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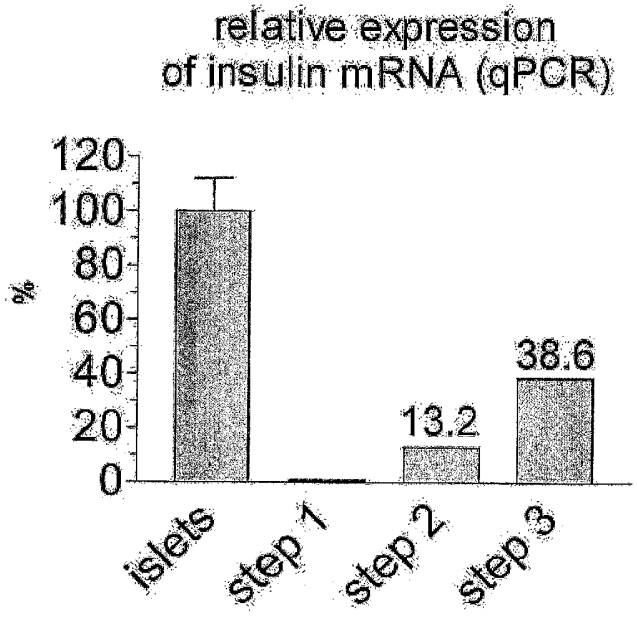

(57) Abstract: Methods and compositions are described for the treatment of type I insulin-dependent diabetes mellitus. The methods described permit the generation of large numbers of insulin producing cells for transplant to diabetic individuals from adult pancreatic donor cells. The invention describes novel methods for the *ex vivo* differentiation of pancreatic endocrine cells with enhanced insulin expression from expanded human islet progenitor cells *in vitro*. Methods are also described whereby pancreatic endocrine cells differentiated ex vivo are transplanted finto a patient in need thereof, either allogeneically, isogeneically or xenogenically, to provide replacement for lost or damaged insulin-secreting cells.

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EXPANSION AND DIFFERENTIATION OF ISLET PROGENITOR CELLS

GOVERNMENT SUPPORT

The invention was supported, in whole or in part, using U.S. government funds, grants DK60125 and DK61251 awarded by the National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD OF THE INVENTION

The invention is related to the field of progenitor cells and their differentiation. In particular, it is related to the field of beta cells of the islets of Langerhans and the differentiation of pancreatic islet progenitor cells into insulin-secreting pancreatic islet endocrine cells and their use for the treatment of diabetes mellitus.

BACKGROUND OF THE INVENTION

Diabetes mellitus is increasing at epidemic proportions throughout the populations of the world. It is estimated that 100 million individuals worldwide currently suffer from diabetes, a debilitating disease that results from the progressive failure of the β - cells of the endocrine pancreas (islets of Langerhans) to produce the hormone insulin in the amounts required to maintain nutrient homeostasis. Type I juvenile diabetes is caused by the complete destruction of the β- cells by processes of autoimmunity in which the body's immune system mistakenly attacks and destroys the β- cells. The causation of type 2 adult- onset diabetes is more complex and poorly understood, but the β - cells fail to produce adequate amounts of insulin in the face of the accompanying resistance of peripheral tissues to the actions of insulin. In general, a lack of insulin results in elevations in blood glucose levels (hyperglycemia) and the subsequent development of premature cardiovascular disease, stroke, and kidney failure. Although blood glucose levels can be somewhat controlled by daily insulin shots or, in moderate cases of diabetes, by oral hypoglycemic (blood glucose- lowering) drugs, there is no currently available permanent cure for diabetes.

Patients transplanted with pancreatic islets can become insulin dependent (Rajotte RV et al. Diabetes 51: 2148– 2157, 2002; Ryan et al. Diabetes (2001) 50: 710– 719). However, the feasibility of islet transplants or whole organ transplants is limited by the availability of tissues and organs. Methods of *Ex vivo* differentiation of pancreatic progenitor cells into pancreatic islet-like clusters, known in the prior art, are sub-optimal and result in only low levels of insulin

gene expression (Zulewski et al., 2001, Diabetes, 50:521-533; Abraham et al., 2002. Endocrinology, 143:3142-3161).

There is therefore a need in the art for an improved method of *ex vivo* differentiation of pancreatic progenitor cells into islet endocrine cells with increased insulin gene expression.

SUMMARY OF THE INVENTION

It is an object of the invention to provide an improved method for differentiating pancreatic progenitor cells *ex vivo* to obtain cells that produce increased amounts of insulin. It is also an object of the invention to provide methods for transplantation into a mammal that utilize *ex vivo* differentiated mammalian pancreatic islet endocrine cells for the treatment of diabetes. 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. Islet progenitor cells (IPCs) from a pancreatic islet of a donor are isolated, cultured ex vivo to permit cell division (preferably in the presence of epithelial growth factor (EGF) and beta nerve growth factor (βNGF)) and cultured on an extracellular matrix to produce islet-like clusters (ILCs). Islet-like clusters cultured in suspension mature into insulin secreting islet endocrine cells that are then transferred into the patient suffering from diabetes. Preferably ILCs produce insulin at a level that is at least 1% of that of native islets. Preferably IECs produce insulin at a level that is at least 5% of that of native islets.

Another embodiment of the invention provides a method of treating a patient with diabetes mellitus, comprising the steps of isolating islet progenitor cells (IPCs) from a pancreatic islet of a donor, culturing the cells *ex vivo* to permit cell division (preferably in the presence of EGF and βNGF), culturing the progenitor cells *ex vivo* on an extracellular matrix to produce islet-like clusters (ILCs), and transplanting the islet-like clusters (ILCs) into a patient, wherein the islet-like clusters (ILCs) differentiate into insulin-producing cells. Preferably ILCs produce insulin at a level that is at least 1% of that of native islets.

In one embodiment of the method of treating a patient with diabetes mellitus, the expansion of the cells *ex vivo* occurs in the presence of NGF or EGF.

In one embodiment of the method of treating a patient with diabetes mellitus, the expansion of the cells *ex vivo* occurs in the presence of NGF and EGF.

In a further embodiment of the method of treating a patient with diabetes mellitus, the patient is treated with GLP-1 prior to transplantation.

In a further embodiment of the method of treating a patient with diabetes mellitus, the patient is treated with GLP-1 prior to, during and post-transplantation.

In another embodiment of the method of treating a patient with diabetes mellitus, the differentiation of said islet progenitor cells (IPCs) into islet endocrine cells (IECs) increases the expression of insulin.

In another embodiment of the method of treating a patient with diabetes mellitus, the islet progenitor cells (IPCs) express at least one of the markers as set out in Table 1.

In another embodiment of the method of treating a patient with diabetes mellitus, the invention also provides for islet progenitor cells (IPCs) comprising 80% or more (i.e., 81, 85, 90, 95, 99, 100% ore more) cuboidal epithelial cells.

In another embodiment of the method of treating a patient with diabetes mellitus, the invention also provides for islet progenitor cells (IPCs) comprise 20% or less (i.e., 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, or 0%) spindle-shaped fibroblast-like cells. Preferably, the method of the invention includes a step wherein IPCs are separated from mesenchymal/fibroblast-like cells by culturing them in suspension.

In another embodiment of the method of treating a patient with diabetes mellitus, the invention also provides for cuboidal epithelial cells that express at least one of the genes selected from the group consisting of cytokeratin 19, E-cadherin, PDX-1 and nestin.

In another embodiment of the method of treating a patient with diabetes mellitus, the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs) up regulates the expression of at least one of the genes selected from the group of genes consisting of PDX-1, BETA2/NEUROD, NGN3, GLUCOKINASE, GLUT2, PC1/3 and CK19.

In another embodiment of the method of treating a patient with diabetes mellitus, the islet-like clusters (ILCs) express at least one of the genes selected from the group consisting of C-peptide, glucagon and the ductal cell marker cytokeratin 19.

In another embodiment of the method of treating a patient with diabetes mellitus, the differentiation of islet-like clusters (ILCs) on extracellular matrix occurs in serum free media.

In a preferred embodiment of the method of treating a patient with diabetes mellitus, the extracellular matrix is Matrigel™.

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, 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.

A further embodiment provides a method of inducing the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs), comprising the steps of culturing the islet progenitor cells (IPCs) *ex vivo* to permit cell division (preferably in the presence of EGF and βNGF), culturing the progenitor cells *ex vivo* on an extracellular matrix to produce islet-like clusters (ILCs) and culturing the islet-like clusters (ILCs) in suspension, to produce islet endocrine cells (IECs). Preferably ILCs produce insulin at a level that is at least 1% of that of native islets. Preferably IECs produce insulin at a level that is at least 5% of that of native islets.

A further embodiment provides a method of inducing the differentiation of islet progenitor cells (IPCs) into islet cluster-like cells (ILCs), comprising the steps of culturing the islet progenitor cells (IPCs) *ex vivo* to permit cell division (preferably in the presence of EGF and βNGF), culturing the progenitor cells *ex vivo* on an extracellular matrix to produce islet-like clusters (ILCs), wherein the culturing promotes the differentiation of islet progenitor cells (IPCs) into islet cluster-like cells (ILCs). Preferably ILCs produce insulin at a level that is at least 1% of that of native islets.

In one embodiment of the method of inducing the differentiation of islet progenitor cells (IPCs) into islet cluster-like cells (ILCs), the method further comprises culture of the islet-like clusters (ILCs) in suspension to produce islet endocrine cells (IECs). Preferably IECs produce insulin at a level that is at least 5% of that of native islets.

In one embodiment the expansion step occurs in the presence of NGF, EGF or NGF and EGF.

In one embodiment of the method of inducing the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs), the differentiation of the islet progenitor cells (IPCs) into islet endocrine cells (IECs) increases the expression of insulin.

In one embodiment of the method of inducing the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs), the islet progenitor cells (IPCs) express at least one of the markers as set out in Table 1.

In one embodiment of the method of inducing the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs), the islet progenitor cells (IPCs) comprise 80% or more (i.e., 81, 85, 90, 95, 99, 100% ore more) cuboidal epithelial cells.

In another embodiment of the method of treating a patient with diabetes mellitus, the invention also provides for islet progenitor cells (IPCs) comprise 20% or less (i.e., 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, or 0%) spindle-shaped fibroblast-like cells. Preferably, the method of the invention includes a step wherein IPCs are separated from mesenchymal/fibroblast-like cells by culturing them in suspension.

In another embodiment of the method of inducing the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs), the cuboidal epithelial cells express at least one of the genes selected from the group consisting of cytokeratin 19, E-cadherin, PDX-1 and nestin.

In another embodiment of the method of inducing the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs), the differentiation of the islet progenitor cells (IPCs) up regulates the expression of at least one of the genes selected from the group of genes consisting of PDX-1, BETA2/NEUROD, NGN3, GLUCOKINASE, GLUT2, PC1/3 and CK19.

In another embodiment of the method of inducing the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs), the islet-like clusters (ILCs) express C-peptide, glucagon and the ductal cell marker cytokeratin 19.

In another embodiment of the method of inducing the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs), the differentiation on extracellular matrix occurs in serum free media.

In one embodiments of the method of inducing the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs), the extracellular matrix is Matrigel^{TM}.

In one embodiment, the invention provides for an isolated islet progenitor cell (IPC) that differentiates to form insulin-producing islet endocrine cells (IECs).

In another embodiment, the isolated islet progenitor cell (IPC) expresses at least one of the markers as set out in Table 1.

In another embodiment, the isolated islet-like clusters (ILCs) differentiate to form insulin-producing islet endocrine cells (IECs).

In another embodiment, the isolated islet-like clusters (ILCs) express at least one of the genes selected from the group consisting of C-peptide, glucagon and the ductal cell marker cytokeratin 19.

In one embodiment, the invention provides for an isolated islet endocrine cell (IEC) that differentiates to form a pancreatic β islet cell.

In another embodiment, the isolated islet endocrine cell (IEC) expresses insulin.

In another aspect, the invention provides a method of generating insulin-producing cells, comprising: a) dissociating a preparation of pancreatic islets from a donor into a cell suspension and culturing the dissociated cells in serum-containing medium, to permit cell division; b) culturing cells resulting from the culture of step (a) in essentially serum-free medium, whereby a cell in the culture differentiates into an insulin-producing cell.

In one embodiment, cells resulting from cell division in step (a) produce less than 1% of the insulin of cells prepared from native islets.

In another embodiment, the culturing of step (b) results in the formation of islet like clusters comprising insulin-producing cells.

In another embodiment, an islet-like cluster resulting from step (b) produces insulin at a level that is at least 1% of that produced by native islets.

In another embodiment, an islet-like cluster resulting from step (b) produces insulin at a level that is at least 10% of that produced by native islets. In another embodiment, an islet-like cluster resulting from step (b) produces insulin at a level that is at least 20% of that produced by

native islets. The method of claim 43 wherein an islet-like cluster resulting from step (b) produces insulin at a level that is at least 30% of that produced by native islets.

In another embodiment, the serum-containing medium is supplemented with EGF. In another embodiment, the serum-containing medium is supplemented with EGF and bFGF or β-NGF. In another embodiment, the serum-containing medium is supplemented with EGF, bFGF and β-NGF.

In another embodiment, the serum-containing medium comprises a low glucose concentration.

In another embodiment, the essentially serum-free medium comprises a high glucose concentration.

In another embodiment, culturing step (b) is performed in culture dishes comprising an extracellular matrix material. In another embodiment, the extracellular matrix material comprises Matrigel™.

In another embodiment, a cell resulting from the cell division in step (a) expresses nestin.

In another aspect, there is provided a method of generating insulin-producing cells, the method comprising: a) placing pancreatic islet cells from an adult donor in culture under conditions that permit a cell in the culture to dedifferentiate and proliferate, thereby producing an expanded culture of dedifferentiated cells; b) placing cells from the expanded culture of dedifferentiated cells from step (a) under conditions that permit re-differentiation of a cell in the culture, thereby generating an insulin-producing islet like cluster.

In one embodiment, the cell that dedifferentiates is a mature pancreatic islet beta cell.

In another embodiment, an ancestor of the cell that re-differentiates is a mature beta cell that de-differentiated in step (a).

In another embodiment, insulin production by the insulin-producing islet like cluster is at least 1% the level produced by native islets.

In another embodiment, the essentially serum-free medium comprises a high glucose concentration.

In another embodiment, step (a) comprises proteolytic digestion of isolated islets to produce a single cell suspension.

In another embodiment, step (b) comprises culture on an extracellular matrix to produce islet-like clusters that produce insulin at a level that is at least 1% that of native islets.

In another embodiment, the method further comprises the step of placing islet-like cell clusters prepared in step (b) into suspension culture in essentially serum-free medium.

In another aspect, a method is provided for treating diabetes, the method comprising the steps of: a) administering a preparation of insulin-producing cells produced by the methods described above to a diabetic individual, whereby the diabetes is treated.

In another aspect, a method of generating islet progenitor cells is provided, the method comprising: a) expressing a Snail family or ZEB polypeptide in a pancreatic beta cell from an adult donor, wherein the expression permits the mature pancreatic beta cell to divide, thereby generating an islet progenitor cell.

In another aspect, a method of inducing the differentiation of islet progenitor cells is provided, the method comprising contacting the cells with an inhibitor of the activity or expression of a member of the Snail family or a ZEB polypeptide.

