccac3' for GAPDH, 5'agaggggaattcctggag3' and 5'ctgaggaccaggactctcta3' for nestin, 5'tcctggagcggttctacgac3' and 5'gggcttcttggacaacctttca3' for MDR1 and 5'gctggggttctcttcttcctgacg3' and 5'ctaccccagccagtgtcaac3' for ABCG2. PCR products for ABCG2 were transferred to nylon membranes (Hybond N+; Amersham Pharmacia; Little Chalfont; GB). Blots were hybridized with radiolabeled, cloned ABCG2 or MDR1 probes using Rapid Hyb Buffer (Amersham) following the manufacturers protocol. Primers for the full open reading frame of human ABCG2 were 5'tattaagctgaaaagataaaaactctcc3' and 5'atgtgaggataaatcatactgaat3' (base pairs 174-202 and 2184-2207 of Genbank sequence XM_032424).

EXAMPLE 8

<u>1.5 to 2% of cultured cells have a side population phenotype in the Hoechst 33342 dye exclusion assay.</u>

NIPs, linked to neural stem cells by their expression of nestin, also show characteristics of bone marrow SP stem cells, by virtue of their expression of ABCG2 in a substantial subpopulation of NIPs identified by the Hoechst 33342 exclusion assay. This side population of NIPs also expresses MDR-1, contributing to their continued expansion *in vitro*.

To investigate whether the expression of ABCG2 lends a side population (SP) phenotype

to some of the cultured NIPs, Hoechst 33342 dye exclusion assays were performed following the published protocol (Goodell et al., 1996, supra) with slight modifications. The exclusion of the Hoechst 33342 dye, which defines the pluripotential side population (SP) of hematopoietic stem cells, is mediated by the ATP-binding cassette transporter, ABCG2. Verapamil was used as a specific inhibitor of H33342 transport (Goodell et al., 1996, supra).

The Hoechst dye exclusion assay was done following the protocol of Goodell *et al.* 1996, supra, with the modification that staining was performed while the cells were still attached to the culture dish. Cell viability with this change in protocol was significantly higher compared to the original protocol in which cells are stained while in suspension. Hoechst 33342 (Sigma) was added to the growth media at a final concentration of 5μg/ml and cells were incubated for 90 minutes at 37°C. Where indicated, verapamil (Sigma) was added 15 minutes prior to the start of the assay at a final concentration of 50 μmol/l. For analysis cells were trypsinized, poured through a 40 μm cell strainer, and resuspended in ice cold Hanks' balanced salt solution containing propidium iodide (Sigma) at a final concentration of 2 μg/ml for the discrimination of dead cells. Flow cytometry was done on a Vantage and a Moflow cell sorter using a UV laser for excitation of Hoechst 33342 and propidium iodide. 450/20 BP (blue) and 630/20 (red) filters were used for analysis in linear mode.

Fluorescence activated cell sorting (FACS) showed a clearly visible side population (Fig. 19B; 2.1% gated cells) which was absent in the presence of the inhibitor verapamil (Fig. 19C; 0.1% gated cells). In analyses of two other NIP cultures independently derived, the percentage of SP-positive cells was 1.5 and 2 percent, respectively (data not shown). We also amplified the full open reading frame of ABCG2 from NIPs. Cloning into an expression vector and transfection into INS-1 insulinoma cells resulted in effective Hoechst 33342 exclusion in transfected cells (data not shown).

EXAMPLE 9

<u>The SP phenotype correlates with the expression of ABCG2, MDR1, and nestin</u>

SP cells and non-SP control cells were isolated from cultured NIPs by FACS (Fig. 20A; gates R1 and R2 respectively). Expression of ABCG2 correlated well with the SP phenotype as shown by RT-PCR (Fig. 20B) and Southern blot hybridization with a cloned ABCG2 probe. RT-PCR and Southern blot hybridization demonstrated expression of MDR1 ((P-glycoprotein),

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another ATP-binding cassette transporter present in hematopoietic stem cells (Chaudhary et al., 1991, *Cell* 66:85)) in the SP-fraction of cultured NIPs (Fig. 20B). The SP fraction also expressed nestin at a markedly higher level than the non-SP controls (Fig. 20B).

Nestin-positive cells derived from human pancreatic islets contain 1.5 to 2% of SP cells, which express ABCG2 and nestin at high levels compared to non-SP control cells. The correlation of ABCG2 expression with the SP phenotype confirms the finding that ABCG2 activity is a major component of the SP dye efflux (Kim et al., supra; Scharenberg et al., supra; Zhou et al., supra). The coexpression of nestin and ABCG2 indicates a broader role for nestin as a general marker of stem/progenitor cells.

The SP cell fraction of the bone marrow in different mammalian species including humans contains the vast majority of multipotential hematopoietic stem cells (HSCs) (Goodell et al., 1997, supra). The differentiation of bone marrow-derived SP cells is not limited to blood lineages; in animal models SP cells also differentiate to skeletal and cardiac muscle as well as endothelial cells (Gussoni et al., *Nature* 401:390; Jackson et al., 2001, *J Clin Invest* 107:1395). In addition, bone marrow-derived stem cells differentiate into functional brain and liver cells (Brazelton et al., 2000, *Science* 290:1775; Lagasse et al., 2000, *Nat Med* 6:1229).

The portion of SP cells in the NIP cultures (1.5 to 2%) is at least 20-fold higher than that found in the bone marrow (0.05 %) (Goodell et al., 1996, supra). However, a comparable percentage of SP cells is found in cultures of muscle satellite cells, which are considered to be myogenic progenitors (Jackson et al., 1999, *Proc Natl Acad Sci* 96:14482). In addition to generating differentiated myofibres muscle SP cells have been shown to reconstitute the bone marrow of lethally irradiated mice, thus exhibiting an unexpected degree of plasticity (Gussoni et al., 1999, supra). Some evidence exists that these SP cells also come from the bone marrow before taking up residence in the muscle (Kawada et al., 2001, *Blood* 98:2008). An SP population has also been demonstrated in mouse embryonic stem cells (Zhou et al., 2001, supra). The expression on ABCG2 neural stem cells has been shown to be significantly higher than it is in more differentiated neural cells(Geschwind et al., 2001, *Neuron* 29:325).

MDR1 (P-glycoprotein, ABCB1) is another ATP-binding cassette transporter expressed in hematopoietic stem cells (among several other cell types) (Zhou et al., 2001, supra; Chaudhary et al., 1991, supra). Overexpression of MDR1 in bone marrow cells leads to an expansion of the SP population, their prolonged survival in culture and an enhanced repopulating activity after transplantation into mice (Bunting et al., 2000, supra). In some of the transplanted animals it ultimately results in a myeloproliferative syndrome resembling chronic myelogenous leukemia

(Bunting et al., 1998, *Blood* 92:2269). Therefore MDR-1 expression has been implicated in the expansion of hematopoietic stem cells and was suggested as a characteristic of proliferating stem cells in contrast to quiescent cells, only expressing ABCG2 (Bunting et al., 2002, supra). MDR1 is expressed in the SP fraction of pancreatic islet derived NIPs.