In one embodiment, the inhibitor comprises a siRNA that targets expression of a Snail family member or a ZEB polypeptide.

In another embodiment, the inhibitor comprises a Src kinase inhibitor.

In another embodiment, the Src kinase inhibitor is PP2.

In another embodiment, the invention provides for a pharmaceutical composition comprising the isolated islet progenitor cell (IPC) admixed with a physiologically compatible carrier.

In another embodiment, the invention provides for a pharmaceutical composition comprising the isolated islet-like clusters (ILCs) admixed with a physiologically compatible carrier.

In another embodiment, the invention provides for a pharmaceutical composition comprising the isolated islet endocrine cell (IEC) admixed with a physiologically compatible carrier.

In another aspect, methods for the restoration of insulin-producing cells involve inducing mature pancreatic islet cells, e.g., insulin-producing beta cells, to dedifferentiate to a proliferative phenotype that permits an expansion in cell numbers, followed by differentiation of expanded cells to an insulin-producing phenotype. Ex vivo dedifferentiation, expansion and re-differentiation are preferred, although one or more of the processes can occur in vivo.

Definitions:

The term "pancreas" is art recognized, and refers generally to a large, elongated, racemose gland situated transversely behind the stomach, between the spleen and duodenum. The pancreatic exocrine function, e.g., external secretion, provides a source of digestive enzymes. Indeed, "pancreatin" refers to a substance from the pancreas containing enzymes, principally amylase, protease, and lipase, which substance is used as a digestive aid. The exocrine portion of the pancreas is composed of several serous cells surrounding a lumen. These cells synthesize and secrete digestive enzymes such as trypsinogen, chymotrypsinogen, carboxypeptidase, ribonuclease, deoxyribonuclease, triacylglycerol lipase, phospholipase A_2, elastase, and amylase.

As used herein, "pancreatic islets" refer to the islets of Langerhans within the endocrine portion of the pancreas. The islets of Langerhans appear as rounded clusters of cells embedded within the exocrine pancreas. Four different types of cells α, β, δ, ϕ and have been identified in the islets. The α cells constitute about 20% of the cells found in pancreatic islets and produce the hormone glucagon. Glucagon acts on several tissues to make energy available in the intervals between feeding. In the liver, glucagon causes breakdown of glycogen and promotes gluconeogenesis from amino acid precursors. The δ cells produce somatostatin which acts in the pancreas to inhibit glucagon release and to decrease pancreatic exocrine secretion. The hormone pancreatic polypeptide (PP) is produced in the ϕ cells. This hormone inhibits pancreatic exocrine secretion of bicarbonate and enzymes, causes relaxation of the gallbladder, and decreases bile

secretion. The most abundant cell in the islets, constituting 60-80% of the cells, is the β cell, which produces insulin. Insulin is known to cause the storage of excess nutrients arising during and shortly after feeding. The major target organs for insulin are the liver, muscle, and fat-organs specialized for storage of energy.

As used herein, the term "insulin-producing beta cell" refers to any cell which can produce and secrete insulin in an amount equal to at least 1%, for example , 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 99, 100% or more of the amount of insulin produced and secreted by native β 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.

As used herein, "express" relates to the level of insulin gene expression as compared to the level of insulin gene expression found in a native β cell in the islets of Langerhans in the pancreas. In a preferred embodiment, the level of insulin gene expression is at least 1%, (for example, 1, 2, 3, 4, 5, 6, 7, 8, 9%, preferably 10%, 25%, 50%, 75%, 90%, 99%, 100% or more, or at least 2-fold or more, for example 2, 3, 4, 5, 6, 7, 8, 9, 10, 25, 100, 1000-fold or more of the level of insulin gene expression found in a native β cell in the islets of Langerhans in the pancreas.

"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/ progenitor 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/ progenitor cells, *e.g.*, a culture of stem/ progenitor cells, it is understood that it is practically impossible to obtain a collection of stem/ progenitor cells which is 100% pure. Therefore, an isolated stem/ progenitor cell can exist in the presence of a small fraction of other cell types which do not interfere with the utilization of the stem/ progenitor cell for analysis or production of other, differentiated cell types. Isolated stem/ progenitor cells will generally be at least 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 98%, or 99% pure. Preferably, isolated stem/ progenitor cells according to the invention will be at least 98% or at least 99% pure.

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. A stem cell is pluripotential, and given the appropriate signals from their environment, can differentiate into any tissue in the body.

As used herein, 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.

As used herein, "islet progenitor cells" (IPCs) refer to cells that have been isolated from pancreatic tissue and/or a cell that is capable of long-term proliferation and differentiation into islet-like clusters (ILCs) in culture. As used herein, single cell suspensions of human islet preparations or pancreatic stem cells differentiate into pancreatic "islet progenitor cells" (IPCs). In a preferred embodiment, IPCs are seeded at a density of 2.5 X 10^4 cells/cm^2. In a preferred embodiment, "islet progenitor cells" or IPCs of the invention express at least one of the genes listed in Table 1 at a level that is at least 2-fold greater than fresh pancreatic islet tissue, and at least 2-fold greater than an islet-like cluster (ILC). "At least 2-fold greater" refers to 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, 500, or 1000-fold or more. In another preferred embodiment, islet progenitor cells (IPCs) comprise at least 80%, 90%, 95%, 99% or more cuboidal epithelial cells. In another embodiment, islet progenitor cells (IPCs) comprise at most 20%, 10%, 5%, 1% or less spindle-shaped fibroblast-like cells.

As used herein, pancreatic "cuboidal epithelial cells" refer to cells that are present in pancreatic islet progenitor cell preparations that express at least one of cytokeratin 19, E-cadherin as well as (mainly cytoplasmic) PDX1 by immunostaining.

As used herein, pancreatic "fibroblast-like cells" refer to cells that are of spindle-shaped morphology that are present in pancreatic islet progenitor cell preparations and that do not express cytokeratin 19, E-cadherin as well as (mainly cytoplasmatic) PDX1 by immunostaining.

As used herein, "differentiation" or "differentiating" refers to the process by which a cell undergoes a change to a particular cell type, e.g. to a specialized cell type. "Differentiation" in the present context also means the formation of cells expressing markers known to be associated with cells that are more specialized and closer to becoming terminally differentiated cells incapable of further division or differentiation. For example, in a pancreatic context, differentiation can be seen in the production of islet-like cell clusters containing an increased proportion of beta-epithelial cells that produce increased amounts of insulin.

As used herein, "differentiate into pancreatic islet progenitor cells (IPCs)" refers to the differentiation of a fresh pancreatic islet tissue or a pancreatic stem cell into an IPC that expresses at least one of the genes listed in Table 1 at a level that is at least 2-fold greater (for example, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, 500, or 1000-fold or more, than the level of expression of a pancreatic stem cell or a native pancreatic islet. Preferably, "differentiation into IPCs" occurs by culturing single cell suspensions of islets in the presence of NGF and EGF.

As used herein, "islet-like clusters" or ILCs refer to the group of about 100 to 1,000 cells that form after culturing a single cell suspension of "islet progenitor cells" (IPCs) on an extracellular matrix, as defined herein, in serum-free medium. Preferably, at least 20%, more preferably 20-50% and most preferably, 50% or more, of a population of islet progenitor cells (IPCs) produce ILCs. In a preferred embodiment, the extracellular matrix is Matrigel™ (Collaborative Research, Catalog No. 40234). ILCs express insulin mRNA at a level that is at least 1%, preferably, 1-5%, for example, 1, 2, 3, 4, or 5%, and more preferably, 5-25% (for example, 5, 6, 7, 8, 9, 10, 15, 20 or 25%) or more (for example, 26, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99, 100% or more) of that of native islets. ILCs also express at least one of the genes listed in Figure 8, at a level that is at least 2-fold greater (for example 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, 500,

or 1000-fold or more) than the level of expression of IPCs. ILCs stain positive for C-peptide, glucagon and the ductal cell marker cytokeratin 19. ILCs do not express at least one of the genes in Table 1 at a level that is 2-fold or more greater (for example 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, 500, or 1000-fold or more) than the level of expression of a pancreatic stem cell or fresh pancreatic islet tissue. Preferably, ILCs are prepared by culturing single cell suspensions of islets in the presence of NGF and EGF to form IPCs, and then culturing the cells on an ECM. Preferably, ILCs are separated from mesenchymal/fibroblast-like cells by culturing the ILCs in suspension.

ILCs include islet endocrine cells (IECs) including insulin-producing beta cells and glucagon producing alpha cells, as well as cytokeratin 19-positive ductal cells and stromal cells. Preferably ILCs comprise less than 10% ductal cells and less than 20% stromal cells

ILCs form islet endocrine cells (IECs) when cultured in suspension. As used herein, "islet endocrine cells" or IECs refer to "islet-like clusters" (ILCs) that have been cultured in suspension. Preferably, 20%, and more preferably, 50% or more a population of ILCs form IECs when cultured in suspension. Preferably, the ILCs are allowed to differentiate in a three-dimensional culture system when they are cultured in suspension , following their removal from matrigel.

In a preferred embodiment, "islet endocrine cells" are produced from ILCs that have been cultured for one, two, three, four or five days or more in suspension. IECs express at least one of the islet-specific genes as set forth in Figure 3, at a level that is at least 2-fold greater (for example, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, 500, or 1000-fold or more) than the level of expression of an IPC. IECs according to the invention also express insulin mRNA at a level that is at least 1%, preferably 1-20%, (for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 115, 20) and more preferably, 20-50% (for example, 20, 25, 30, 35, 40, 45, 50) or more (for example. 51, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99, 100 or more) of that of native islets. IECs also express at least one of the genes listed in Figure 8, at a level that is at least 2-fold greater than the level of expression of IPCs. ILCs secrete C-peptide at a level that is at least 2%, or more, preferably 2-5% for example, 2%, 3%, 4%, 5%, and more preferably 5% or more, for example, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100% or more, of that of native islets.

As used herein, the term "adult donor" is used to differentiate between a fetal or neonatal donor of pancreatic tissue. Thus, an "adult donor," as the term is used herein, is generally an individual from about 6 months of age to a fully grown individual.

As used herein, the term "de-differentiation" refers to a process whereby an insulin-producing pancreatic islet beta cell regains the ability to proliferate. In this regard, the term is equivalent to the term "differentiate into pancreatic islet progenitor cells" as that term is applied to the differentiation of a fresh pancreatic islet cell, e.g., a mature beta cell, into an islet progenitor cell. The process of "de-differentiation" is marked by the essential loss of insulin expression and by the expression of one or more markers of a pancreatic stem cell phenotype as described herein, preferably at least by the expression of nestin, but also preferably by the expression of a Snail family member or a ZEB polypeptide. "De-differentiated cells" refers to cells that have undergone de-differentiation, and to their progeny, so long as the progeny have not undergone re-differentiation to an insulin-producing phenotype.

As used herein, the term "re-differentiation" refers to a process whereby a cell resulting from the de-differentiation and proliferative expansion of cells derived from an insulin producing pancreatic islet beta cell (the de-differentiated cell and its expanded progeny having an essentially non-insulin-producing phenotype) gains an insulin-producing phenotype. The term "re-differentiation" can be applied to a process that occurs in vitro or in vivo. Re-differentiation in vitro can be characterized by the formation of islet-like clusters, especially when cells are cultured in the presence of an extracellular matrix.

As used herein, "conditions that permit a cell to de-differentiate and proliferate" comprise, at a minimum, culture in serum-containing medium supplemented with EGF. Such conditions preferably also include culture in serum-containing medium supplemented with EGF and one or both of bFGF and beta-NGF.

As used herein, "conditions that permit a cell to re-differentiate and produce insulin" comprise, at a minimum, culture in essentially serum-free medium on a support comprising an extracellular matrix material.

As used herein, the term "essentially serum-free," when used in reference to culture medium, means medium that comprises less than or equal to 2% serum, preferably less than or equal to 1% serum, more preferably less than or equal to 0.5% serum, and more preferably still, no serum.

As used herein, the term "serum containing medium" refers to culture medium that comprises at least 5% serum, preferably about 5% to 15% serum, but possibly higher, e.g., 20% or more. "Serum containing medium" supports the de-differentiation of pancreatic islet beta cells in culture.

As used herein, the term "high glucose," when used in reference to culture medium, means medium in which the glucose concentration is at least 17.5 mM, e.g., 17.5 mM, 18 mM, 20 mM, 22 mM or more. Essentially serum free medium containing "high glucose" promotes the re-differentiation of expanded islet precursor cell cultures to a differentiated insulin-producing phenotype, e.g., in islet like cell clusters.

As used herein, the term "low glucose," when used in reference to culture medium, means medium in which the glucose concentration is less than about 12 mM, preferably less than 10 mM, 8 mM, 7 mM, 6 mM, 5 mM, 4 mM, 3 mM, 2 mM, 1 mM or below. Low glucose, in conjunction with serum promotes the in vitro de-differentiation of pancreatic islet cells.

By "ex vivo" is meant cells that have been taken from a body, temporarily cultured in vitro, and returned to a body.

As used herein in reference to comparisons of an amino acid, amino acid sequence, or protein domain, the term "similar" refers to amino acids or domains that although not identical, represent "conservative" differences. By "conservative" is meant that the differing amino acid has like characteristics with the amino acid in the corresponding or reference sequence. Typical conservative substitutions or variations are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. In calculating the degree (most often as a percentage) of similarity between two polypeptide sequences, one considers the number of positions at which identity or similarity is observed between corresponding amino acid residues in the two polypeptide sequences in relation to the entire lengths of the two molecules being compared.

By "extracellular matrix (ECM)" is meant compounds, whether natural or synthetic, which support production of ILCs form IPCs. IPCs cultured on an ECM of the invention produce ILCS that express insulin. Preferably, an ILC of the invention expresses insulin mRNA at a level that is at least 1%, preferably, 1-5%, for example, 1, 2, 3, 4, or 5%, and more preferably, 5-25% (for

example, 5, 6, 7, 8, 9, 10, 15, 20 or 25%) or more (for example, 26, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99, 100% or more) of that of native islets. mRNA is detected, for example, by RT-PCR, as described herein. ILCs produced on an ECM of the invention also express at least one of the genes listed in Figure 8, at a level that is at least 2-fold greater (for example 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, 500, or 1000-fold or more) than the level of expression of IPCs. Gene expression is detected for example by RT-PCR, as described herein. ILCs produced on an ECM of the invention stain positive for C-peptide, glucagon and the ductal cell marker cytokeratin 19. Expression of C-peptide, glucagon and cytokeratin 19 is detected by immunostainting or radioimmunoassay, as described herein. ILCs do not express at least one of the genes in Table 1 at a level that is 2-fold or more greater (for example 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 200, 300, 400, 500, or 1000-fold or more) than the level of expression of a pancreatic stem cell or fresh pancreatic islet tissue. Preferably, at least 20%, more preferably, 20-50% and most preferably, 50% or more of a population of IPCs produce ILCs when cultured on an ECM of the invention.

In one embodiment, an ECM according to the invention comprises a biologically active polymerizable extract (for example, prepared as described in U.S. 4,829,000, incorporated herein by reference) containing in parts by weight about 60-85% laminin, 5-30% collagen IV, 1-10% nidogen, 1-10% heparan sulfate proteoglycan and 1-5% entactin. The term "biologically active" as used herein means capable of supporting normal growth and differentiation of various cell types when cultured including epithelial cells.