EXAMPLE 10

Immunocytochemical identification of GLP-1R-positive human pancreatic stem cells

Nestin-positive pancreatic islet stem cells were analyzed for GLP-1R expression. Human islet tissue was obtained from the Juvenile Diabetes Research Center for Islet Transplantation, Harvard Medical School. NIPs were isolated as described previously (Zulewski, et al., 2001, *Diabetes* 50: 521). Briefly, islets were washed and cultured in RPMI 1640 medium containing serum, 11.1 mM glucose, antibiotics, sodium pyruvate, β-mercaptoethanol, and growth factors. Within several days, nestin-positive cells (identified immunocytochemically as described above) grew out from the islets. Later, these cells were cloned and expanded in medium containing 20 ng/ml of basic fibroblast growth factor and epidermal growth factor or 1000 units of recombinant human leukemia inhibitory factor. Incubation with GLP-1 was administered in the absence of serum and fresh peptide was added every 48h without changing the medium.

Immunocytochemical detection of GLP-1R was performed using rabbit polyclonal antisera (Heller et al., *supra*) as follows. Cells cultured on Lab-Tek chamber slides (Nunc, Naperville, IL) or gridded coverslips (Bellco Glass, NJ) were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. After several rinses in PBS, cells were blocked with normal donkey serum for 30 minutes and incubated with primary antisera or pre-immune sera at 4° C. The following day, cells were rinsed with PBS and incubated with secondary antisera (donkey anti-rabbit and donkey anti-guinea pig) labeled with Cy-3 or Cy-2 for 1 hour at room temperature. After several washes, coverslips containing cells were mounted onto slides in mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) which stains nuclei. Fluorescence images were obtained using a Zeiss epifluorescence microscope equipped with an Optronics TEC-470 CCD camera (Optronics Engineering, Goleta, CA) interfaced with a Powermac 7100. IP lab Spectrum software (Signal Analytics, Vienna, VA) was used to acquire and analyze images.

GLP-1R immunoreactivity was detected in the majority of NIPs (at least 60%) examined (Figure 22A).

EXAMPLE 11

Identification of GLP-R1-positive human stem cells by RT-PCR

To confirm the immunocytochemical identification of GLP-1R expression in pancreatic islets (NIPs), RT-PCR was performed using of total RNA prepared from NIPs. RT-PCR was performed as described above in example 6, for the identification of nestin mRNA with the difference being that the following GLP-1R-specific primers were used: 5' gtgtggcggccaattactac 3' (Forward); 5' cttggcaagtctgcatttga 3' (Reverse). Amplification of NIP mRNA produced the expected 346 bp product (Figure 22B) indicating that, in addition to expressing the GLP-1R protein, NIPs have the biosynthetic ability to produce GLP-1R. GLP-1R, therefore, in addition to nestin, is useful in the present invention as a marker for pancreatic stem cells.

EXAMPLE 12

In vitro proliferation of nestin positive stem cells

The ability of nestin-positive stem cells to proliferate in vitro was determined.

Islets prepared from 60 day-old rats or a normal adult human were first plated on concanavalin-A-coated dishes and cultured in modified RPMI 1640 medium containing 10% fetal bovine serum for four days to purge the islet preparation of fibroblasts and other non-islet cells that adhered to the ConA-coated plates. The islets that did not adhere to the plates under these culture conditions were collected and transferred to 12-well plates (without ConA coating) containing the same modified RPMI 1640 medium now additionally supplemented with bFGF and EGF (20 ng/mL each). The growth factors bFGF and EGF together were selected because they are known to stimulate the proliferation of neural stem cells derived from ependyma of the brain (Reynolds and Weiss, 1996, Dev. Biol., 175:1-13). The islets attached to the plates and cells slowly grew out of the islet as a monolayer (estimated cell doubling time 40-45 hrs in human cells). The outgrowing monolayer of cells were phenotypically homogenous (Fig. 9A, panel 1) and expressed nestin (Fig. 9A, panel 2). Rat

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cells were picked from the monolayer (batches of at least 20-30 cells), subcloned into 12-well plates, and incubated with the modified RPMI 1640 medium (11.1 mM glucose) containing bFGF and EGF. The subcloned cells grew rapidly and became confluent at six days with an estimated cell doubling time of 12-15 hrs (Fig. 9A, panel 3), and by 12 days formed wave-like structures. After 15-17 days of culture, the cells formed islet-like clusters (ILCs) (Fig. 9A, panel 4). Similar cells were cloned from human islets (Fig. 9B). Upon reaching confluence (Fig. 9B, panel 1), the human cells migrated to form large vacuolated structures in the dish (Fig. 9B, panels 2 and 3). The cells lining the large spaces then changed morphology, rounded, and aggregated together forming three dimensional ILCs (Fig. 9B, panels 4-6).

Indicators of differentiation of these nestin-positive islet progenitor cells (NIPs) that formed these ILCs were characterized by RT-PCR and Southern blot and found that they express the endocrine marker NCAM (neural cell adhesion molecule) (Cirulli et al., 1994, J. Cell Sci., 107:1429-36) (Fig. 9C, right panel) and the ductal cell marker CK19 (cytokeratin 19) (Bouwens et al., 1998, J. Pathol., 184:234-9; Bouwens et al., 1995, J. Histochem. Cytochem., 43:245-53; Bouwens et al., 1994, Diabetes, 43:1279-93) (Fig. 9C, left panels). At this stage of the studies it was concluded that when the NIPs became confluent and aggregated into islet-like cell clusters, they began to express pancreatic genes (NCAM and CK19), but were limited in expression of islet genes because of the absence of growth factors essential for their differentiation to endocrine cells. It was also recognized that the differentiation of a progenitor cell population typically requires first a proliferative phase and then quiescence of proliferation in the presence of differentiation-specific morphogen growth factors. Therefore the culture conditions were modified in some instances by replacing the media containing 11.1 mM glucose, bFGF and EGF, which induces proliferation of cells, with media containing lower glucose (2.5 mM), which is less proliferative, and the factors HGF/Scatter Factor or betacellulin and Activin A. Glucose is a known proliferative factor for pancreatic islet β-cells (Swenne, 1992, Diabetologia, 35:193-201; Bonner-Weir, 1989, Diabetes, 38:49-53) and both HGF/Scatter Factor and Activin A have been shown to differentiate the pancreatic ductal cell line AR42J into an endocrine phenotype that produces insulin, glucagon, and other pancreatic endocrine cell proteins (Mashima et al., 1996, Endocrinology, 137:3969-76; Mashima et al., 1996, J. Clin. Invest., 97:1647-54).

Cultures containing ILCs expressed the pancreas-specific homeodomain protein IDX-1 by immunocytochemistry (Fig. 10A, upper panel), RT-PCR and Southern blot (Fig. 10B), and

by Western immunoblot (Fig. 10C). The ILCs also expressed the mRNA encoding proglucagon as seen by RT-PCR (Fig. 10D) and produced immunoreactive glucagon, glucagon-like peptide-1, and insulin. Radioimmunoassays of media obtained following 72-96 h of culture of islet-like clusters in several wells gave values of 40-80 pg/ml GLP-1, 30-70 pg/ml glucagon, 29-44 pg/ml insulin. Radioimmunoassays were performed as follows.

Insulin and glucagon concentrations in culture media were determined by ultra sensitive radioimmunoassay kits purchased from Linco Research Inc. and DPC Inc., respectively. The antisera supplied in the respective kits are guinea pig anti-human insulin and rabbit anti-human glucagon. GLP-1 secretion was measured with an anti-human GLP-1(7-36)amide rabbit polyclonal antiserum raised by immunization of a rabbit with a synthetic peptide CFIAWLVKGR amide conjugated to keyhole limpet hemocyanin. The antiserum is highly specific for the detection of GLP-1(7-36)amide and only weakly detects proglucagon. The sensitivity levels for these assays are 6 pg/mL, 13 pg/mL and 10.2 pg/mL, respectively.