An "extracellular matrix" also functions as a substrate for cell attachment, cell migration and growth. In one embodiment, an ECM provides a physical support for organization of the cells. An ECM of the invention can provide tensile strength for the cells that are cultured on the ECM. An ECM of the invention can modulate cells shape, cell migration, cell differentiation and cell growth. An ECM of the invention may regulate cellular differentiation, and metabolic function, for example by modulation of cell growth by binding growth factors.

According to the invention, an "extracellular matrix" also functions to facilitate terminal differentiation of IPCs, as evidenced by the expression of markers characteristic of beta cells and enhanced insulin expression, by promoting the removal of inhibitory influences (i.e. cell-cell interaction with mesenchymal/ fibroblast like cells and exposure to growth factors secreted by these cells). An "extracellular matrix" of the invention further facilitates the 3D organization of

the ILCs and subsequent deposition of endogenous ECM by islet progenitor cells as well as cell-cell interaction between islet progenitor cells and their derivatives within a given islet-like cluster in isolation from inhibitory factors and/ or cells. Matrigel, or ECMs defined herein, also inhibit adherence and spreading of the cell clusters to plastic dishes as well as fusion of the clusters to each other.

An ECM useful according to the invention can be any one of laminin, fibronectin, concanavalin A, collagen (for example collagen IV), heparan sulfate proteoglycans, nidogen, entactin or any combination thereof including those components described in U.S. 4,829,000 and U.S. 6, 326,201.. Additional extracellular matrix components useful according to the invention include, without limitation, fibrin, thrombin, vitronectin, elastin, glycosaminoglycans, proteoglycans, gelatins, gel-forming peptidomimetcis of the parent compounds and combinations of some or all of these components (e.g., Matrigel™., Collaborative Research, Catalog No. 40234, Kleinman et al., 1986, Biochemistry, 25:312-318).

ECM is prepared according to methods known in the art. For example, Matrigel is thawed overnight at 4C and plated directly undiluted onto hydrophobic plates and incubated at 37 degrees celsius at least 4- 5 hours for gelling. . In other embodiments one or more ECM components described herein at the appropriate concentration are mixed together in serum free media and plated onto hydrophobic plates for gelling. An IPC suspension is then seeded onto the Matrigel or ECM component(s).

In another embodiment hydrophobic plates are coated with one or more ECM components, washed with serum free media and then seeded with an IPC suspension

An ECM according to the invention also includes plastic, for example, hydrophobic plastic.

As used herein, hydrophobic plates are non-tissue culture plates, i.e., plastic Petri dishes that are not treated for tissue culture.

An ECM according to the invention also includes a support matrix. In one embodiment the support matrix comprises a three-dimensional polymer scaffold, for example, as described in Levenberg et al., 2003, Proc. Natl. Acad. Sci. USA 100:12741-12746.

As used herein, a "scaffold" refers to porous biodegradeable polymers that facilitate the growth, differentiation and three dimensional organization of ILCs into islet like endocrine tissue with enhanced insulin gene expression as described herein. In a preferred embodiment, IPCs are seeded onto porous biodegradeable polymers in hydrophobic plates and having the dimensions of the plate. In another embodiment, the scaffold 'sponges', described below, are cut into rectangular pieces for example with dimensions of approx 5 X 4 X 1 mm3. In another embodiment, IPCs are seeded onto porous biodegradeable polymers in the presence of Matrigel or other purified ECM component as described in U.S. 4, 829,000.

Support matrices in which the progenitor cells can be incorporated or embedded include matrices which are recipient-compatible and which degrade into products which are not harmful to the recipient. Natural and/or synthetic biodegradable matrices are examples of such matrices. Natural biodegradable matrices include plasma clots, e.g., derived from a mammal, and collagen matrices. Synthetic biodegradable matrices include synthetic polymers such as polyanhydrides, polyorthoesters, and polylactic acid. Other examples of synthetic polymers and methods of incorporating or embedding cells into these matrices are known in the art. See e.g., U.S. Pat. No. 4,298,002 and U.S. Pat. No. 5,308,701. These matrices provide support and protection for the fragile progenitor cells in vivo and are, therefore, the preferred form in which the progenitor cells are introduced into the recipient subjects.

In one embodiment of the invention, porous biodegradeable polymer scaffolds are used either alone, or in conjunction with Matrigel (Levenberg et al., 2003, Proc. Natl. Acad. Sci. USA 100:12741-12746). In one embodiment, a scaffold consists of a 50/50 blend of poly(lactic- co-glycolic acid) (PLGA) (Boehringer Ingelheim Resomer 503H, Ingelheim, Germany, Mn ~ 25,000) and poly(L-lactic acid) (PLLA) (Polysciences, Warrington, PA, Mn~ 300,000). Sponges are then fabricated by a salt- leaching process as described by Levenberg et al (2002) Proc. Natl. Acad. Sci. 99, 4391-4396). In another embodiment, scaffolds are soaked in 50 μg/ ml fibronectin (Sigma) for 1 h and washed in PBS before direct cell seeding.

In another embodiment, a scaffold, e.g. PLGA, is selected to degrade quickly in order to facilitate cellular ingrowth.

In another embodiment, a scaffold, e.g. PLLA, is selected to provide mechanical stiffness and support for 3D strcuctures.

In another embodiment a scaffold with a pore size of 50-1000 nM, and preferably, 200-500mM is chosen to facilitate the seeding and ingrowth of cells.

In another embodiment, polymer scaffolds facilitate the differentiation and organization of blood vessels.

In another embodiment, a scaffold supported structure of the invention contracts by only 0.2 % under a cellular compressive stress of 110Pa using the methods described in Levenberg et al., 2003, Proc. Natl. Acad. Sci. USA 100:12741-12746.

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. A "progenitor cell" can also arise by de-differentiation of a more differentiated cell type to generate a cell having renewed capacity to proliferate.

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 culture conditions which support cell viability or propagation. "Culturing" 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 mitogens, differentiation factors, growth factors or other signaling factors that may or may not influence the differentiation state of the cells being cultured.

As used herein, "suspension" refers to a type of culture comprising at least one or more non-adherent cells, wherein the one or more cells are suspended in liquid medium. "Single-cell suspension" refers to a suspension in which the majority of cells are present as single cells, as opposed to aggregates of cells. A single cell suspension is preferably at least 70% single cells, more preferably at least 70%, 80%, 85%, 90%, 95% or, most preferably, 100% single cells.

As used herein, "increases", "up regulated" or "up regulation" refers to differential expression of marker gene expression. For example, marker gene mRNA in a first sample is expressed in greater amounts as compared with a second sample and includes increased differential expression of 2 fold, 2.5 fold, 3 fold, 5 fold, 10 fold, 20 fold, 50 fold or more.

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. Differentiation factors useful according to the invention include but are not limited to βNGF and EGF.

As used herein, EGF refers to epithelial growth factor. EGF useful according to the invention can be from any species, including murine, bovine, ovine, porcine, equine, avian, and preferably human, in native sequence or in genetically engineered variant form, and from any source, whether natural, synthetic, or recombinantly produced. Epidermal Growth Factor (EGF) and it analogs represent a family of polypeptides having a variety of biological activities. Human EGF itself is a 53 amino acid polypeptide. Its analogs vary in the number of amino acids in the polypeptide chain. A variety of these have been described in the literature. For example, see U.S. Pat. No. 3,917,824 issued Nov. 4, 1975. Preferably, EGF is recombinantly produced (for example human recombinant EGF available from Sigma). In a preferred method, the EGF is cloned and its DNA expressed, e.g., in mammalian cells, in bacterial cells. EGF and equivalents and methods of producing them are described in US 4,528,186; US 4,717,717; US 4,621,052; US4,743,679; US20030171269, US20030199103.

As used herein, βNGF refers to beta nerve growth factor from any species, including murine, bovine, ovine, porcine, equine, avian, and preferably human, in native sequence or in genetically engineered variant form, and from any source, whether natural, synthetic, or recombinantly produced. Preferably, NGF is recombinantly produced. In a preferred method, the beta NGF is cloned and its DNA expressed, e.g., in mammalian cells, in bacterial cells.

The term "beta-NGF" refers to the pure, active, mature beta subunit of 7S NGF, and its equivalents. NGF-beta is a dimer of two identical 118 amino acid (aa) chains (MW 26KD) and is apparently solely responsible for the observed biological activity of NGF as described in US 5,683,894. The isolation of the beta.-subunit of hNGF and its expression as a heterologous protein in E. coli is described in European Patent Application 0,121,338. Preferred for human use is human native-sequence, mature beta NGF. Methods for the production of human beta NGF are described in US 5,272,063. In one embodiment, βNGF is human recombinant bNGF (Sigma).

According to the invention, beta nerve growth factor (β-NGF) and epithelial growth factor (EGF) are used at a ratio of 1/10, 1/5, ¼, ½, 1/1, 2/1, 4/1..etc Alternatively, EGF and βNGF are used at a ratio of 1/10, 1/5, ¼, ½, 1/1, 2/1, 4/1..etc

In a preferred embodiment of the invention, IPCs are cultured in 100ng/ml beta nerve growth factor (β-NGF), and 25ng/ml epithelial growth factor (EGF).

A "signaling factor" as used herein is an agent secreted by a cell which has an effect on the same or a different cell. 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.

"Proliferation" indicates an increase in cell number.

A stem/progenitor cell is "expanded" when it is propagated in culture and gives rise by cell division to other stem cells and/or progenitor cells. As used herein, "expanded" refers to the proliferation of stem/progenitor cells under culture conditions that maintain the differentiation state of the stem/progenitor cell. Expansion of stem/ progenitor 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 mitogens, growth factors or other signaling factors that maintain the differentiation state of the cell.

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. 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.

As used herein, "treating" refers to the transfer of pancreatic stem/ progenitor cells, islet progenitor cells, islet-like clusters or pancreatic endocrine cells produced by the *ex vivo* differentiation of pancreatic stem/ progenitor cells into a patient for the treatment of diabetes.

As used herein, cells are "transplanted" into a mammal when they are transferred from a culture vessel into a patient. Transplantation, as used herein, can include the steps of isolating a progenitor cell according to the invention, expansion in vitro, differentiation *ex vivo* into islet endocrine cells and transfer into a mammal or a patient. Transplantation can involve transferring

cells 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 cells 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.

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.

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.

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

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

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

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, "Nestin" refers to an intermediate filament protein having a sequence disclosed in Genbank Access No. X65964.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a 3-step protocol (Expansion, Differentiation, Maturation) to induce the differentiation of endocrine cells from expanded human islet progenitor cells *in vitro*.

Figure 2 shows the relative expression of insulin mRNA in different islet preparations. qPCR: quantitative RT-PCR.

Figure 3 shows the global gene expression of fresh human pancreatic islets (Islets), expanded IPCs (Step 1) and differentiated ILCs/ IECs (Step 3). All samples were from islet prep A of Figure 2. Gene cluster analysis of the microarray data using self organizing maps demonstrated that a number of islet-specific genes were upregulated after IPC differentiation.

Figure 4 shows the gene expression profiles of the expanded IPCs from the successful prep A and prep B (see Figure 2), which gave no differentiation at all. Cluster analysis using self organizing maps revealed a set of 124 genes that is exclusively expressed at high levels during IPC expansion (Step 1) of prep A. These genes are absent or expressed at low levels during the expansion of prep B as well as in native pancreatic islets and after induction of differentiation from IPCs *in vitro*.

Figure 5 depicts the two major distinct cell types that are present in early expansion cultures (IPCs): spindle-shaped chymal/fibroblast-like cells and cuboidal epithelial cells growing in patches.

Figure 6 shows that the cuboidal epithelial cells are positive for cytokeratin 19, E-cadherin as well as (mainly cytoplasmatic) PDX1 by immunostaining. A small subpopulation of the epithelial cells also expresses nestin.

Figure 7 shows the significant increase in insulin mRNA expression (close to 40% of that of fresh native islets cultures) obtained from IPC cultures enriched with epithelial cells (~80%).

Figure 8 shows that differentiation of IPC cultures enriched with epithelial cells results in the increased expression of a number of genes that are also expressed in pancreatic β-cells.

Figure 9 shows that differentiation of IPC cultures enriched with epithelial cells results in secretion of C-peptide (2-5% the amount secreted by fresh human islets).

Figure 10 shows that IPC cultures enriched with epithelial cells stain positive for C-peptide, Glucagon and the ductal cell marker cytokeratin 19.

Figure 11 shows rtPCR analyses for the expression of beta cell genes from cultures of islet-depleted, duct-enriched tissue put through the same regimen as the islet-rich preparations. The number of PCR cycles was titrated to a minimum to stay in the linear range of amplification. Compared to the panel in Fugure 7, 3-5 PCR cycles more were needed to amplify the target cDNA from the duct samples. Insulin expression for these samples, as measured by quantitative rtPCR, is given at the bottom. A nested PCR was done to amplify ngn3 mRNA, which was expressed only at a low level.

Figure 12 shows the results of C-peptide secretion assays. Static incubations of step 3 clusters derived from two different islet (**A**, **B**) and one duct preparation ©. Panel **D** shows C-peptide

secretion from freshly isolated human islets is shown for comparison. Measurements were normalized for DNA content of each sample. 3 samples per condition were measured for **A**, **B** and **D** (* $p<0.05$; ** $p<0.001$ in comparison to baseline condition (2.8mM Glucose); one-way ANOVA). Only one sample per condition was measured for panel **C**.

Figure 13 shows immunocytochemical analyses of expanded and differentiated cultures: **A-F.** Immunofluorescent staining of expansion cultures (step 1). Two major cell populations were identified: E-cadherin/cytokeratin 19 (CK19) positive epithelial cells growing in patches (**A, D**) and nestin/vimentin-positive spindle-shaped cells growing separate from each other (**A, B**). Many of the spindle-shaped cells also co-expressed smooth muscle actin (**Figure 13 C**). Occasional cells with epithelial characteristics (E-cadherin and CK19-positive) also stained positive for nestin (arrows in **E, F**). **G-L.** Immunofluorescent and H&E staining of step 3 cell clusters. **G** and **H** show adjacent sections through a cluster from a culture with high insulin expression (34.4%). **H** is a negative control stained in parallel . **I** to **L** show clusters with lower insulin expression (5.5%). All four major islet hormones (C-peptide/insulin, glucagon, somatostatin, pancreatic polypeptide/PP) are expressed in distinct cells in these clusters (**K, L**), but many vimentin-positive, hormone-negative cells are also found (**I**). **J** shows a hematoxylin/eosin stain of a section adjacent to **K**.

DETAILED DESCRIPTION

The invention describes a new protocol to induce the *in vitro* differentiation of expanded human pancreatic progenitor cells into islet endocrine cells with improved insulin gene expression.