Incubation of the ILCs for 7 days in 10 mM nicotinamide, as described by Ramiya et al. (Ramiya et al., 2000, Nat. Med., 6:278-282), increased insulin secretion by 2- to 3-fold.

Several additional pancreatic markers were expressed in differentiated NIPs such as glucose transporter-2 (Wang et al., 1998), synaptophysin, and HGF (Menke et al., 1999) as shown in Fig. 15. To determine whether the differentiating NIPs may have properties of pancreatic exocrine tissue, we used RT-PCR and detected the expression of amylase and procarboxypeptidase (Fig.15).

Some cultures of NIPs containing stem cells also expressed the mRNA encoding proglucagon and insulin as seen by RT-PCR (Fig 16A and B).

The expression of IDX-1 is of particular importance because it is recognized to be a master regulator of pancreas development, and particularly to be required for the maturation and functions of the pancreatic islet β-cells that produce insulin (Stoffers et al., 1997, Trends Endocrinol. Metab., 8:145-151).

Because the neogenesis of new islets is also known to occur by differentiation of cells in pancreatic ducts, particularly during the neonatal period (rats and mice) but to some extent throughout adult life (Bonner-weir et al., 1993, Diabetes, 42:1715-1720; Rosenberg, 1995, Cell Transplant, 4:371-383; Bouwens et al., 1996, Virchows Arch., 427:553-560), nestin expression was analyzed in the pancreatic ducts of adult rats. By dual fluorescence immunocytochemistry with antisera to nestin and to cytokeratin 19, a marker of ductal

epithelium, nestin is strongly expressed in localized regions of both the large and small ducts, as well as in some centrolobular ducts within the exocrine acinar tissue (Figs. 11A and 11B). Remarkably, the localized regions of nestin expression in the ducts are mostly devoid of staining with the anti CK19 antiserum. Further, the nestin-positive cells in the ducts appear to have a morphology that is distinct from that of the epithelial cells. The epithelial cells consist of a homogenous population of cuboidal, rounded cells, whereas the nestin-positive cells are nucleated, serpiginous and appear to reside in the interstices among or around epithelial cells (Fig. 11C).

Thus, CK19 is not expressed in the majority of ductal cells that express nestin suggesting that these nestin-expressing cells located within the pancreatic ducts are a passenger population of cells distinct from the ductal epithelial cells and are stem cells that have not yet differentiated into a ductal or endocrine phenotype. The finding of localized populations of nestin-expressing cells within the pancreatic ducts and islets of the adult rat pancreas further supports the idea that rat pancreatic ducts contain cells that are progenitors of islet cells (neogenesis), but these progenitors are not a subpopulation of ductal epithelial cells *per se*.

EXAMPLE 13

Transplantation of pancreatic stem cells engineered to express IDX-1 in human subjects with diabetes mellitus

Islets isolated from pig or human donor pancreata, or from pancreatic biopsy of eventual human transplant recipient are cultured *ex vivo* in conditions that stimulate outgrowth of stem cells. Stem cells are then isolated away from islets (cloned), expanded *in vitro* in proliferation media containing bFGF-2, EGF, and 11.1 mM glucose, transfected/injected with an expression vector containing DNA encoding transcription factor IDX-1, and transplanted into a diabetic recipient. Alternatively, IDX-1-transfected stem cells are treated with GLP-1, or other differentiation morphogens or growth factors for 1-3 days before transplantation to initiate processes of differentiation of engineered stem cells to β-cells. In one embodiment, stem cells are neither expanded or differentiated prior to administration to the recipient or are only expanded or differentiated prior to administration to the recipient. In one embodiment, GLP-1 is administered to the recipient during and for several days after transplantation to stimulate differentiation of stem cells and encourage successful engraftment. According to this method, xenographs (pig islets) or allographs (human islets from a human donor that is not the recipient),

as well as isographs (islets derived from the recipient) are carried out. It is hypothesized that when transplanted to a host recipient the stem cell genetic repertoire is reprogrammed so that the host recognizes the stem cells (in the case of xenographs or allographs) as self, such that immune intolerance and graft rejection and destruction by autoimmunity (type 1 diabetes) does not occur.

EXAMPLE 14

Transplantation of pancreatic stem cells cultured to stimulate expression of IDX-1 in human subjects with diabetes mellitus

Islets isolated as described are cultured *ex vivo* for several days in conditions that stimulate first the expansion (proliferation) of stem cells that exist within the islets and then the expression of transcription factor IDX-1. The proliferation of stem cells is achieved by culturing the islets in media containing bFGF-2, EGF, and 11.1 mM glucose. Induction of the expression of IDX-1 is achieved by incubation in the presence of GLP-1 and 2 mM glucose. The islets so preconditioned by the treatments described are transplanted to the host recipient. Additionally, the host recipient may be administered GLP-1 during and for several days after the transplantation to further expand and differentiate stem cells to insulin-producing cells to enhance success of engraftment.

According to this method, xenographs (pig islets), allographs (human islets from a human donor that is not the recipient), as well as isographs (islets derived from the recipient) are effectuated.

EXAMPLE 15

Xenogeneic transplantation of pancreatic stem cells into the kidney

Human nestin-positive-islet progenitor cells (NIPS) were isolated as described, and transplanted under the renal capsules of eight C57B16 mice that were not immunosuppressed. The transplanted human cells were not rejected by the mouse recipient. Current understanding is that a xenograft, such as human tissue, would be rejected by the mouse within 5-10 days. Contrary to current understanding, we found that in 8 of the 8 non-immunosuppressed mice tested to date, all of the transplants successfully engrafted and proliferated into large masses of tissue engulfing the pole of the kidney by one month (30-38 days) after a transplantation of approximately 10^5 to 10^6 cells.

One C57B16 mouse was sacrificed and determined to have a large area of new growth at

the site of transplantation. A section of the kidney that included the new tissue was divided into two pieces; one piece was frozen for frozen section histology, and the other piece was fixed in paraformaldehyde for paraffin section histology. Frozen sections were prepared and stained with hematoxylin and eosin (H&E) and antisera to various islet cell antigens.

Examination of the H&E stained kidney section demonstrated the presence of a new growth that was not part of the kidney, exhibiting a pleiomorphic morphology consisting of a mixed mesenchymal and epithelial tissue containing hepatic, neural, ductal, adipodipic and hematopoetic components. Photomicrographs of the kidney section demonstrated that the new growth seemed to be invading the renal parenchyme, and the glomeruli. Specific immunostaining with human-specific (not mouse) antisera revealed cords of immunopositive cells staining for human-specific keratins, vimentin, and the CD45 leukocyte antigen specific for human hematapoetic lymphocytes. The kidney of a second C57Bl6 mouse also had a similar looking new growth at the site of the NIP transplantation.

The paraffin section of the NIP-engrafted kidney of a C57Bl6 mouse (the first mouse to be sacrificed) was examined. The tissue block that was examined was from the top of the kidney and showed the foreign tissue to be well contained under the renal capsule with no signs of "invasion" into the renal parenchyma. Notably, amongst the pleiomorphic-looking graft tissues were areas that resembled renal parenchyma. Without being bound to theory one hypothesis is that the graft consists of stem cells trying to differentiate and that the stem cells are not "invading" but simply migrating and proliferating and looking for a niche, i.e. mesenchymal instructions. They may be receiving cues from the kidney and may be attempting to differentiate into kidney. The graft cells may not be malignant, but may be just stem cells attempting to carry out their function.