In one embodiment, the invention provides differentiated pancreatic progenitor 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 differentiated pancreatic progenitor cells of the invention can also be used to carry out gene therapy of endocrine pancreas in isograft, allograft or xenograft transplantations. Further, the differentiated pancreatic progenitor 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 differentiated pancreatic progenitor cells, described herein, can be used in various diagnostic, pathological, or investigative procedures to identify, localize, and quantitate stem/ progenitor 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, J. Neurosci. 5:3310; Lendahl et al., 1990, Cell 60:585). 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., supra). Nestin was initially found exclusively in stem cells of the embryonic developing brain and skeletal muscle (Lendahl et al., supra). Later studies identified nestin-positive neural stem cells in the subependymal layer of the adult mammalian forebrain (Morshead et al., 1994, Neuron 13:1071). Nestin-positive stem cells have been shown to be pluripotential even when isolated from adult mouse 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, Dev. Bio. 175:1). 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, Cell 96:25) and restore hematopoietic cells of the bone marrow after infusion into irradiated mice (Bjornson et al., 1999, Science 283:534). Nestin is increasingly thought of as a characteristic marker of cells having the capacity to differentiate along multiple lineages. Nestin-containing intermediate filaments are closely involved with establishing and maintaining cell shape, which is critical to maintenance or modification of the differentiated state of the cell. The nestin protein has also been shown to mediate the disassembly of vimentin intermediate filaments during mitosis, which may influence intracellular trafficking of cellular components during that process (Chou et al., 2003, Mol. Biol. Cell. 14: 1468-1478). Consistent with its expression in immature cells, nestin has been proposed to play a role in the uneven partitioning of key cellular components during cell divisions of progenitor cells (Chou et al., supra). That is, nestin may play a role in the uneven distribution of cellular factors that permits a cell division event to result in a daughter cell with a more differentiated phenotype and a parent cell that retains its undifferentiated phenotype. Nestin and vimentin expression are extinguished when cells become terminally differentiated.

Characterization of Pancreatic 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 ¾ (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 anti rat 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' (SEQ ID NO: 1);

Reverse, 5'aggcaaggggaagagaaggatgt3' (SEQ ID NO: 2);

Hybridization, 5'aagctgaagccgaatttccttgggataccagagga3' (SEQ ID NO: 3).

Rat keratin 19: Forward, 5'acagccagtacttcaagacc3' (SEQ ID NO: 4);

Reverse, 5'ctgtgtcagcacgcacgtta3' (SEQ ID NO: 5);

Hybridization, 5'tggattccacaccaggcattgaccatgcca3' (SEQ ID NO: 6).

Rat NCAM: Forward, 5'cagcgttggagagtccaaat3' (SEQ ID NO: 7);

Reverse, 5'ttaaactcctgtggggttgg3' (SEQ ID NO: 8);

Hybridization, 5'aaaccagcagcggatctcagtggtgtggaacgatgat3' (SEQ ID NO: 9).

Rat IDX-1 Forward, 5'atcactggagcagggaagt3' (SEQ ID NO: 10)

Reverse, 5'gctactacgtttcttatct3' (SEQ ID NO: 11)

Hybridization, 5'gcgtggaaaagccagtggg3' (SEQ ID NO: 12)

Human nestin: Forward, 5'agaggggaattcctggag3' (SEQ ID NO: 13);

Reverse, 5'ctgaggaccaggactctcta3' (SEQ ID NO: 14);

Hybridization, 5'tatgaacgggctggagcagtctgaggaaagt3' (SEQ ID NO: 15).

Human keratin: Forward, 5'cttttcgcgcgcccagcatt3' (SEQ ID NO: 16);

Reverse, 5'gatcttcctgtccctcgagc3' (SEQ ID NO: 17);

Hybridization, 5'aaccatgaggaggaaatcagtacgctgagg3' (SEQ ID NO: 18).

Human glucagon: Forward, 5'atctggactccaggcgtgcc3' (SEQ ID NO: 19);

Reverse, 5'agcaatgaattccttggcag3' (SEQ ID NO: 20);

Hybridization, 5'cacgatgaatttgagagacatgctgaaggg3' (SEQ ID NO: 21);

Human E-Cadherin Forward, 5' agaacagcacgtacacagcc 3' (SEQ ID NO: 22)

Reverse, 5'cctccgaagaaacagcaaga 3' (SEQ ID NO: 23)

Hybridization, 5' tctcccttcacagcagaactaacacacggg 3' (SEQ ID NO: 24)

Human transthyretin Forward, 5' gcagtcctgccatcaatgtg 3' (SEQ ID NO: 25)

Reverse, 5' gttggctgtgaataccacct 3' (SEQ ID NO: 26)

Hybridization, 5' ctggagagctgcatgggctcacaactgagg 3' (SEQ ID NO: 27)

Human Pancreatic Amylase Forward, 5'gactttccagcagtcccata 3' (SEQ ID NO: 28)

Reverse, 5' gtttacttcctgcagggaac 3' (SEQ ID NO: 29)

Hybridization, 5' ttgcactggagaaggattacgtggcgttcta 3' (SEQ ID NO: 30)

Human procarboxypeptidase

Forward, 5' tgaaggcgagaaggtgttcc 3' (SEQ ID NO: 31)

Reverse, 5' ttcgagatacaggcagatat 3' (SEQ ID NO: 32)

Hybridization, 5' agttagacttttatgtcctgcctgtgctca 3' (SEQ ID NO: 33)

Human Synaptophysin Forward, 5' cttcaggctgcaccaagtgt 3' (SEQ ID NO: 34)

Reverse, 5' gttgaccatagtcaggctgg 3' (SEQ ID NO: 35)

Hybridization, 5' gtcagatgtgaagatggccacagacccaga 3' (SEQ ID NO: 36)

Human Hepatocyte Growth Factor (HGF)

Forward, 5' gcatcaaatgtcagccctgg 3' (SEQ ID NO: 37)

Reverse, 5' caacgctgacatggaattcc 3' (SEQ ID NO: 38)

Hybridization, 5' tcgaggtctcatggatcatacagaatcagg 3' (SEQ ID NO: 39)

Human cMET (HGF-receptor) Forward, 5' caatgtgagatgtctccagc 3' (SEQ ID NO: 40)

Reverse, 5' ccttgtagattgcaggcaga 3' (SEQ ID NO: 41)

Hybridization, 5' ggactcccatccagtgtctccagaagtgat 3' (SEQ ID NO: 42)

Human XBP-1 Forward, 5'gagtagcagctcagactgcc 3' (SEQ ID NO: 43)

Reverse, 5' gtagacctctgggagctcct 3' (SEQ ID NO: 44)

Hybridization, 5' cgcagcactcagactacgtgcacctctgca 3' (SEQ ID NO: 45)

Human Glut-2 Forward, 5' gcagctgctcaactaatcac 3' (SEQ ID NO: 46)

Reverse, 5' tcagcagcacaagtcccact 3' (SEQ ID NO: 47)

Hybridization, 5' acgggcattcttattagtcagattattggt 3' (SEQ ID NO: 48)

Human Insulin Forward, 5' aggcttcttctacaca3' (SEQ ID NO: 49)

Reverse, 5' caggctgcctgcacca 3' (SEQ ID NO: 50)

Hybridization, 5' aggcagaggacctgca 3' (SEQ ID NO: 51)

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.

The above-noted approaches to the identification of pancreatic stem cells is also useful for the identification of cells derived from the pancreas that have stem cell character yet arise from mature islet cells.

Isolated Pancreatic Progenitor/ Stem Cells from Pancreatic Islets and Their Use

Progenitor/ 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 (see Example 1). The progenitor/ 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 β cells to replace β 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, the use of isografted progenitor/ 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 progenitor/ stem cells over whole islets is that transplanted progenitor/ 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.

The invention further contemplates the use of partially differentiated stem cells *ex vivo*, for example, to form islet progenitor cells (IPCs), islet-like clusters (ILCs) and pancreatic islet endocrine cells (IECs), 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 islet-like clusters (ILCs) and pancreatic islet endocrine cells is preferred, another embodiment contemplates the use of an allograft of stem cells, progenitor cells, or islets-like clusters obtained from another individual or from a mammal of another species.

In yet another embodiment of the invention, the stem cells, progenitor cells, or islet-like clusters (ILCs) or pancreatic islet endocrine cells are immunologically blind or immunoprivileged. In one embodiment of this aspect of the invention, immunoprivileged stem cells, progenitor cells, or islet-like clusters (ILCs) and pancreatic islet endocrine 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, progenitor cells, or islet-like clusters (ILCs) or pancreatic islet endocrine 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, progenitor cells, or islet-like clusters (ILCs) or pancreatic islet endocrine cells do not express class I MHC antigens and/or class II MHC antigens. These 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, progenitor cells, or islet-like clusters (ILCs) or pancreatic islet endocrine 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.

Differentiation of Stem /Progenitor Cells to Islet-like clusters

In one aspect, the invention provides a 3-step protocol to induce the differentiation of endocrine cells from expanded human islet progenitor cells (IPCs) *in vitro* that results in an increase in the expression of insulin by ILCs derived from IPCs, compared to native human islets (see Example 2). Using this protocol the efficiency of differentiation of insulin-producing cells increased to close to 10% in select islet preparations and the resulting ILCs were shown to express high levels of β-cell specific genes and secrete significant quantities of C-peptide / insulin (see Example 3). However, differentiation is highly variable and some islet preparations gave no differentiation at all (for example, prep B in Figure 2). To remedy this variation in the

efficiency of differentiation, expansion cultures from human islets were analyzed and shown to contain different proliferating cell types, mainly one type of mesenchymal-like and one type of epithelial cells. Evidence presented herein suggests the epithelial cells may function as IPCs *in vitro* and that favoring the expansion of this cell type improves the differentiation success and results in a more efficient expression of insulin. A subpopulation of epithelial cells is nestin-positive, a previously identified marker of the pancreatic stem cell phenotype. The observations of the present invention suggest these nestin-positive epithelial cells could represent a more immature cell type. The mesenchymal/fibroblast-like cells may also be stem-like cells that express stem cell markers such as nestin, SSEA, Oct ¾, and may arise in *in vitro* conditions by epithelial to mesenchymal transition (EMT).

In one aspect, mature pancreatic islet cells, e.g., beta cells and/or ductal cells from adult donors are the source of pancreatic cells having stem-cell-like character. Such stem-cell like cells express only low levels of insulin, if any, and rapidly proliferate in culture. These cells expand from mature islet cells cultured in serum-containing medium and express markers, such as nestin, that are more characteristic of stem cells or islet progenitor cells than mature islet cells. Stem-like cells of this origin are included in the class of cells herein referred to as "islet progenitor cells." These stem-like cells that arise by de-differentiation of adult mature islet cells, e.g., from mature beta cells, have the capacity to re-differentiate to form insulin producing cells in islet-like clusters when cultured in serum-free medium.

The de-differentiation of pancreatic beta cells during in vitro expansion is well known, and is a major problem on the way to cell expansion for transplantation. One approach that has been taken to limit this problem was to maintain islet architecture by expansion in a three dimensional gel matrix. In contrast to this approach, the method provided herein in this aspect embraces the de-differentiation approach as a way to expand the number of cells ex vivo. In this aspect, islets are disrupted to form single cell suspensions, and are then plated in serum-containing medium. Adherent cells proliferate efficiently under these conditions; this expansion is aided in the presence of EGF (e.g., at 25 ng/ml). Additional factors that may enhance the transition of differentiated epithelial cells to the de-differentiated progenitor cell phenotype include β–NGF (e.g., at 100 ng/ml) and/or bFGF (e.g., at 20 ng/ml).

A population of cells that emerge from these expansion cultures comprises nestin-positive cells, which comports with their less differentiated, stem-cell-like character. Other sub-populations express cytokeratin 19, and a smaller sub-population expresses both nestin and

cytokeratin 19. Importantly, cells in the expanded cell population retain the ability to be re-differentiated when placed under conditions that permit such re-differentiation. For example, when the expanded cells are transferred to serum-free medium, they form islet-like clusters that express insulin at levels that can be as high as 30-40% of the level of freshly isolated islet cells. The re-differentiation process can be aided by culture on an extracellular matrix material, e.g., Matrigel™ or other matrix materials described herein or known in the art. The process can be further aided by culture in the presence of a high glucose concentration (e.g., at least 17.5 mM or more) in the serum-free medium. Even higher insulin expression can be achieved by subsequent removal of the cell clusters from the matrix material and their incubation in suspension culture in serum-free, high glucose medium.

As noted above, the early expansion cultures comprise two primary cell types: a cuboidal, epithelial phenotype; and a fibroblastoid or "spindle shaped" phenotype. Without wishing to be bound by any single theory regarding mechanism, it is contemplated that epithelial beta cells from the single cell dispersion of islets de-differentiate, undergoing epithelial to mesenchymal transition, to a fibroblastoid phenotype, perhaps under the influence of continued exposure to EGF in the medium. It is further contemplated that the population of fibroblastoid cells that trace their heritage back to epithelial cells in the original culture, particularly back to islet beta cells, is more likely than fibroblastoid cells of other origins in the culture to re-differentiate back to the epithelial phenotype when conditions favor that transition. If correct, this would tend to explain the data showing that cultures rich in epithelial cells from the start tend to produce more insulin-producing cells after re-differentiation. Under this theory, fibroblastoid cells in the expansion cultures that trace their heritage back, instead, to non-beta cells will be less likely to differentiate to insulin-producing cells.

The present invention shows that culturing/growing human IPCs on and within a synthetic three-dimensional scaffold configured in the form of 200-500 nm spheres with the presence of appropriate surface matrix treatment of the polymer spheres, and subsequent treatment with selected, sequential use of soluble growth factor/morphogens in the cell culture media, enhances the efficiency of the differentiation of IPCs into glucose, nutrient, and increase hormone-responsive ILCs. These observations further suggest differentiation of ILCs in three dimensional suspension cultures is favored because potentially inhibitory influences of the mesenchymal/fibroblast-like cells within the two dimensional in vitro culture plate are effectively removed. This is consistent with previous reports indicating the importance of a

three-dimensional configuration to achieve more terminal differentiation of stem cells (Levenberg et al. PNAS 100: 12741, Oct. 28, 2003).

Cluster analysis of gene expression within IPCs (see Example 3) revealed a set of 124 genes that is exclusively expressed at high levels during IPC expansion. These genes are absent or expressed at low levels in non differentiating IPCs as well as in native pancreatic islets and after induction of differentiation from IPCs *in vitro* (Figure 4). Based on this pattern of expression it appears that these genes are activated specifically in functional IPCs and thus could be used as markers for the isolation of such cells in the future (Table 1).

Methods of Transplantation

The invention provides for methods of transplantation into 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.

In one embodiment, cells derived from one mammal, for example a mouse, are transplanted into a different mammal, for example a human.

Transplantation, according to the invention can include the steps of isolating a progenitor/ stem cell according to the invention and transferring the progenitor/ stem cell into a mammal or a patient. Transplantation according to the invention can involve transferring a stem cell, Islet progenitor cell, Islet-like cluster or Islet endocrine 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 progenitor/ 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 progenitor/ stem cell according to the invention, and culturing and transferring the progenitor/ stem cell into a mammal or a patient. In another embodiment, transplantation, as used herein, can include the steps of isolating a progenitor/ stem cell according to the invention, expanding and differentiating the progenitor/ stem cell according to the protocol described herein, and

transferring differentiated pancreatic islet endocrine cells into a mammal or a patient. Transplantation, as used herein, can include the steps of isolating a islet progenitor cell according to the invention, expanding the islet progenitor cell, differentiating the islet progenitor cell into islet-like clusters and islet endocrine cells and transferring the islet endocrine cells into a mammal or a patient.