EXAMPLE 16

Xenogeneic transplantation of pancreatic stem cells into the pancreas

Human nestin-positive-islet progenitor cells (NIPS) are isolated as described, and transplanted into the pancreas of mice that are not immunosuppressed and are (a) injured by streptozotocin (to produce streptozotocin induced diabetes) treatment or (b) NOD mice in which there is an ongoing islet is with inflammation.

The pancreas of the transplanted animals is examined to determine if the NIPs find their proper niche, receive instructions from the islet region, and differentiate into islet (β-cell) cells.

EXAMPLE 17

Treatment of diabetes by xenogeneic transplantation of pancreatic stem cells

Human islets are isolated as described and cultured for several days *in vitro* to expand the stem cell population. Human NIPS are transplanted to the liver via the portal vein (according to conventional procedures well known in the art for transplantation to the liver.

Alternatively, a population of human NIPs (isolated as described) are introduced into the blood stream. In certain embodiments, the human NIPS are introduced via the pancreatic artery, to direct them to the diabetic pancreas.

A population of control (untransplanted animals) and transplanted animals are analyzed for amelioration of the symptoms of diabetes (e.g. blood glucose levels, insulin levels, number of pancreatic β–cells.

EXAMPLE 18

Identification of Nestin positive stem cells in the liver

Rat livers were isolated and frozen section were prepared according to methods known in the art and described herein.

Frozen sections of rat liver (6μM) were immunostained with a rabbit polyclonal anti-nestin serum. The immunofluorescent signal was developed by reaction of anti-donkey IgG serum tagged with the fluorophore, Cy3 (yellow-orange color. Nestin-positive cells surrounding a possible large biliary duct are depicted in figure 13A. Clusters of nestin positive cells surrounding several small biliary ducts are depicted in figure 13B.

EXAMPLE 19

Differentiation of NIPs toward hepatic phenotype

Because of the reported apparent commonalties between hepatic stem cells (oval cells), hepatic stellate cells, and progenitor cells in the pancreas, and the observations that following some injuries, the regenerating pancreas undergoes liver metaplasia (Slack, 1995; Reddy et al., 1991; Bisgaard et al., 1991; Rao et al., 1996), we performed RT-PCR to detect liver-

expressed genes in the stem cells. PCR products were obtained for XBP-1, a transcription factor required for hepatocyte development (Reimold et al., 2000), and transthyretin, a liver acute phase protein. Several other liver markers were also expressed such as α-fetoprotein (Dabeva et al., 2000), E-Cadherin (Stamatoglou et al., 1992), c-MET (Ikeda et al., 1998), HGF (Skrtic et al., 1999) and synaptophysin (Wang et al., 1998); see Fig. 15)) The expression of proteins shared by the pancreas and liver, such as HGF and synaptophysin, may reflect their common origin from the embryonic foregut endoderm, and represent differentiation toward either pancreatic or hepatic phenotypes.

EXAMPLE 20

GLP-1R signalling in NIPs

The application of GLP-1 amide to single, isolated NIPs elevates levels of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Cells were plated onto gridded coverslips to permit subsequent immunohistochemical staining of the same cells to test for nestin expression. All cells from which $[Ca^{2+}]_i$ recordings were made were shown to express nestin. In contrast to adult β-cells, GLP-1 stimulates $[Ca^{2+}]_i$ levels at basal (5.6 mM) glucose but was ineffective in the presence of high (20 mM) glucose (Figure 23, panel A). These effects were reproduced by forskolin (Figure 23, panel B) suggesting that the effects of GLP-1 are mediated through the activation of Gs and cAMP production, the same signalling pathway used in normal β-cells. The pretreatment of single isolated NIPs with the peptide Extendin (9-39), a specific antagonist of GLP-1, prevents the increase in $[Ca^{2+}]_i$ mediated by GLP-1 (Figure 23, panels C and D). The effects of GLP-1R antagonist Extendin (9-39) suggests that the same isoform of GLP-1R is expressed in NIPs as in β-cells. The $[Ca^{2+}]_i$ increase mediated by GLP-1 was inhibited by exprecellular La^{3+} (5 μM) suggesting that GLP-1 is activating $[Ca^{2+}]_i$ influx, consistent with its known role to depolarize β-cells (Figure 23, panel E). We further demonstrate that tolbutamide (100 μM) stimulates $[Ca^{2+}]_i$ elevation in NIPs indicating that they express ATP-sensitive K^+ channels (Figure 23, panel F). These data are consistent with GLP-1 induced membrane depolarization and activation of voltage-dependent Ca^{2+} channels in NIPs, consistent with its mechanism of action in β-cells.

EXAMPLE 21

GLP-1 induces differentiation of NIPs into insulin-secreting cells

Previous studies have demonstrated the insulinotropic action of GLP-1 as well as its ability to stimulate β-cell neogenesis in partial pancreactectomized rats (Xu et al., 1999, *Diabetes* 48:2270). NIPs were picked from islet cultures and expanded in growth medium containing bFGF plus EGF (passage 01) for 3-10 days (Zulewski et al., 2001 *Diabetes* 50: 521). In certain instances NIPOs that were expanded for 3-5 days were fixed and subjected to immunocytochemical detection of Nestin (Cy-3) and Insulin (Cy-2) as shown in Figure 24A. It was found that at this stage they are nestin positive and insulin negative. When NIP cultures were expanded for 7-10 days and then treated with either GLP-1 or its stable analog extendin-4, a subset of cells became insulin positive (Cy-2; Figure 24B, panel 3, 5, and 6) and Idx-1 positive (Cy-3; Figure 24B, panel 7) and nestin negative (Cy-3; Figure 23B, panel 4). There was an overall decrease in nestin expression when cultures were treated with GLP-1 (Figure 24B, panel 2 vs. 4). Accordingly, treatment with Extendin-4 induced a 2- to 3- fold increase in insulin secretion (Figure 25). However, in some culture wells, confluence alone was sufficient to initiate small amounts of insulin secretion.

The homeodomain protein Idx-1 is critical for pnacreas development and plays a major role in the transcriptional regulation of the insulin gene. Haploindufficiency of Idx-1 expression results in a form of early onset type-2 diabetes (MODY4) and inherited amino acid changes in Idx-1 are associated with late onset type-2 diabetes. Idx-1 is expressed in differentiated NIP cell populations.

Human NIPs that had been repeatedly passaged lose their ability to secrete insuling in response to GLP-1. However, transfection of these cells with an expression vector encoding the human Idx-1 cDNA rendered them responsive to GLP-1 and induced the synthesis of insulin in a subset of NIPs (Figure 26, panel 1). Interestingly, transfection of NIPs (passage 9) with Idx-1 in the absence of GLP-1 treatment did not induce insulin biosynthesis (Figure 26, lower panel B), but did stimulate Idx-1 expression levels (Figure 26, upper panel B). Taken together, these results suggest that GLP-1 stimulates levels of Idx-1 expression in NIP cells and that a critical threshold concentration of Idx-1 is required for NIPs to convert into insulin-producing cells, thus suggesting a role for the GLP-1R in islet cell differentiation.