In one embodiment, transplant recipients can be treated with NGF, EGF, GLP-1, other growth factors or combinations thereof, prior to, during and post-transplantation or any combination thereof. In another embodiment, cells or cell aggregates i.e. islet-like clusters or islet endocrine cells are pretreated with growth factors e.g. NGF and/or EGF prior to transplantation.

In one embodiment, a patient is treated prior to transplantation, during transplantation, post-transplantation, or any combination thereof, with an agent including but not limited to EGF, NGF, 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 embodiment, the cells to be transplanted are cultured, prior to transplantation, for example, 1, 6, 12, 24 hours or 2, 3, 4, 5 or more days in the presence of an agent including but not limited to EGF, NGF, 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.

Preferably, transplantation according to the invention, is under the kidney capsule, into the spleen which allows for access to the liver or in the tail vein which is systemic.

Methods of Treating Insulin-Dependent Diabetes

Islet endocrine cells resulting from ex vivo differentiation of pancreatic islet progenitor 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 progenitor/ stem cells, progenitor cells, or islet-like clusters or islet endocrine cells. Progenitor/ 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 as described herein. Progenitor/ stem cells can be proliferated and isolated by the methods described herein within 2-3 weeks. Islet progenitor cells can be transplanted back into the

patient directly following isolation or after a period of differentiation as described herein. 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, progenitor/ 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 Stem/Progenitor Cell Transfection

A variety of methods are available for gene transfer into pancreatic progenitor/ stem cells. Calcium phosphate precipitated DNA has been used but provides a low efficiency of transformation, especially for non-adherent 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, Int. J. Cell Cloning 8:80). 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 progenitor/ stem cells would improve the feasibility of the microinjection approach to targeted gene modification of pancreatic progenitor/ 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 progenitor/ 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.

In these embodiments, the treated progenitor/ stem cells can be expanded and differentiated ex vivo prior to transplantation into a recipient animal according to the differentiation protocol described herein.

Retroviral Transformation Of Pancreatic progenitor/ 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 are 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 progenitor/ stem cell transformation are co culture and supernatant infection. Supernatant infection involves repeated exposure of progenitor/ stem cells to the viral supernatant. Co culture involves the commingling of progenitor/ 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 progenitor/ stem cell transformation. After co culture, infected progenitor/ 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 progenitor/ stem cells are harvested may be treated with 5-fluorouracil (5-FU) prior to extraction. 5-FU treated

progenitor/ stem cells are more susceptible to retroviral infection than untreated cells. 5-FU progenitor/ stem cells dramatically reduce the number of clonogenic progenitors, however.

In another embodiment, harvested progenitor/ stem cells may be exposed to various growth factors, such as those employed to promote proliferation or differentiation of pancreatic progenitor/ 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 increases transformation efficiency from 30 to 80%.

In these embodiments, the treated progenitor/ stem cells can be expanded and differentiated ex vivo prior to transplantation into a recipient animal according to the differentiation protocol described herein.

Typical Retroviral Transformation Protocol

The ex vivo transduction of mammalian pancreatic progenitor/ 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 into the recipient.

Specifically, bone marrow progenitor/ 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 are 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 is 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 progenitor/ stem cells are 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 progenitor/ stem cells. The progenitor/ stem cells are 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). In another embodiment the transduced progenitor/ stem cells are expanded and differentiated ex vivo prior to transplantation into a recipient animal according to the differentiation protocol described herein.

Successful progenitor/ stem cell transduction and engraftment of progenitor/ 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.

Factors influencing differentiation state of pancreatic islet-derived cells

Nestin-positive islet-derived stem/progenitor cells (NIPs) are a population of monolayer cells that grow out and away from islets when isolated islets are cultured in vitro in the presence of EGF and bFGF. NIPS are also referred to as islet-derived progenitor cells or IPCs. The NIPs have a fibroblastoid morphology and uniformly express the intermediate filament proteins nestin and vimentin. NIP cultures grown to near confluence and treated with appropriate morphogens express markers of fat, liver, cartilage, neurons and glia (Zulewski et al., 2001, Diabetes 50: 521-533). Transplantation of human NIPs under the kidney capsule of immunocompetent C57bl/6 mice results in progressive growth of the graft, without oncogenicity, and the differentiation into multiple tissue types, including smooth muscle, adipose, liver, and endocrine pancreas expressing Pdx-1, insulin, and glucagon (Abraham, 2004, Am. J. Physiol. Endocrinol. Metab. 164: 817-830). NIPS thus have the characteristics of multipotent stem-like cells that can differentiate into islet like clusters that resemble, morphologically and functionally, native islets. NIPS are thought to arise from epithelial to mesenchymal transition, or EMT.

The inventors have discovered that the zinc-finger transcriptional repressor family members Snail 1, Snail 2, Slug, Zeb 1 (also referred to as delta EF1) and Zeb 2 (also known as Sip1) are expressed in mouse NIPs. These factors are markers of EMT. Snails 1 and 2 are detected by rtPCR in NIPs and postnatal day 1 mouse pancreas but only Snail 1 faintly in adult pancreas. Zeb1 is expressed in postnatal day 1 pancreas, but neither Zeb 1 nor Zeb 2 is expressed in adult pancreas. Removal of EGF and bFGF from the NIP culture medium for three days results in a decrease in Slug in the nuclei of NIPs. Snail and Slug function as repressors of the insulin gene, as demonstrated by co-transfection experiments using an insulin-1-luciferase reporter and a snail expression vector in Ins-1 cells (data not shown). Also in Ins-1 cells, the inventors have found that the inhibition of Slug/Snail expression with PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine), a Src kinase inhibitor, stimulated the

differentiation of undifferentiated Ins-1 cells into insulin-producing cells. Additional Src kinase inhibitors are expected to have similar effects against Snail family members. There are many other Src kinase inhibitors, which include, for example, 4-(4′-Phenoxyanilino)-6,7-dimethoxyquinazoline (Src kinase inhibitor I), Src kinase inhibitor II, SU6656, Genistein, Herbimycin A, and PP3 (each available from, e.g. Calbiochem).

Members of the Snail superfamily of transcriptional repressors are characterized by the presence of a C-terminal zinc-finger domain that binds E-box DNA sequences (an E-box has the core sequence CANNTG (SEQ ID NO: 98); examples include, e.g., CAGGTG (SEQ ID NO: 52) and CACCTG (SEQ ID NO: 53)) and by an N-terminal repressor domain termed the SNAG domain, each of which is necessary for repressor function. Thus, a Snail family member as the term is used herein comprises a C-terminal zinc finger domain that binds an E-box sequence, and a SNAG domain. The Snail family member will repress transcription from a promoter that is responsive to factor binding to an E-box motif. A listing of known Snail family members, including Accession Numbers is provided in Table 2.

The primary motifs in Snail superfamily proteins are arranged in the following order from amino to carboxy terminal: SNAG domain, Scratch domain, slug domain, zinc finger domain. (The Scratch and Slug domains are signature domains that permit a gene to be unambiguously ascribed to a subfamily within the Snail superfamily.) The zinc-finger domain of the Snail family comprises five to six zinc finger motifs. Consensus sequences for family proteins with five zinc fingers are reported by Nieto, M., 2002, Nature Reviews 3: 155-166. Briefly, the zinc finger consensus sequences reported there are as follows: I Amino-terminal - - C- - C – K – Y – T - - L - - H - - - H carboxy terminal (SEQ ID NO: 54); II Amino terminal - - C - - C – K – Y – S - - AL – MH - - TH carboxy terminal (SEQ ID NO: 55); III Amino terminal - - C - - CGK – FSRPWLLQGH – R – H carboxy terminal (SEQ ID NO: 56); IV Amino terminal F – C – HC - - AFADRSNLRAH – QTH carboxy terminal (SEQ ID NO: 57); and V Amino terminal - - C - - C - - - F - - - S – L – KH - - - - carboxy terminal (SEQ ID NO: 58). The Nieto reference also provides an alignment of Slug domain sequences, Scratch domain sequences and SNAG domain sequences from various species.

The SNAG domain of members of the Snail family has the sequence MPRSFLVKK (SEQ ID NO: 59) in all vertebrates, except in murine and *Homo sapiens* Snail which have the SNAG sequence MPRSFLVRK (SEQ ID NO: 60), and the murine Smuc, which has the SNAG sequence MPRSFLVKT (SEQ ID NO: 61). Sequences from other species that correspond

include those from sea urchin Snail (MPRSFLIKK (SEQ ID NO: 62)), *Patella vulgata* Snail 2 (MPRAFLIKK (SEQ ID NO: 63)) and *Drosophila melanogaster* Scratch (MPRCLIAKK (SEQ ID NO: 64)). A "SNAG domain" as the term is used herein will have one of these sequences or a conservative variant of the MPRSFVLKK (SEQ ID NO: 59) sequence that predominates in vertebrates. A conservative SNAG variant, in addition to comprising a conservative mutation, will have repressor activity against transcription from a promoter that responds to factors binding to an E-box motif. Further, a conservative variant will preferably vary at 4 amino acid positions or fewer, more preferably 3 positions or fewer, 2 positions or fewer, or only one position, relative to the MPRSFVLKK (SEQ ID NO: 59) sequence.

Another protein that represses transcription from E-box-containing regulatory sequences is ZEB, the vertebrate homolog of the Drosophila zinc finger/homeodomain protein Zfh-1, a member of the *zfh* family of *Drosophila* genes, which is expressed in muscle precursors and is critical for the proper development of muscle. ZEB has been identified as a negative regulator of muscle differentiation (Postigo & Dean, EMBO Journal Vol. 16 No. 13 pp. 3935-3943, 1997). As noted above, ZEB is also expressed in NIPs. ZEB (sequence information available at GenBank Accession Nos. NM 011546 and AL117340) has zinc finger domains on either end that flank a repressor domain that actually comprises two separate repressor domains that act upon different subsets of promoters (Postigo & Dean, 1999, Mol. Cell. Biol. 19: 7961-7971).

As used herein, a ZEB polypeptide is a homeodomain-containing polypeptide comprising, at a minimum, two sets of zinc finger motifs, one near the amino-terminus of the polypeptide, the other near the carboxy-terminus of the polypeptide, a homeodomain motif located between the two sets of zinc finger motifs, and at least one, and preferably at least two PLDLS (SEQ ID NO: 65) or PLNLS (SEQ ID NO: 66) motifs that bind the CtBP co-repressor protein (see Postigo et al., 1999, Proc. Natl. Acad. Sci. U.S.A. 96: 6683-6688). A ZEB polypeptide as used herein binds an E-box DNA motif and represses transcription from a promoter comprising that E-box motif. A ZEB polypeptide is preferably at least 70% identical to human ZEB polypeptide having the sequence at GenBank Accession No. U19969.

A ZEB polypeptide that acts upon E-box containing promoters can induce the de-differentiation of pancreatic islet cells, permitting them to proliferate. Activators of ZEB polypeptide activity or ZEB expression constructs can thus be used to induce the expansion of islet progenitor cells from adult islet tissue. Conversely, agents that inhibit ZEB expression (e.g.,

siRNAs active against ZEB polypeptide expression) or activity can also be used to induce the differentiation of expanded islet progenitor cells.

In one aspect, the expression of Snail family members or a ZEB polypeptide is exploited to generate islet progenitor cells from mature adult pancreatic islet cells. In this aspect, islet cells are placed in culture and treated to induce the expression of a Snail family member. Expression can be induced, for example, by introducing a construct that drives the expression of a Snail, Slug or Zeb polypeptide, e.g., by transfection or infection with a viral vector. Resulting proliferative cells will be nestin positive and have the capacity to re-differentiate upon inhibition or cessation of repressor expression or function.

In one embodiment, the transfection can be transient transfection. The temporary expression of the construct in this instance would permit cells to re-enter the cell cycle and proliferate to generate islet progenitor cells, yet also permit the down-regulation of the expression necessary to re-differentiate the resulting expanded cells. In another embodiment, the expression from the construct is conditional, e.g., induced by the addition of an agent or a change in culture conditions such as pH or temperature. Conditional expression systems are well known to those of skill in the art. Conditional expression permits control over the level and/or duration of construct expression that can be advantageous in permitting later re-differentiation of the cells.

In another aspect, a method is provided for inducing islet-derived progenitor cells expanded from islet tissue to differentiate to an insulin-producing phenotype by inhibiting the expression or activity of a member of the Snail, Slug or Zeb family of transcription factors. In this embodiment, adult pancreatic islet cells are expanded ex vivo as described herein, and the expanded cells are then contacted with an inhibitor of one or more members of this family, thereby de-repressing insulin expression and permitting expanded islet progenitor cells to differentiate to an insulin-producing phenotype. In addition to PP2 or another chemical compound which inhibits repressor expression or function, the repressors can also be targeted, for example, using RNAi technology.

RNAi or "RNA interference" is the phenomenon by which short, double-stranded RNA molecules, in which one strand corresponds to or is complementary to the transcript for the transcript one wishes to target, inhibit the expression of the protein encoded by the target gene. The exact mechanism of RNAi is not fully understood, but the technique is reliably able to reduce the expression of a target gene. Briefly, without wishing to be bound by any one mechanism or theory of action, RNAi is believed to involve the processing of longer dsRNA

molecules into 19-23 nucleotide (nt) dsRNAs (referred to as short interfering RNAs or "siRNAs") that serve as guides for enzymatic cleavage of complementary RNAs (Elbashir, S. M. et al. (2001) Genes Dev 15, 188-2000; Parrish, S. et al. (2000) Mol Cell 6, 1077- 87; Nykanen, A. et al. (2001) Cell 107, 309- 21; Elbashir, S. M. et al. (2001) Embo J 20, 6877-88; Hammond, S. M. et al. (2000) Nature 404, 293-6; Zamore, P. D. et al. (2000) Cell 101, 25-33; Bass, B. L. (2001) Nature 411, 428-9; and Yang, D. et al. (2000) Curr Biol 10, 1191-200). The generation of siRNAs has been attributed to an RNAse III enzyme called *Dicer* in Drosophila that is also implicated in the processing of small temporal RNAs in *C. elegans* and similar small RNAs in human cells. Synthetic siRNAs can trigger RNAi in *C. elegans*, Drosophila, and cultured mammalian cells.

There are several different approaches to the inhibition of target genes using RNAi. In one approach, siRNAs can be microinjected injected into the cell or organism. However, for the methods described herein it is preferred that the siRNAs be introduced by expression from a recombinant construct or by direct introduction of the siRNAs into cells by, e.g., liposome-mediated transfer.

Where the siRNA is to be generated by expression, there are several approaches. In one approach, the transgene construct encodes an RNA transcript which forms a hairpin loop, where the stem of the hairpin comprises the dsRNA corresponding to the target transcript. Following expression of the transcript, the dsRNA in the stem is processed to the 19-23 nt siRNAs which function to reduce protein expression of the target gene. This approach is described, for example, by Kennerdell and Carthew, 2000, Nature Biotechnology 18: 896-898.

Another approach is to introduce a construct in which a portion of the target gene sequence is flanked on each end by a different promoter oriented in opposite orientations to each other, such that the transcription products are complementary to each other and include the target gene segment. When this construct is introduced to a cell in which the promoters are active, transcripts are generated that hybridize to each other and permit processing to siRNAs. Other approaches, including approaches involving the use of conditional RNAi expression, are known to those of skill in the art.