EXAMPLE 22

A method for the use of NIPs to pre-induce a state of immune tolerance in a prospective host recipient of a tissue/organ graft

NIPs are used to pre-induce a state of tolerance toward a potential organ donor. Recent published reports indicate that the transplantation of stem cells from a donor into a transplant recipient can induce tolerance to transplants from the donor without any requirement for immunosuppressive drugs (see for example Fandrich et al., Nat. Med., Feb 2002, 8:171; Quaini et al., N. Engl. J. Med., 3 Jan 2002, 346:5; Korbling et al., N. Engl. J. Med., 7 Mar 2002, 346:738. The procedure described herein corroborates these observations and demonstrates a novel use of NIPs in the pre-induction of a state of immune tolerance toward a potential organ donor, prior to organ transplantation from the donor into the recipient

According to this method, a tissue biopsy is obtained from the pancreas or liver of a prospective human donor. Human nestin positive cells (NIPs) are isolated and expanded in vitro, as described, to generate NIPs or NIP equivalent stem/progenitor cells. These NIPs are then administered to an immunologically competent recipient either via subcutaneous or intravenous injection. After 4-8 weeks, tissue specimens are obtained by needle or punch biopsy. Genomic PCR and in situ hybridization using donor-specific probes indicate that the transplanted human NIPs colonize multiple organs of the recipient and created a state of micro/mixed chimerism which induced a condition of immune tolerance. Acceptance of a human tissue graft by a recipient is believed to result from the differentiation of the transplanted stem/progenitor cells into dendritic antigen-presenting cells and so induces the immune system of the recipient to recognize the engrafted human tissue as self. The success of the induction of immune tolerance in the recipient toward the donor tissue is confirmed by taking a skin biopsy from the donor and engrafting it on the skin of the recipient and showing that the recipient does not reject the donor skin graft. After verification of the induction of a state of immune tolerance toward the donor, the NIP transplant is surgically removed. Organs from the donor (kidney, heart, liver, pancreas) are then transplanted into the recipient without incurring rejection of the grafted organ. This organ pre-transplantation protocol commandeers the recipient's immune system to recognize the grafted organ as self and therefore obviates the need for powerful but toxic immunosuppressive drugs.

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OTHER EMBODIMENTS

Other Embodiments are within the claims that follow.

WE CLAIM:

1. A nestin-positive pancreatic stem cell for use in the treatment of a patient with diabetes mellitus comprising:
   (a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor; and
   (b) transferring the stem cell into the patient, wherein the stem cell differentiates into an insulin-producing cell.

2. Use of a nestin-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with diabetes mellitus comprising:
   (a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor; and
   (b) transferring the stem cell into the patient, wherein the stem cell differentiates into an insulin-producing cell.

3. Use of stem cell according to claim 1 or 2, wherein said nestin-positive pancreatic stem cell is also ABCG2- positive.

4. Use of stem cell according to claim 1 or 2, wherein said nestin-positive pancreatic stem cell is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits $\alpha 6$ and $\beta 1$, C-kit, MDR-1, SUR-1, or Kir 6.2.

5. Use of stem cell according to claim 1 or 2, wherein said nestin-positive pancreatic stem cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

6. An ABCG2-positive pancreatic stem cell for use in the treatment of a patient with diabetes mellitus comprising:
   (a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor; and
   (b) transferring the stem cell into the patient, wherein the stem cell differentiates into an insulin-producing cell.

7. Use of an ABCG2-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with diabetes mellitus comprising:
   (a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor; and
   (b) transferring the stem cell into the patient, wherein the stem cell differentiates into an insulin-producing cell.

8. Use of stem cell according to claim 1, 2, 6 or 7, wherein the patient serves as the donor for said stem cells of step a.

9. Use of stem cell according to claim 1, 2, 6 or 7, wherein, prior to the step of transferring, the stem cell is treated ex vivo with an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, GLP-1, exendin-4, IDX-1, a nucleic acid molecule encoding IDX-1, betacellulin, activin A, TGF-β, and combinations thereof.

10. Use of stem cell according to claim 1, 2, 6 or 7, wherein the step of transferring is performed via endoscopic retrograde injection.

11. Use of stem cell according to claim 1, 2, 6 or 7, additionally comprising the step of:
(c) treating the patient with an immunosuppressive agent.

12. Use of stem cell according to claim 11, wherein the immunosuppressive agent is selected from the group consisting of FK-506, cyclosporin, and GAD65 antibodies.

13. A nestin-positive pancreatic stem cell for use in the treatment of a patient with diabetes mellitus comprising:
(a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell *ex vivo* to produce a progenitor cell; and
(c) transferring the progenitor cell into the patient, wherein the progenitor cell differentiates into an insulin-producing beta cell.

14. Use of a nestin-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with diabetes mellitus comprising:
(a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell *ex vivo* to produce a progenitor cell; and
(c) transferring the progenitor cell into the patient, wherein the progenitor cell differentiates into an insulin-producing beta cell.

15. Use of stem cell according to claim 13 or 14, wherein said nestin-positive pancreatic stem cell is also ABCG2- positive.

16. Use of stem cell according to claim 13 or 14, wherein said nestin-positive pancreatic stem cell is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SUR-1, or Kir 6.2.

17. Use of stem cell according to claim 13 or 14, wherein said nestin-positive pancreatic stem cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

18. An ABCG2-positive pancreatic stem cell for use in the treatment of a patient with diabetes mellitus comprising:

(a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell *ex vivo* to produce a progenitor cell; and
(c) transferring the progenitor cell into the patient, wherein the progenitor cell differentiates into an insulin-producing beta cell.

19. Use of an ABCG2-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with diabetes mellitus comprising:

(a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell *ex vivo* to produce a progenitor cell; and
(c) transferring the progenitor cell into the patient, wherein the progenitor cell differentiates into an insulin-producing beta cell.

20. Use of stem cell according to claim 13, 14, 18 or 19, wherein the patient serves as the donor for said stem cells of step a.

21. Use of stem cell according to claim 13, 14, 18 or 19, wherein the step of expanding is performed in the presence of an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, GLP-1, exendin-4, IDX-1, a nucleic acid molecule encoding IDX-1, betacellulin, activin A, TGF-β and combinations thereof.

22. Use of stem cell according to claim 13, 14, 18 or 19, wherein the step of transferring is performed via endoscopic retrograde injection.

23. Use of stem cell according to claim 13, 14, 18 or 19 additionally comprising the step of:

(d) treating the patient with an immunosuppressive agent.

24. Use of stem cell according to claim 23, wherein the immunosuppressive agent is selected from the group consisting of FK-506, cyclosporin, and GAD65 antibodies.

25. A nestin-positive pancreatic stem cell for use in the treatment of a patient with diabetes mellitus comprising:

(a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell to produce a progenitor cell;
(c) differentiating the progenitor cell in culture to form pseudo-islet like aggregates; and
(d) transferring the pseudo-islet like aggregates into the patient.

26. Use of an nestin-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with diabetes mellitus comprising:
(a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell to produce a progenitor cell;
(c) differentiating the progenitor cell in culture to form pseudo-islet like aggregates; and
(d) transferring the pseudo-islet like aggregates into the patient.

27. Use of stem cell according to claim 25 or 26, wherein said nestin-positive pancreatic stem cell is also ABCG2- positive.

28. Use of stem cell according to claim 25 or 26, wherein said nestin-positive pancreatic stem cell is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits $\alpha 6$ and $\beta 1$, C-kit, MDR-1, SUR-1, or Kir 6.2.

29. Use of stem cell according to claim 25 or 26, wherein said nestin-positive pancreatic stem cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

30. An ABCG2-positive pancreatic stem cell for use in the treatment of a patient with diabetes mellitus comprising:
(a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell to produce a progenitor cell;
(c) differentiating the progenitor cell in culture to form pseudo-islet like aggregates; and
(d) transferring the pseudo-islet like aggregates into the patient.