While RNAi is reliable for the inhibition of protein expression for a given gene, the exact part of the target gene sequence one should express as a dsRNA is not always definite. Promoter and intron sequences are not likely to efficiently inhibit target gene expression when expressed as RNAi dsRNAs. Often, however, the region about the translation initiation codon provides an effective RNAi effect when expressed as a dsRNA. Given the target transcript sequence, one of

skill in the art can generate an effective RNAi targeting construct by making a series of constructs, each progressively moving down the transcript from near the initiation codon. In order to efficiently determine the target gene sequence to include in a construct for RNAi, the targeting sequence can be optimized using a cell culture system, e.g., cultured cells that express the Snail/Slug/Zeb family member being targeted. That is, a series of RNAi expression constructs targeting progressively different regions of the target gene can be transfected into a cell culture system, followed by monitoring for target gene protein expression (either directly, e.g., by immunoblotting, or indirectly, through use of a reporter system). The success of an RNAi approach can be gauged by the level to which expanded islet progenitor cells become insulin positive.

Because islet cells tend to continue to further differentiate upon transplantation into an individual, it is expected that cells in which re-differentiation is induced by RNAi will remain differentiated once they are re-introduced to a patient. That is, it is not expected that withdrawal of the RNAi that will accompany transplantation will result in de-differentiation of the transplanted cells. This can be readily confirmed using an animal model of diabetes as described herein.

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 islet progenitor or islet endocrine 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 pancreatic islet progenitor or islet endocrine 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 pancreatic islet progenitor or islet endocrine 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 an islet progenitor cell or islet-like clusters 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 1 mM-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.

EXAMPLE 1

Isolation of Human 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).

EXAMPLE 2

Ex vivo Differentiation of Pancreatic Progenitor Islets

A 3-step protocol was used to induce the differentiation of endocrine cells from expanded human islet progenitor cells (IPCs) *in vitro*. This protocol resulted in an increase in the expression of insulin from <1% to approximately 40% by ILCs derived from IPCs, as compared to protocols described in Abraham et al. supra, and Zulewski et al. supra. Cultures are initiated from single cell suspensions of human islet preps rather than from whole islets. At the end of the expansion phase cells are trypsinized off the dish and seeded at a defined density of 25,000 IPCs/square centimeter on an extracellular matrix (Matrigel™) in serum-free medium. After approximately 48 hours, the cells migrate to form clusters of about 100 to 1,000 cells (islet-like clusters, ILCs). After 5 days of culture, the cell clusters are removed from the matrix by hand-picking and cultured free-floating for another 5 days (Figure 1). Using this protocol the efficiency of differentiation of insulin-producing cells increased from less than 1% to close to 10% in select islet preparations as compared to the method of Example 1. However, differentiation is still highly variable and some islet preparations gave no differentiation at all (Figure 2).

Methods

Expansion protocol

Single cell suspensions of Human pancreatic islet preparations were made by digestion with 5mg/ml Trypsin in PBS at 37°C and passage through a glass pipette. 10,000 to 30,000 islet equivalents are trypsinized . at 37 degrees for approximately 10min in 5 mg/ml trypsin in PBS. . A glass pipette was used to separate the islets into a single cell suspension which was filtered through a 40 micron cell strainer. Living cells were counted by trypan blue exclusion and 2,000 live cells / square centimeter were seeded in expansion medium on regular tissue culture dishes. About 1 in 1,000 islet cells initiates an IPC colony. Media is changed after 24 hours and every 3 days thereafter. IPCs are expanded to ~ 80% confluency (~10 days) before they are transferred onto matrigel.

Viable cells were counted by Trypan Blue exclusion and seeded at 10,000 to 100,000 cells per well in CMRL 1066 medium with 10% fetal calf serum, 100ng/ml beta nerve growth factor (β-NGF), and 25ng/ml epithelial growth factor (EGF). Cells were expanded for 10-14 days and medium was changed every 2-3 days.

Differentiation protocol

Expanded cells were trypsinized, washed with serum-free medium and seeded on hydrophobic culture dishes coated with undiluted Matrigel™ (BD Biosciences). Seeding density is $2.5x10^4$ cells/cm^2. The differentiation medium was serum-free DMEM/F12 plus ITS+1 (Insulin, Transferrin, Selenium, lipids; from Sigma). After 5 days on Matrigel the gel layer was disrupted with a cell scraper. Cell clusters (approx 100-1000 cells) and matrigel pieces were transferred to a larger volume of medium and individual clusters were handpicked. These cell clusters (ILCs) were further cultured (100-500 clusters/ml) for five days in suspension in hydrophobic dishes and produced islet endocrine cells (IECs) that secreted insulin. The medium was also DMEM/F12 plus ITS+1.

EXAMPLE 3

Analysis of Islet Progenitor Cells

The starting material (human islet preparations) is heterogeneous and always contains a number of different epithelial and mesenchymal cell types suggesting that the differentiation capacity of a culture is determined by the ratio of IPCs to other cell types. To examine this question in more detail, early expansion cultures were examined microscopically. Two major distinct cell types were found to be present: spindle-shaped chymal/fibroblast-like cells and cuboidal epithelial cells growing in patches (Figure 5). The latter cell type is positive for cytokeratin 19, E-cadherin as well as (mainly cytoplasmic) PDX1 by immunostaining. A small subpopulation of epithelial cells also expressed nestin (Figure 6).

Under the original expansion conditions, the fibroblast-like type cells progressively take over the cultures and the epithelial cells diminish rapidly. Culture conditions were optimized to favor the growth of cultures with ~ 80% epithelial cells. Ex vivo differentiation of these cultures was performed according to the protocol of Example 2. RNA of islet endocrine cells was then isolated with the Rneasy Mini Kit (Qiagen) and treated with DNASE1. cDNA was synthesized using the Superscript First Strand Kit (Invitrogen). Quantitative PCRs for insulin and gapdh were done using Taqman® probes. Relative levels of insulin gene expression were calculated using the ΔΔCt method. Insulin expression of native islets was averaged from 3 different preparations. Regular PCR was analyzed by agarose gel electrophoresis and all PCR products were sequenced to confirm their identity. This analysis showed that ex vivo differentiation of islet progenitor cells resulted in an increase in insulin mRNA expression to approximately 40% of that of fresh native islets (Figure 7). Differentiation also induced the expression of a number of other β-cell

genes (Figure 8) including secreted C-peptide (2-5% the amount secreted by fresh human islets) (Figure 9). ILCs also stained positive for C-peptide, Glucagon and the ductal cell marker cytokeratin 19 (Figure 10).

Methods

Immunostaining

Cells were grown on tissue culture treated plastic slides, fixed with 4% paraformaldehyde and stained using standard techniques. ILCs were embedded in OCT and snap frozen on dry ice. Cryosections were fixed with 4% paraformaldehyde and stained using standard techniques.

C-peptide secretion assay

ILCs were washed 3 times with PBS. 10 ILCs/tube were handpicked and incubated at 37°C for 1 hour in RPMI medium with different glucose concentrations. Each concentration was done in triplicate. The C-peptide concentration in the medium was measured by radioimmunoassay (Linco).

EXAMPLE 4

Analysis of Islet-like cluster (ILC) gene expression

Global gene expression of fresh human pancreatic islets, expanded IPCs and differentiated ILCs was compared. All samples were from islet prep A, the most successful preparations at that time. Gene expression profiling was performed according to the following procedure. RNA was isolated with the Rneasy Mini Kit (Quiagen) and treated with DNASE1. cDNA was synthesized using the Superscript First Strand Kit (Invitrogen), labeled and hybridized to Affymetrix U95A chips. Data analysis was done with Dchip and GeneCluster 2. Gene cluster analysis of the microarray data using self organizing maps (as described in Tamayo et al., 1999, Proc. Natl. Acad. Sci. USA 96:2907) demonstrated that a number of islet-specific genes were upregulated after IPC differentiation further indicating that IPCs can be efficiently differentiated into pancreatic endocrine cells using the novel ex vivo differentiation protocol described herein (Figure 3).

To address the variability between different islet preparations the gene expression profiles of the expanded IPCs was compared from the successful prep A and prep B, which gave no differentiation at all. Cluster analysis using self organizing maps revealed a set of 124 genes

that is exclusively expressed at high levels during IPC expansion (Step 1) of prep A. These genes are absent or expressed at low levels during the expansion of prep B as well as in native pancreatic islets and after induction of differentiation from IPCs *in vitro* (Figure 4). Based on this pattern of expression, these genes may be activated specifically in functional IPCs and thus could be used as markers for the isolation of such cells in the future (Table 1).

EXAMPLE 5

Differentiation of Insulin-Producing Cells:

The following describes another example of the generation of insulin-producing islet-like clusters from single cell suspensions of adult donor human islets.

Cell culture:

Isolated human pancreatic islets from six independent donors and one preparation of islet depleted, duct-enriched tissue were obtained through the JDRF Human Islet Distribution Program from the following centers: The Joslin Diabetes Center, Boston, the Pacific Northwest Tissue Center Islet and Cell Processing Laboratory, Seattle, and the Islet Distribution Center at the Diabetes Research Institute, Miami. These experiments used unselected preparations of pancreatic cells as a starting material. The human donor islet preparations consisted of endocrine cells and other tissues such as vasculature, interstitial and adherent exocrine acinar and ductal cells, blood vessels and stroma. The islet tissue preparations were not further purified.

Culture conditions were as follows:

Step 1: The original islet preparations (n=6) were dissociated into single cells and seeded at low density in expansion medium (as in Figure 1). Single cell suspensions of Human pancreatic islet preparations were made by digestion with 5mg/ml Trypsin in PBS at 37°C and trituration by repeated passages through a glass pipette. Viable cells were counted by Trypan Blue exclusion and seeded at approximately 10,000 cells/cm^2 on tissue culture treated plastic dishes (Corning, Corning, NY). CMRL 1066 medium (5.5 mmol/l Glucose (this qualifies as "low glucose") containing 10% FBS, 1x penicillin/streptomycin, 100ng/ml beta nerve growth factor (β-NGF; R&D Systems, Minneapolis, MN), and 25ng/ml EGF was used during expansion.

Upon culture initiation only a few cells (10% or less) attached to the dish and some of the attached cells began to proliferate and formed small colonies. Twenty-four to 48 hours after seeding, non-adherent cells were removed by a media change and one wash with PBS. Thereafter

cells were expanded for 10-14 days (estimated 20 to 30 doublings) during which time they reached confluence. The culture medium was changed every 3 days during this rapid expansion phase.

At this time, the level of insulin mRNA in the cultured cells had markedly decreased compared to the starting material and ranged from 0.02 to 0.8% of the average of fresh islet preparations (as in Figure 7). Immunostaining revealed only occasional insulin-positive cells within the monolayer of expanded cells (data not shown), thus confirming the PCR data.

Step 2: When the expansion cultures reached confluence, they were detached from the dish by trypsinization and transferred onto a variety of growth substrates in different types of media to identify conditions that would induce endocrine differentiation. It was found that seeding the expanded cells at, for example, $2.5x10^4$ cells/cm^2, on hydrophobic culture dishes coated with undiluted Matrigel™ (BD Discovery Labware, Franklin Lakes, NJ) in serum-free medium resulted in efficient formation of cell clusters within 24-48 hours. In the majority of cultures, a substantial up-regulation of insulin gene expression occurred (as in Figure 7). The differentiation medium was serum-free DMEM/F12 (17.5 mmol/l glucose (this qualifies as "high glucose", which is preferred for differentiation)) plus ITS+1 (Insulin, Transferrin, Selenium, linoleic acid BSA; from Sigma).

Step 3: Insulin gene expression was further enhanced by picking cell clusters from the Matrigel™ and maintaining them in suspension culture for another five days in the same serum-free medium. Final insulin expression after this step ranged as high as 34.3% of the average of fresh islet preparations. Compared to the end of step 1 (expansion phase) insulin expression at the end of step 3 was increased by as much as 64.9-fold. It was noted that final insulin expression in cultures from the different islet preparations did not correlate with the insulin expression of aliquots of the human islets used to initiate the cultures or with the remnant low levels of insulin expression after cell expansion (step 1; data not shown).

Samples for rtPCR, gene expression profiling, C-peptide secretion, and immunostaining were collected at the end of each culture step.

Throughout these studies, quantitative rtPCR was used to assess insulin gene expression as a screening tool for beta cell differentiation. For relative quantification the mean insulin expression of five fresh native islet preparations was set to 100%. For gene expression analyses by reverse transcription polymerase chain reaction, RNA was isolated with the Rneasy Mini Kit

(Qiagen, Valencia, CA) and treated with DNASE1 (Qiagen). cDNA was synthesized using the Superscript First Strand Kit (Invitrogen). Quantitative PCRs for insulin and gapdh were done on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) using Faststart Taq polymerase (Roche, Indianapolis, IN) and gene-specific Taqman® probes. The level of insulin gene expression was calculated relative to gapdh using the $2^{\Delta\Delta CT}$ method (Livak & Schmittgen, 2001, Methods 25: 402-408). Regular rtPCR was analyzed by agarose gel electrophoresis and all PCR products amplified from in vitro differentiated cells were sequenced to confirm their identity. Controls without reverse transcriptase were done for all PCR reactions and were always negative. PCR primers, probes and conditions were:

qPCR: insulin: f:ccgcagcctttgtgaaccaa (SEQ ID NO: 67), r:ctcgttccccgcacactagg (SEQ ID NO: 68), probe:agcttccaccaggtgtgagccgca (SEQ ID NO: 69);

gapdh: f:ggacctgacctgccgtctag (SEQ ID NO: 70), r:tagcccaggatgcccttgag (SEQ ID NO: 71), probe:cctccgacgcctgcttcaccacct (SEQ ID NO: 72);

cytokeratin 19: f:gatcactacaacaatttgtctgcc (SEQ ID NO: 73), r:cccttccttcccatccctcta (SEQ ID NO: 74), probe:ccaaggtcctctgaggcagcaggc (SEQ ID NO: 75);

vimentin: tgtccaaatcgatgtggatgtttc (SEQ ID NO: 76), r:ttgtaccattcttctgcctcctg (SEQ ID NO: 77), probe:ctgacctcacggctgccctgcg (SEQ ID NO: 78);

nestin: f:ggcagacatcattggtgttaatgg (SEQ ID NO: 79), r:ccttgccccacttcctcaga (SEQ ID NO: 80), probe:tgctccagcccgttcatcactccc (SEQ ID NO: 81).

Annealing temperature for all qPCRs was 60°C.

Regular rtPCR:

gapdh: f:tgaaggtcggagtcaacggatttggt (SEQ ID NO: 82), r:catgtgggccatgaggtccaccac (SEQ ID NO: 83) (annealing temperature 58°C; 30 cycles);

pdx1: f:gcgcaccttcaccaccacctc (SEQ ID NO: 84), r:cccgccacccccgacag (SEQ ID NO: 85) (68°C, 32 cycles);

Beta2/neuroD: f:ggaggccccagggttatgagacta (SEQ ID NO: 86), r:caggttggtggtgggttgggataag (SEQ ID NO: 87) (60°C, 32 cycles);

ngn3: first round: f:cccacggccctcgctgctcatc (SEQ ID NO: 88), r:tcacagaaaatctgagaaagcca (SEQ ID NO: 89) (30 cycles), second round: f:tgcgccggtagaaaggatgac (SEQ ID NO: 90), r:gctcgcggtcgttggccttctttc (SEQ ID NO: 91) (30 cycles);

glut2: f:ggtgtgcgagccatccttca (SEQ ID NO: 92), r:tcgccctgccttctccaca (SEQ ID NO: 93) (30 cycles);

glucokinase: f:ccggcgagctggacgagttc (SEQ ID NO: 94), r:ccagccccgccgagcacat (SEQ ID NO: 95) (30 cycles);

pc1/3: f:tttgattttgcccactcctaacact (SEQ ID NO: 96), r:gctctgcaaacaccctctacacaa (SEQ ID NO: 97) (54°C, 30 cycles)

Immunofluorescent staining:

In vitro generated cell clusters were either frozen in OCT (for cryosections) or embedded in a fibrin clot prior to fixation (for paraffin sections). Clotting was achieved by mixing solutions of human fibrinogen (80 mg/ml in PBS, Sigma, St. Louis, MO) and human thrombin (50 units/ml in 40 mmol/l $CaCl_2$, Sigma). Clots were fixed in 10% buffered formalin, dehydrated, embedded in paraffin and cut into 4μm sections. Frozen sections were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Slides were then blocked with normal donkey serum in PBS/0.1%Triton for 1 hour at RT, incubated with primary antibodies over night at 4°C, washed, incubated with Cy2/Cy3 labeled secondary antibodies for 1 hour at RT, washed again and mounted. Paraffin sections were dewaxed in xylene, hydrated, boiled for 10 minutes in a microwave oven in 10mmol/l sodium citrate, pH 6 for antigen retrieval and stained as described above without the use of Triton. Nuclei were counterstained with DAPI. Antibodies used were rabbit anti C-peptide (1:1,000), guinea pig anti-insulin (1:2,000), guinea pig anti-glucagon (1:2,000), rabbit anti-somatostatin (1:2,000), guinea pig anti-pancreatic polypeptide (1:2,000; all from Linco, St. Charles, MS), mouse anti-vimentin (1:100, Signet, Dedham, MA), rabbit anti-nestin (1:500, Chemicon, Temecula, CA), mouse anti- smooth muscle actin (1:100, Sigma), mouse anti-E-cadherin (1:100, Zymed, South San Francisco, CA), mouse anti-cytokeratin 19 (1:100, Sigma) and mouse anti-pan-cytokeratin (Chemicon).

Static C-peptide secretion assay:

Step 3 cell clusters or freshly isolated human islets were washed 3 times with assay buffer (1x Gey & Gey buffer: 111 mmol/l NaCl, 27 mmol/l $NaHCO_3$, 5 mmol/l KCl, 1 mmol/l

$CaCl_2$, 1 mmol/l $MgCl_2$, 0.3 mmol/l $MgSO_4$, 1.18 mmol/l Na_2HPO_4, 0.29 mmol/l KH_2PO_4, 2.8 mmol/l Glucose, gassed to pH7.4 with CO_2/O_2 (5%/95%)) and preincubated for 30 minutes at 2.8 mmol/l Glucose. Then 500 µl of fresh buffer with different glucose concentrations, or additional KCl (30 mmol/l final), Arginine (10 mmol/l) or Tolbutamide® (100 µmol/l) were added. After 30 minutes at 37°C 300 µl of buffer were removed and C-peptide concentration was measured with the Human C-Peptide RIA Kit (Linco). Culture media and assay buffer were also measured and showed undetectable levels of C-peptide. DNA was extracted from the cell clusters in each sample using the Dneasy Tissue Kit (Quiagen) and quantified by spectrophotometry.

Statistics were done with Prism. One-way ANOVA and Dunnett's Multiple Comparison Test were used in comparisons of stimulated C-peptide secretion with baseline secretion at 2.8 mmol/l Glucose.

In successful cultures that showed high expression of insulin (30-40% that of native islets), the expression of several additional beta cell genes paralleled the level of insulin mRNA, as demonstrated by rtPCR. For example, during step 2, expression of the transcription factor neurogenin 3 (ngn3) was detected. Neurogenin 3 has been shown to be transiently expressed in endocrine-specific precursor cells destined to become mature beta cells (Schwitzgebel et al., 2000, Development 127: 3533-3542). Also demonstrated is the expression of a number of beta cell genes important for insulin gene transcription (pdx1, neuron), proinsulin processing (pc1/3), and glucose sensing (glut 2, glucokinase).

In an additional experiment a preparation of islet-depleted, duct-enriched tissue was analyzed. The duct preparation was from the same donor in which isolated islets were studied. The duct cells were subjected to the same three-step expansion and differentiation protocol as was used for islets and also resulted in a substantial up-regulation of the expression of insulin and other beta cell genes (Figure 11). Insulin expression after step 3 was 20 times higher than in the original duct-enriched preparation, but about 10 times lower than that achieved from the islets of the same donor. In this sample of ducts we were unable to detect ngn3, possibly due to a low expression level (Figure 11).

To test whether islet-like clusters containing insulin-producing cells generated *in vitro* showed functional characteristics of pancreatic beta cells static C-peptide secretion assays were performed. Because of the controversy surrounding insulin uptake into cells from media

supplements (Rajagopal et al., 2003, Science 299: 363; Hansson et al., 2004, Diabetes 53: 2603-2609), C-peptide was measured instead of insulin for these assays. *In vitro* generated cell clusters showed stimulated C-peptide secretion in response to the depolarizing agents KCl and Arginine, but little or no response to high glucose or Tolbutamide® (Figure 12 A, B). The lack of glucose or Tolbutamide responsiveness could be due to desensitization by high glucose in the differentiation media or to an immature cell phenotype. Glucose responsiveness would be preferred for clinical use, but it has been shown that somewhat immature tissue can also differentiate further after transplantation: Fresh neonatal pig islets respond poorly to glucose in vitro, but attain full glucose responsiveness *in vivo* after transplantation (Omer et al., 2003, Diabetes 52: 69-75).

Cells derived from ductal cultures also secreted C-peptide, however, at a lower level (Figure 12 C). Compared to one random preparation of freshly isolated human islets (Figure 12 D), the maximal stimulated insulin secretion of two samples of in vitro-generated cell clusters was 19% and 14% respectively. The corresponding levels of insulin mRNA expression were 5.5 and 1.1% of the average of fresh human islets. The duct-derived cell clusters showed a maximum secretion of 3% with an insulin expression of 0.6%. The islet preparation used for comparison of secretion (the only one available at the time) also showed a blunted glucose response and therefore probably does not represent the full secretory capacity of fresh human islets.

Immunostaining of cell clusters generated in vitro showed the expression of C-peptide, glucagon, somatostatin, and pancreatic polypeptide in distinct cells of the cell clusters (Figure 13 G-L). Clusters with high insulin expression consisted mainly of endocrine cells (Figure 13 G, H) whereas less well differentiated clusters also contained many hormone-negative, vimentin-positive cells, probably of mesenchymal origin (Figure 13 I-L).

Immunostaining revealed that expansion cultures consisted of two major types of cells: E-cadherin/cytokeratin 19 (CK19) positive epithelial cells growing in patches and nestin/vimentin-positive spindle-shaped cells growing separate from each other (Figure 13 A, B). Many of the spindle-shaped cells also co-expressed smooth muscle actin (Figure 13 C). Occasional cells with epithelial characteristics (E-cadherin and CK19-positive) also stained positive for nestin (Figure 13 E, F).

This example demonstrates a protocol that allows for the expansion of adult human islet tissue *in vitro* and for the generation of redifferentiated cells into islet-like clusters containing

insulin-secreting cells after their expansion. The final levels of insulin expression in the best samples was over 30% that of average freshly isolated human islets.

These data are consistent with the de-differentiation and subsequent re-differentiation of mature endocrine cells. Supporting the concept of the *in vitro* differentiation of de-differentiated cells into beta cells are the following:

(1) Cell expansion from cultured pancreatic islets was accompanied by a marked decrease in overall insulin expression, on average of over a 100-fold decrease. In accordance with this, immunostaining of expanded cells (Step 1) revealed only occasional insulin-positive cells. During steps 2 and 3, insulin-expression increased again by up to 64-fold and final in vitro-generated cell clusters comprised up to over 80% of endocrine cells, as demonstrated by immunostaining. It seems unlikely that such changes can be explained by the segregation of endocrine cells that persist throughout the culture period.

(2) Final insulin expression in cultures from the different islet preparations did not correlate with the insulin expression of the starting material or with the remnant expression after cell expansion. Therefore factors other than "left-over" beta cells likely determine the final number of insulin-producing cells.

(3) Insulin-secreting cells were also found in cell clusters generated from an islet-depleted, duct-enriched fraction of the human pancreas. Here the final amount of insulin produced was 20-fold higher than what was found in the starting material, again arguing for the differentiation of new beta-like cells *in vitro*.

(4) The expression of ngn3 during step 2 of the culture protocol (differentiation phase) also supports the notion that new endocrine cells are formed. Ngn3 is absent from mature cells but marks an endocrine precursor that is an intermediate between a common endocrine/exocrine progenitor and fully differentiated endocrine cells (Schwitzgebel et al., supra). Ngn3 is essential for the development of the endocrine pancreas during embryogenesis (Gradwohl et al., 2000, Proc. Natl. Acad. Sci. U.S.A. 97: 1607-1611).

In these studies, the formation of three-dimensional cell clusters along with growth arrest in serum-free medium seem to be critical factors for endocrine differentiation. Dedifferentiation and redifferentiation in vitro have also been observed in other systems, *i.e.* neural (Kondo &

Raff, 2000, Science 289: 1754-1757) and skin progenitor cells (Li et al., 2004, J. Clin. Invest. 113: 390-400).

EXAMPLE 6

Animal Model of Diabetes Mellitus

Treatments for diabetes mellitus 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.

A diabetic mouse model useful according to the invention is the NOD/SCID immunocompetent mouse that has been rendered diabetic by treatment with streptozotocin which selectively destroys the beta cells (see Gerling et al., 1994, Diabetes, 43:433).

The non-obese diabetic (NOD) mouse is also 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/ islet progenitor or islet endocrine cell preparation according to the invention. As such, treatment via administration of stem cell/ islet progenitor or islet endocrine cell cells are tested in the NOD mouse for their effect on type I diabetes.

The stem cell/ islet progenitor cells, islet-like clusters (ILCs) or islet endocrine cells (IECs) 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). Similar methods are applicable to the measurement of diabetes and the effectiveness of diabetes treatment in human subjects. Diabetes is "treated" when there is a clinically relevant improvement in the regulation of blood sugar. 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.

Immunocytochemical staining of transplanted pancreatic islets

Transplanted pancreatic islets were analyzed for marker gene expression. Islets were isolated as described above. 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).

Table 1:

List of new potential IPC markers (NCBI sequence ID; name):

NM_002914; replication factor C 2 (40kD) isoform 2 NM_181471; replication factor C 2 (40kD) isoform 1

NM_002916; replication factor C 4, 37kDa

NM_012145; deoxythymidylate kinase (thymidylate kinase)

NM_006572; guanine nucleotide binding protein (G protein), alpha 13

NM_002828; protein tyrosine phosphatase, non-receptor type 2 isoform 1 NM_080422; protein tyrosine phosphatase, non-receptor type 2 isoform 2 NM_080423; protein tyrosine phosphatase, non-receptor type 2

NM_000878; interleukin 2 receptor beta precursor

NM_002737; protein kinase C, alpha

NM_002849; protein tyrosine phosphatase, receptor type, R isoform 1 precursor NM_130846; protein tyrosine phosphatase, receptor type, R isoform 2

NM_002849; protein tyrosine phosphatase, receptor type, R isoform 1 precursor NM_130846; protein tyrosine phosphatase, receptor type, R isoform 2

NM_003503; CDC7-like 1

NM_005585; MAD, mothers against decapentaplegic homolog 6

NM_005292; G protein-coupled receptor 18

NM_147156; mob protein

NM_002891; Ras protein-specific guanine nucleotide-releasing factor 1 isoform 1 NM_153815; Ras protein-specific guanine nucleotide-releasing factor 1 isoform 2

NM_001174; Rho GTPase activating protein 6 isoform 2 NM_006125; Rho GTPase activating protein 6 isoform 3 NM_013422; Rho GTPase activating protein 6 isoform 5 NM_013423; Rho GTPase activating protein 6 isoform 4 NM_013427; Rho GTPase activating protein 6 isoform 1

NM_003202; transcription factor 7 (T-cell specific, HMG-box)

NM_003578; sterol O-acyltransferase 2

NM_000331; serum amyloid A1

NM_015703; CGI-96 protein

NM_006142; stratifin

NM_014435; N-acylsphingosine amidohydrolase (acid ceramidase)-like

NM_002591; phosphoenolpyruvate carboxykinase 1 (soluble)

NM_002591; phosphoenolpyruvate carboxykinase 1 (soluble)

NM_014388; novel putative protein similar to YIL091C yeast hypothetical 84

NM_031246; pregnancy specific beta-1-glycoprotein 2

NM_001724; 2,3-bisphosphoglycerate mutase

NM_005458; G protein-coupled receptor 51

NM_003631; poly (ADP-ribose) glycohydrolase

NM_000882; interleukin 12A precursor

NM_021013; type I hair keratin 4

NM_002990; small inducible cytokine A22 precursor

NM_003441; zinc finger protein 141 (clone pHZ-44)

NM_001504; chemokine (C-X-C motif) receptor 3

NM_005807; proteoglycan 4

NM_005807; proteoglycan 4

NM_001412; eukaryotic translation initiation factor 4C

NM_006208; ectonucleotide pyrophosphatase/phosphodiesterase 1

NM_004982; potassium inwardly-rectifying channel J8

NM_000728; calcitonin-related polypeptide, beta

NM_003108; SRY-box 11

NM_024854; hypothetical protein FLJ22028

NM_005727; tetraspan 1

NM_001657; amphiregulin preproprotein

NM_004227; pleckstrin homology, Sec7 and coiled/coil domains 3

NM_003608; G protein-coupled receptor 65

NM_001977; glutamyl aminopeptidase (aminopeptidase A)

NM_007317; kinesin family member 22

NM_015973; galanin preproprotein

NM_024411; prodynorphin

NM_014260; HLA class II region expressed gene KE2

NM_024570; hypothetical protein FLJ11712

NM_003720; Down syndrome critical region protein 2

NM_005312; guanine nucleotide-releasing factor 2

NM_002362; melanoma antigen, family A, 4

NM_001390; dystrobrevin, alpha isoform 1 NM_001391; dystrobrevin, alpha isoform 3 NM_001392; dystrobrevin, alpha isoform 7 NM_032975; dystrobrevin, alpha isoform 2 NM_032978; dystrobrevin, alpha isoform 4 NM_032979; dystrobrevin, alpha isoform 5 NM_032980; dystrobrevin, alpha isoform 6 NM_032981; dystrobrevin, alpha isoform 8

NM_002216; inter-alpha (globulin) inhibitor, H2 polypeptide

NM_002165; inhibitor of DNA binding 1 isoform a NM_181353; inhibitor of DNA binding 1 isoform b

NM_002853; RAD1 homolog isoform 1 NM_133282; RAD1 homolog isoform 2 NM_133377; RAD1 homolog isoform 1