31. Use of an ABCG2-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with diabetes mellitus comprising:
(a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell to produce a progenitor cell;
(c) differentiating the progenitor cell in culture to form pseudo-islet like aggregates; and
(d) transferring the pseudo-islet like aggregates into the patient.

32. Use of stem cell according to claim 25, 26, 30 or 31, wherein the patient serves as the donor for said stem cells of step a.

33. Use of stem cell according to claim 25, 26, 30 or 31, wherein the step of expanding is performed in the presence of an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, GLP-1, exendin-4, IDX-1, a nucleic acid molecule encoding Bd-1, betacellulin, activin A, TGF-β, and combinations thereof.

34. Use of stem cell according to claim 25, 26, 30 or 31, wherein the step of transferring is performed via endoscopic retrograde injection.

35. Use of stem cell according to claim 25, 26, 30 or 31 additionally comprising the step of:
(e) treating the patient with an immunosuppressive agent.

36. Use of stem cell according to claim 35, wherein the immunosuppressive agent is selected from the group consisting of FK-506, cyclosporin, and GAD65 antibodies.

37. A method of isolating a stem cell from a pancreatic islet of Langerhans, comprising the steps of:
(a) removing a pancreatic islet from a donor;
(b) culturing cells from the pancreatic islet; and
(c) selecting a nestin-positive clone from the culture.

38. The method of claim 37, wherein said nestin-positive clone is also an ABCG2-positive clone.

39. The method of claim 37, wherein said nestin-positive clone is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SUR-1, or Kir 6.2.

40. The method of claim 37, wherein said nestin-positive clone does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

41. A method of isolating a stem cell from a pancreatic islet of Langerhans, comprising the steps of:
(a) removing a pancreatic islet from a donor;
(b) culturing cells from the pancreatic islet; and
(c) selecting an ABCG2-positive clone from the culture.

42. The method of claim 37 or 41, wherein the culturing is first performed in a vessel coated with concanavalin A and then again performed in a vessel not coated with concanavalin A.

43. The method of claim 37 or 41, comprising the additional step of:

(d) expanding the nestin-positive clone by treatment with an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, GLP-1, exendin-4, IDX-1, a nucleic acid molecule encoding IDX-1, betacellulin, activin A, TGF-β, and combinations thereof.

44. The method of claim 41 comprising the additional step of:

(d) expanding the ABCG2-positive clone by treatment with an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, GLP-1, exendin-4, IDX-1, a nucleic acid molecule encoding IDX-1, betacellulin, activin A, TGF-β, and combinations thereof.

45. A method of inducing the differentiation of a nestin-positive pancreatic stem cell into a pancreatic progenitor cell, comprising the step of:

treating a nestin-positive pancreatic stem cell with an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, IDX-1, a nucleic acid molecule encoding IDX-1, GLP-1, exendin-4, betacellulin, activin A, TGF-β, and combinations thereof, whereby the stem cell subsequently differentiates into a pancreatic progenitor cell.

46. The method of claim 45, wherein said nestin-positive pancreatic stem cell is also an ABCG2-positive clone.

47. The method of claim 45, wherein said nestin-positive pancreatic stem cell is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SUR-1, or Kir 6.2.

48. The method of claim 45, wherein said nestin-positive pancreatic stem cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

49. A method of inducing the differentiation of an ABCG2-positive pancreatic stem cell into a pancreatic progenitor cell, comprising the step of:

treating an ABCG2-positive pancreatic stem cell with an agent selected from the group consisting of EGF, bFGF-2, high glucose, KGF, HGF/SF, IDX-1, a nucleic acid molecule encoding IDX-1, GLP-1, exendin-4, betacellulin, activin A, TGF-β, and combinations thereof, whereby the stem cell subsequently differentiates into a pancreatic progenitor cell.

50. The method of claim 45 or 49, wherein the pancreatic progenitor cell subsequently forms pseudo-islet like aggregates.

51. An isolated, nestin-positive human pancreatic or liver stem cell that is not a neural stem cell.

52. The isolated stem cell of claim 51, wherein said cell is also ABCG2-positive.

53. The isolated stem cell of claim 51, wherein said cell is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits $\alpha 6$ and $\beta 1$, C-kit, MDR-1, SUR-1, or Kir 6.2.

54. The isolated stem cell of claim 51, wherein said cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

55. An isolated, ABCG2-positive human pancreatic or liver stem cell that is not a neural stem cell.

56. The isolated cell of claim 55, wherein said cell is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits $\alpha 6$ and $\beta 1$, C-kit, MDR-1, SUR1, or Kir 6.2.

57. The isolated cell of claim 55, wherein said cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

58. The isolated stem cell of claim 55, wherein said cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

59. The isolated stem cell of claim 51 or 55, that differentiates to form insulin-producing beta cells.

60. The isolated stem cell of claim 51 or 55, that differentiates to form glucagon-producing alpha cells.

61. The isolated stem cell of claim 51 or 55, that differentiates to form pseudo-islet like aggregates.

62. The isolated stem cell of claim 51 or 55, that differentiates to form hepatocytes.

63. A method of identifying a pancreatic cell as a stem cell, comprising the step of:

contacting a cell with a labeled nestin-specific antibody, whereby if the cell becomes labeled with the antibody the cell is identified as a stem cell.

64. The method of claim 63, further comprising the step of contacting a cell with a labeled antibody that binds to a marker selected from the group consisting of ABCG2, Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR-1, SUR-1, or Kir 6.2, whereby if the cell becomes labeled with the antibody the cell is identified as a stem cell.

65. Use of stem cell according to claim 36 further comprising the step of:

contacting the cell with a labeled cytokeratin-19 specific antibody, whereby if the cell does not become labeled with the antibody the cell is identified as a stem cell.

66. The method of claim 63 further comprising the step of:

contacting the cell with a labeled collagen IV specific antibody, whereby if the cell does not become labeled with the antibody the cell is identified as a stem cell.

67. The method of claim 63, further comprising the step of contacting the cell with a labeled antibody that binds to a marker selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II, whereby if the cell does not become labeled with the antibody the cell is identified as a stem cell.

68. A method of inducing a nestin-positive pancreatic stem cell to differentiate into hepatocytes, comprising the step of:

treating the nestin-positive pancreatic stem cell with an effective amount of an agent that induces the stem cell to differentiate into hepatocytes or into progenitor cells that differentiate into hepatocytes.

69. The method of claim 68, wherein said nestin-positive pancreatic stem cell is also ABCG2-positive.

70. The method of claim 68, wherein said nestin-positive pancreatic stem cell is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits α6 and β1, C-kit, MDR1, SUR-1, or Kir 6.2.

71. The method of claim 68, wherein said nestin positive pancreatic stem cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

72. A method of inducing an ABCG2-positive pancreatic stem cell to differentiate into hepatocytes, comprising the step of:

treating the ABCG2-positive pancreatic stem cell with an effective amount of an agent that induces the stem cell to differentiate into hepatocytes or into progenitor cells that differentiate into hepatocytes.

73. The method of claim 68 or 72, wherein the agent is cyclopamine.

74. A nestin-positive pancreatic stem cell for use in the treatment of a patient with liver disease comprising:

(a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor; and
(b) transferring the stem cell into the patient, wherein the stem cell differentiates into a hepatocyte.

75. Use of a nestin-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with liver disease comprising:

(a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor; and
(b) transferring the stem cell into the patient, wherein the stem cell differentiates into a hepatocyte.

76. Use of stem cell according to claim 74 or 75, wherein said nestin-positive pancreatic stem cell is also ABCG2- positive.

77. Use of stem cell according to claim 74 or 75, wherein said nestin-positive pancreatic stem cell is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits $\alpha 6$ and $\beta 1$, C-kit, MDR-1, SUR-1, or Kir 6.2.

78. The method of claim 74 or 75, wherein said nestin positive pancreatic stem cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

79. An ABCG2-positive pancreatic stem cell for use in the treatment of a patient with liver disease comprising:

(a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor; and
(b) transferring the stem cell into the patient, wherein the stem cell differentiates into a hepatocyte.

80. Use of an ABCG2-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with liver disease comprising:

(a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor; and
(b) transferring the stem cell into the patient, wherein the stem cell differentiates into a hepatocyte.

81. Use of stem cell according to claim 74, 75, 79 or 80, wherein the patient serves as the donor for said stem cells of step a.

82. A nestin-positive pancreatic stem cell for use in the treatment of a patient with liver disease comprising:
(a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell ex vivo to produce a progenitor cell; and
(c) transferring the progenitor cell into the patient, wherein the progenitor cell differentiates into a hepatocyte.

83. Use of a nestin-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with liver disease comprising:
(a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell ex vivo to produce a progenitor cell; and
(c) transferring the progenitor cell into the patient, wherein the progenitor cell differentiates into a hepatocyte.

84. Use of stem cell according to claim 82 or 83, wherein said nestin-positive pancreatic stem cell is also ABCG2- positive.

85. Use of stem cell according to claim 82 or 83, wherein said nestin-positive pancreatic stem cell is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits $\alpha 6$ and $\beta 1$, C-kit, MDR-1, SUR-1, or Kir 6.2.

86. Use of stem cell according to claim 82 or 83, wherein said nestin positive pancreatic stem cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

87. An ABCG2-positive pancreatic stem cell for use in the treatment of a patient with liver disease comprising:
(a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell ex vivo to produce a progenitor cell; and

(c) transferring the progenitor cell into the patient, wherein the progenitor cell differentiates into a hepatocyte.

88. Use of an ABCG2-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with liver disease comprising:

(a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) expanding the stem cell ex vivo to produce a progenitor cell; and
(c) transferring the progenitor cell into the patient, wherein the progenitor cell differentiates into a hepatocyte.

89. Use of stem cell according to claim 82, 83, 87 or 88, wherein the patient serves as the donor for said stem cells of step a.

90. A nestin-positive pancreatic stem cell for use in the treatment of a patient with liver disease comprising:

(a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) differentiating the stem cell ex vivo to produce a hepatocyte; and
(c) transferring the hepatocyte into the patient.

91. Use of a nestin-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with liver disease comprising:

(a) isolating the nestin-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) differentiating the stem cell ex vivo to produce a hepatocyte; and
(c) transferring the hepatocyte into the patient.

92. Use of stem cell according to claim 90 or 91, wherein said nestin-positive pancreatic stem cell is also ABCG2- positive.

93. Use of stem cell according to claim 90 or 91, wherein said nestin-positive pancreatic stem cell is also positive for at least one of the markers selected from the group consisting of Oct3/4, GLP-1 receptor, latrophilin (type 2), Hes-1, Nestin, Integrin subunits $\alpha 6$ and $\beta 1$, C-kit, MDR-1, SUR-1, or Kir 6.2.

94. Use of stem cell according to claim 90 or 91, wherein said nestin positive pancreatic stem cell does not express at least one of the markers selected from the group consisting of CD34, CD45, CD133, MHC class I and MHC class II.

95. An ABCG2-positive pancreatic stem cell for use in the treatment of a patient with liver disease comprising:

(a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) differentiating the stem cell *ex vivo* to produce a hepatocyte ; and
(c) transferring the hepatocyte into the patient.

96. Use of an ABCG2-positive pancreatic stem cell in the manufacture of a composition for the treatment of a patient with liver disease comprising:

(a) isolating the ABCG2-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) differentiating the stem cell *ex vivo* to produce a hepatocyte; and
(c) transferring the hepatocyte into the patient.

97. Use of stem cell according to claim 90, 91, 95 or 96, wherein the patient serves as the donor for said stem cells of step a.

98. A pharmaceutical composition comprising the isolated stem cell of claim 51 admixed with a physiologically compatible carrier.

99. A pharmaceutical composition comprising the isolated stem cell of claim 52 admixed with a physiologically compatible carrier.

100. A pharmaceutical composition comprising the isolated stem cell of claim 55 admixed with a physiologically compatible carrier.

101. A method of pre-inducing a state of immune tolerance prior to organ transplantation in a mammal, comprising the steps of:

(a) isolating a nestin-positive pancreatic stem cell from a pancreatic islet of a donor;
(b) transplanting said nestin-positive pancreatic stem cells into a transplant recipient;
(c) inducing a state of immune tolerance in the recipient toward the donor's stem cells;
(d) transplanting an organ from said donor to said recipient without the administration of immunosuppressive drugs.

102. A method of isolating a stem cell from a pancreatic islet of Langerhans, comprising the steps of:

(a) removing a pancreatic islet from a donor;
(b) culturing cells from the pancreatic islet under conditions wherein said cultured cells comprise nestin-positive cells which have been migrated from said islet; and
(c) selecting said nestin-positive cells from the culture.

103. The method of claim 102, wherein said migrated cells from step b form a monolayer.

104. A method of isolating a stem cell from a pancreatic islet of Langerhans, comprising the steps of:

(a) removing said stem cell from a pancreatic islet of Langerhans; and
(b) separating said stem cell from cells which are not stem cells.

105. A method of isolating a stem cell from a pancreatic islet of Langerhans, comprising the steps of:

(a) removing a pancreatic islet from a donor;
(b) culturing cells from the pancreatic islet in a first vessel coated with concanavalin A;
(c) transferring cells from step b to a second vessel not coated with concanavalin A;
(d) culturing cells from step c in said second vessel to produce a cell culture;
(e) selecting a nestin-positive cell from step d to produce an isolated stem cell.

106. The isolated stem cell of claim 51 or 55, isolated by the method of claim 41, 102, 104 or 105.

107. A pharmaceutical composition comprising the isolated stem cell of claim 106 admixed with a physiologically compatible carrier.

24 MAR 2006

SEQUENCE LISTING

<110> Massachusetts General Hospital

<120> Stem Cells of the Islets of Langerhans and Their Use in Treating Diabetes Mellitus

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<151> 2001-09-26

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<151> 2002-05-02

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<170> PatentIn version 3.1

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Gly Met  Glu Asp Ala Gly Pro  Gly Ala Asp Ile Ile  Gly Val Asn
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Gly Gln  Gly Pro Asn Leu Glu  Gly Lys Ser Gln His  Val Asn Gly
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Gly Val  Met Asn Gly Leu Glu  Gln Ser Glu Glu Ser  Gly Ala Arg
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Asn Ala  Leu Val Ser Glu Gly  Asp Arg Gly Ser Pro  Phe Gln Glu
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Glu Glu  Gly Ser Ala Leu Lys  Arg Ser Ser Ala Gly  Ala Pro Val
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35 40 45

Ser Leu Thr Glu Asp Pro Pro Pro Ala Thr Asp Leu Phe Cys Asn Arg
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Thr Phe Asp Glu Tyr Ala Cys Trp Pro Asp Gly Glu Pro Gly Ser Phe
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Val Asn Val Ser Cys Pro Trp Tyr Leu Pro Trp Ala Ser Ser Val Pro
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Gln Gly His Val Tyr Arg Phe Cys Thr Ala Glu Gly Leu Trp Leu Gln
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Lys Asp Asn Ser Ser Leu Pro Trp Arg Asp Leu Ser Glu Cys Glu Glu
        115                 120                 125

Ser Lys Arg Gly Glu Arg Ser Ser Pro Glu Glu Gln Leu Leu Phe Leu
    130                 135                 140

Tyr Ile Ile Tyr Thr Val Gly Tyr Ala Leu Ser Phe Ser Ala Leu Val
145                 150                 155                 160

Ile Ala Ser Ala Ile Leu Leu Gly Phe Arg His Leu His Cys Thr Arg
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Asn Tyr Ile His Leu Asn Leu Phe Ala Ser Phe Ile Leu Arg Ala Leu
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Ser Val Phe Ile Lys Asp Ala Ala Leu Lys Trp Met Tyr Ser Thr Ala
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Ala Gln Gln His Gln Trp Asp Gly Leu Leu Ser Tyr Gln Asp Ser Leu
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Ser Cys Arg Leu Val Phe Leu Leu Met Gln Tyr Cys Val Ala Ala Asn
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Phe Ser Val Phe Ser Glu Gln Trp Ile Phe Arg Leu Tyr Val Ser Ile
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Gly Trp Gly Val Pro Leu Leu Phe Val Val Pro Trp Gly Ile Val Lys
        275                 280                 285

Tyr Leu Tyr Glu Asp Glu Gly Cys Trp Thr Arg Asn Ser Asn Met Asn
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Phe Val Met Asp Glu His Ala Arg Gly Thr Leu Arg Phe Ile Lys Leu
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Phe Thr Glu Leu Ser Phe Thr Ser Phe Gln Gly Leu Met Val Ala Ile
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Leu Tyr Cys Phe Val Asn Asn Glu Val Gln Leu Glu Phe Arg Lys Ser
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Trp Glu Arg Trp Arg Leu Glu His Leu His Ile Gln Arg Asp Ser Ser
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20 25 30

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Glu Gly Ala Val Leu Ser Phe His Asn Ile Cys Tyr Arg Val Lys Leu
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Lys Ser Gly Phe Leu Pro Cys Arg Lys Pro Val Glu Lys Glu Ile Leu
    50                  55                  60

Ser Asn Ile Asn Gly Ile Met Lys Pro Gly Leu Asn Ala Ile Leu Gly
65                  70                  75                  80

Pro Thr Gly Gly Gly Lys Ser Ser Leu Leu Asp Val Leu Ala Ala Arg
                85                  90                  95

Lys Asp Pro Ser Gly Leu Ser Gly Asp Val Leu Ile Asn Gly Ala Pro
            100                 105                 110

Arg Pro Ala Asn Phe Lys Cys Asn Ser Gly Tyr Val Val Gln Asp Asp
        115                 120                 125

Val Val Met Gly Thr Leu Thr Val Arg Glu Asn Leu Gln Phe Ser Ala
    130                 135                 140

Ala Leu Arg Leu Ala Thr Thr Met Thr Asn His Glu Lys Asn Glu Arg
145                 150                 155                 160

Ile Asn Arg Val Ile Gln Glu Leu Gly Leu Asp Lys Val Ala Asp Ser
                165                 170                 175

Lys Val Gly Thr Gln Phe Ile Arg Gly Val Ser Gly Gly Glu Arg Lys
            180                 185                 190

Arg Thr Ser Ile Gly Met Glu Leu Ile Thr Asp Pro Ser Ile Leu Phe
        195                 200                 205

Leu Asp Glu Pro Thr Thr Gly Leu Asp Ser Ser Thr Ala Asn Ala Val
    210                 215                 220

Leu Leu Leu Leu Lys Arg Met Ser Lys Gln Gly Arg Thr Ile Ile Phe
225                 230                 235                 240

Ser Ile His Gln Pro Arg Tyr Ser Ile Phe Lys Leu Phe Asp Ser Leu
                245                 250                 255

Thr Leu Leu Ala Ser Gly Arg Leu Met Phe His Gly Pro Ala Gln Glu
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            260                 265                 270

Ala Leu Gly Tyr Phe Glu Ser Ala Gly Tyr His Cys Glu Ala Tyr Asn
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Asn Pro Ala Asp Phe Phe Leu Asp Ile Ile Asn Gly Asp Ser Thr Ala
    290                 295                 300

Val Ala Leu Asn Arg Glu Glu Asp Phe Lys Ala Thr Glu Ile Ile Glu
305                 310                 315                 320

Pro Ser Lys Gln Asp Lys Pro Leu Ile Glu Lys Leu Ala Glu Ile Tyr
                325                 330                 335

Val Asn Ser Ser Phe Tyr Lys Glu Thr Lys Ala Glu Leu His Gln Leu
            340                 345                 350

Ser Gly Gly Glu Lys Lys Lys Lys Ile Thr Val Phe Lys Glu Ile Ser
        355                 360                 365

Tyr Thr Thr Ser Phe Cys His Gln Leu Arg Trp Val Ser Lys Arg Ser
    370                 375                 380

Phe Lys Asn Leu Leu Gly Asn Pro Gln Ala Ser Ile Ala Gln Ile Ile
385                 390                 395                 400

Val Thr Val Val Leu Gly Leu Val Ile Gly Ala Ile Tyr Phe Gly Leu
                405                 410                 415

Lys Asn Asp Ser Thr Gly Ile Gln Asn Arg Ala Gly Val Leu Phe Phe
            420                 425                 430

Leu Thr Thr Asn Gln Cys Phe Ser Ser Val Ser Ala Val Glu Leu Phe
        435                 440                 445

Val Val Glu Lys Lys Leu Phe Ile His Glu Tyr Ile Ser Gly Tyr Tyr
    450                 455                 460

Arg Val Ser Ser Tyr Phe Leu Gly Lys Leu Leu Ser Asp Leu Leu Pro
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Met Arg Met Leu Pro Ser Ile Ile Phe Thr Cys Ile Val Tyr Phe Met
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Leu Gly Leu Lys Pro Lys Ala Asp Ala Phe Phe Val Met Met Phe Thr
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Leu Met Met Val Ala Tyr Ser Ala Ser Ser Met Ala Leu Ala Ile Ala
        515                 520                 525

Ala Gly Gln Ser Val Val Ser Val Ala Thr Leu Leu Met Thr Ile Cys
    530                 535                 540

Phe Val Phe Met Met Ile Phe Ser Gly Leu Leu Val Asn Leu Thr Thr
545                 550                 555                 560

Ile Ala Ser Trp Leu Ser Trp Leu Gln Tyr Phe Ser Ile Pro Arg Tyr
                565                 570                 575

Gly Phe Thr Ala Leu Gln His Asn Glu Phe Leu Gly Gln Asn Phe Cys
            580                 585                 590

Pro Gly Leu Asn Ala Thr Gly Asn Asn Pro Cys Asn Tyr Ala Thr Cys
        595                 600                 605

Thr Gly Glu Glu Tyr Leu Val Lys Gln Gly Ile Asp Leu Ser Pro Trp
    610                 615                 620

Gly Leu Trp Lys Asn His Val Ala Leu Ala Cys Met Ile Val Ile Phe
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Leu Thr Ile Ala Tyr Leu Lys Leu Leu Phe Leu Lys Lys Tyr Ser
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34