NM_014611; MDN1, midasin homolog

NM_002666; perilipin

NM_000488; serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1

NM_015158; kidney ankyrin repeat-containing protein NM_153186; kidney ankyrin repeat-containing protein

NM_002474; smooth muscle myosin heavy chain 11 isoform SM1 NM_022844; smooth muscle myosin heavy chain 11 isoform SM2 NM_022870;

NM_003355; uncoupling protein 2

NM_000898; monoamine oxidase B

NM_017790; regulator of G-protein signalling 3 isoform 3 NM_021106; regulator of G-protein signalling 3 isoform 2 NM_130795; regulator of G-protein signalling 3 isoform 1 NM_134427; regulator of G-protein signalling 3 isoform 4 NM_144488; regulator of G-protein signalling 3 isoform 6 NM_144489; regulator of G-protein signalling 3 isoform 5

NM_000190; hydroxymethylbilane synthase

NM_001630; annexin A8

NM_002997; syndecan 1

NM_002553; origin recognition complex, subunit 5-like

NM_000662; N-acetyltransferase 1

NM_016369; claudin 18

NM_014251; solute carrier family 25, member 13 (citrin)

NM_019040; elongation protein 4 homolog

NM_000493; collagen, type X, alpha 1 precursor

NM_006087; tubulin, beta, 5

NM_003685; KH-type splicing regulatory protein (FUSE binding protein 2)

NM_004157; protein kinase, cAMP-dependent, regulatory, type II, alpha

NM_004528; microsomal glutathione S-transferase 3

NM_003370; vasodilator-stimulated phosphoprotein

NM_002704; pro-platelet basic protein (includes platelet basic protein, bet

NM_002704; pro-platelet basic protein (includes platelet basic protein, bet

NM_021140; ubiquitously transcribed tetratricopeptide repeat gene, X chromosome

NM_014683; unc-51-like kinase 2

NM_004085; translocase of inner mitochondrial membrane 8 homolog A

NM_002192; inhibin beta A subunit precursor

NM_014391; cardiac ankyrin repeat protein

NM_005924; mesenchyme homeo box 2

NM_006231; polymerase (DNA directed), epsilon

NM_002465; myosin binding protein C, slow type

NM_000259; myosin VA (heavy polypeptide 12, myoxin)

NM_006750; basic beta 2 syntrophin isoform a NM_130845; basic beta 2 syntrophin isoform b

NM_004763; integrin cytoplasmic domain-associated protein 1 isoform 1 NM_022334; integrin cytoplasmic domain-associated protein 1 isoform 2

NM_003864; sin3 associated polypeptide p30

NM_002787; proteasome alpha 2 subunit

NM_003872; neuropilin 2 NM_018534; neuropilin 2

NM_004134; heat shock 70kDa protein 9B precursor

NM_002687; pinin, desmosome associated protein

NM_015341; barren

NM_000258; myosin light chain 3

NM_000258; myosin light chain 3

NM_000270; purine nucleoside phosphorylase

NM_001412; eukaryotic translation initiation factor 4C

NM_002506; nerve growth factor, beta polypeptide

NM_001188; BCL2-antagonist/killer 1

NM_000340; Glucose transporter 2 (Glut-2)

Table 2	Snail superfamily members				
Species	**Common name**	**Gene**	**Synonyms**	**Accession no.**	**Map**
Caenorhabditis elegans	Nematode	ces1* snail-like scratch-like*	 K02D7.2 C55C2.1	AAF01678 T32983 T15225	I:2.9 IV:-26.1 I:-9.3
Helobdella robusta	Leech	snail1 snail2	Hro-sna1 Hro-sna2	AF410864 AF410865	
Patella vulgata	Limpet	snail1 snail2	Pv-sna1 Pv-sna2	AY049727 AY049791	
Drosophila melanogaster	Fruitfly	snail escargot womlu scratch* scratch-like1* scratch-like2*	 CG12605 CG17181	SO6222 AAF12733 S33639 AAA91035 AAF47818 AAF47394	35D2-3 35D1 35D2-3 64A2-3 64A1 61C7
Lytechinus variegatus	Sea urchin	Snail		AAB67715	
Halocynthia roretzi	Ascidia	Snail		BAA75811	
Ciona intestinalis	Ascidia	Snail		AAB61226	
Branchiostoma floridae	amphioxus	Snail		AAC35351	
Takifugu rubripes	Pufferfish	Snail1 Snail2		CAB54535 CAB54536	
Danio rerio	Zebrafish	snail1 snail2 slug scratch*		CAA52795 AAA87196 A1722148 A1883776	
Xenopus laevis	African clawed toad	Snail Slugα Slugβ	Xsna Xslu Xsluβ	P19382 AF368041 AF368043	
Silurana tropicalis	Western clawed frog	Slug	Xslug	AF368038	
Gallus gallus	Chicken	Snail Slug	SnR	CAA71033 CAA54679	
Mus musculus	Mouse	Snail Slug Scratch* Smuc	 Slugh ZIP293	Q02085 AAB38365 AY014997 NP038942	Chr.2-97.0 Chr.16-9.4
Homo sapiens	Human	SNAIL SNAILP SLUG SCRATCH1* SCRATCH2*	SNAIL1, SNAILH SNA11P SLUGH, SNAIL2	AF155233 AF153502 AAC34288 AY014996 AL121758	20q13.1 2q34 8q11 8q24.3 20p12.3-13

The Snail superfamily is subdivided into two families: Snail and Scratch (marked by an asterisk). Accession numbers are from Entrez (http://www.ncbi.nlm.nih.gov/Entrez).

What is claimed is:

1. A method of treating a patient with diabetes mellitus, comprising the steps of:

(a) isolating islet progenitor cells (IPCs) from a pancreatic islet of a donor;

(b) culturing the cells *ex vivo* to permit cell division;

(c) culturing the progenitor cells *ex vivo* on an extracellular matrix to produce islet-like clusters (ILCs) that produce insulin at a level that is 1% or more of that of native islets;

(d) culturing said islet-like clusters (ILCs) in suspension, so as to produce islet endocrine cells (IECs) that produce insulin at a level that is 5% or more of that of native islets; and

(e) transplanting said islet endocrine cells (IECs) into a patient, wherein said cells differentiate into insulin-producing beta cells.

2. A method of treating a patient with diabetes mellitus, comprising the steps of:

(a) isolating islet progenitor cells (IPCs) from a pancreatic islet of a donor;

(b) culturing the cells *ex vivo* to permit cell division;

(c) culturing the progenitor cells *ex vivo* on an extracellular matrix to produce islet-like clusters (ILCs) that produce insulin at a level that is 1% or more of that of native islets;

(d) transplanting the islet-like clusters (ILCs) into a patient, wherein said islet-like clusters (ILCs) differentiate into insulin-producing cells.

3. The method of claims 1 or 2, wherein said expanding occurs in the presence of NGF or EGF.

4. The method of claims 1 or 2, wherein said expanding occurs in the presence of NGF and EGF.

5. The method of claims 1 or 2, wherein said patient is treated with GLP-1 prior to transplantation.

6. The method of claims 1 or 2, wherein said patient is treated with GLP-1 prior to, during and post-transplantation.

7. The method of claim 1 or 2, wherein the differentiation of said islet progenitor cells (IPCs) into islet endocrine cells (IECs) increases the expression of insulin.

8. The method of claim 1 or 2, wherein said islet progenitor cells (IPCs) express at least one of the markers as set out in Table 1.

9. The method of claim 1 or 2, wherein said islet progenitor cells (IPCs) comprise 80% or more cuboidal epithelial cells.

10. The method of claim 1 or 2, wherein said islet progenitor cells (IPCs) comprise 20% or less spindle-shaped fibroblast-like cells.

11. The method of claim 9, wherein said cuboidal epithelial cells express at least one of the genes selected from the group consisting of cytokeratin 19, E-cadherin, PDX-1 and nestin.

12. The method of claim 1 or 2, wherein said differentiation (step b) up regulates the expression of at least one of the genes selected from the group of genes consisting of PDX-1, BETA2/NEUROD, NGN3, GLUCOKINASE, GLUT2, PC1/3 and CK19.

13. The method of claim 1 or 2, wherein said islet-like clusters (ILCs) express at least one of the genes selected from the group consisting of C-peptide, glucagon and the ductal cell marker cytokeratin 19.

14. The method of claim 1 or 2, wherein said differentiation on extracellular matrix occurs in serum free media.

15. The method of claim 1, 2 or 10, wherein said extracellular matrix is Matrigel™.

16. The method of claim 1 or 2, wherein the patient serves as the donor for said islet progenitor cell (IPC) of step a.

17. The method of claim 1 or 2, wherein the step of transferring is performed via endoscopic retrograde injection.

18. The method of claim 1 or 2, additionally comprising the step of: (e) treating the patient with an immunosuppressive agent.

19. The method of claim 18, wherein the immunosuppressive agent is selected from the group consisting of FK-506, cyclosporin, and GAD65 antibodies.

20. A method of inducing the differentiation of islet progenitor cells (IPCs) into islet endocrine cells (IECs), comprising the steps of:

(a) culturing islet progenitor cells (IPCs) *ex vivo* to permit cell division;

(b) culturing the progenitor cells *ex vivo* on an extracellular matrix to produce islet-like clusters (ILCs) that produce insulin at a level that is at least 1% of that of native islets; and

culturing said islet-like clusters (ILCs) in suspension, to produce islet endocrine cells (IECs) that produce insulin at a level that is at least 5% of that of native islets.

21. A method of inducing the differentiation of islet progenitor cells (IPCs) into islet-like clusters (ILCs), comprising the steps of:

(a) culturing islet progenitor cells (IPCs) *ex vivo* to permit cell division;

(b) culturing the progenitor cells *ex vivo* on an extracellular matrix to produce islet-like clusters (ILCs) that produce insulin at a level that is at least 1% of that of native islets; and

wherein said culturing promotes the differentiation of said islet progenitor cells into said islet-like clusters (ILCs).

22. The method of claim 21, further comprising the step of culturing said islet-like clusters (ILCs) in suspension to produce islet endocrine cells (IECs) that produce insulin at a level that is at least 5% of that of native islets.

23. The method of claims 20 or 21, wherein said expanding occurs in the presence of NGF or EGF.

24. The method of claims 20 or 21, wherein said expanding occurs in the presence of NGF and EGF.

25. The method of claim 20, wherein the differentiation of said islet progenitor cells (IPCs) into islet endocrine cells (IECs) increases the expression of insulin.

26. The method of claim 20 or 21, wherein said islet progenitor cells (IPCs) express at least one of the markers as set out in Table 1.

27. The method of claim 20 or 21, wherein said islet progenitor cells (IPCs) comprise 80% or more cuboidal epithelial cells.

28. The method of claim 20 or 21, wherein said islet progenitor cells (IPCs) comprise 20% or less spindle-shaped fibroblast-like cells.

29. The method of claim 27, wherein said cuboidal epithelial cells express at least one of the genes selected from the group consisting of cytokeratin 19, E-cadherin, PDX-1 and nestin.

30. The method of claim 20 or 21, wherein said differentiation (step b) up regulates the expression of at least one of the genes selected from the group of genes consisting of PDX-1, BETA2/NEUROD, NGN3, GLUCOKINASE, GLUT2, PC1/3 and CK19.

31. The method of claim 20 or 21, wherein said islet-like clusters (ILCs) express C-peptide, glucagon and the ductal cell marker cytokeratin 19.

32. The method of claim 20 or 21, wherein said differentiation on extracellular matrix occurs in serum free media.

33. The method of claim 32, wherein said extracellular matrix is Matrigel^{TM}.

34. An isolated islet progenitor cell (IPC) that differentiates to form insulin-producing islet endocrine cells (ILCs) that produce insulin at a level that is at least 1% of that of native islets.

35. The islet progenitor cell (IPC) of claim 34, wherein said cell expresses at least one of the markers as set out in Table 1.

36. An isolated islet-like cluster (ILC) that differentiates to form insulin-producing islet endocrine cells (IECs) that produce insulin at a level that is at least 5% of that of native islets.

37. The isolated islet-like cluster (ILC) of claim 36, wherein said islet-like cluster expresses at least one of the genes selected from the group consisting of C-peptide, glucagon and the ductal cell marker cytokeratin 19.

38. An isolated islet endocrine cell (IEC) that differentiates to form a pancreatic□ islet cell.

39. The isolated islet endocrine cell (IEC) of claim 38, wherein said cell expresses insulin at a level that is at least 5% of that of native islets.

40. A pharmaceutical composition comprising the isolated islet progenitor cell (IPC) of claim 34 admixed with a physiologically compatible carrier.

41. A pharmaceutical composition comprising the isolated islet-like cluster (ILC) of claim 36 admixed with a physiologically compatible carrier.

42. A pharmaceutical composition comprising the isolated islet endocrine cell (IEC) of claim 38 admixed with a physiologically compatible carrier.

43. A method of generating insulin-producing cells, comprising:

a) dissociating a preparation of pancreatic islets from a donor into a cell suspension and culturing the dissociated cells in serum-containing medium, to permit cell division;

b) culturing cells resulting from the culture of step (a) in essentially serum-free medium, whereby a cell in said culture differentiates into an insulin-producing cell.

44 The method of claim 43 wherein cells resulting from cell division in step (a) produce less than 1% of the insulin of cells prepared from native islets.

45. The method of claim 43 wherein culturing of step (b) results in the formation of islet like clusters comprising insulin-producing cells.

46. The method of claim 43 wherein an islet-like cluster resulting from step (b) produces insulin at a level that is at least 1% of that produced by native islets.

47. The method of claim 43 wherein an islet-like cluster resulting from step (b) produces insulin at a level that is at least 10% of that produced by native islets.

48. The method of claim 43 wherein an islet-like cluster resulting from step (b) produces insulin at a level that is at least 20% of that produced by native islets.

49. The method of claim 43 wherein an islet-like cluster resulting from step (b) produces insulin at a level that is at least 30% of that produced by native islets.

50. The method of claim 43 wherein said serum-containing medium is supplemented with EGF.

51. The method of claim 43 wherein said serum-containing medium is supplemented with EGF and bFGF or β-NGF.

52. The method of claim 43 wherein said serum-containing medium is supplemented with EGF, bFGF and β-NGF.

53. The method of claim 43 wherein said serum-containing medium comprises a low glucose concentration.

54. The method of claim 43 wherein said essentially serum-free medium comprises a high glucose concentration.

55. The method of claim 43 wherein culturing step (b) is performed in culture dishes comprising an extracellular matrix material.

56. The method of claim 55 wherein said extracellular matrix material comprises Matrigel™.

57. The method of claim 43 wherein a cell resulting from said cell division in step (a) expresses nestin.

58. A method of generating insulin-producing cells, the method comprising:

a) placing pancreatic islet cells from an adult donor in culture under conditions that permit a cell in said culture to dedifferentiate and proliferate, thereby producing an expanded culture of dedifferentiated cells;

b) placing cells from said expanded culture of dedifferentiated cells from step (a) under conditions that permit re-differentiation of a cell in said culture, thereby generating an insulin-producing islet like cluster.

59. The method of claim 58 wherein the cell that dedifferentiates is a mature pancreatic islet beta cell.

60. The method of claim 58 wherein an ancestor of the cell that re-differentiates is a mature beta cell that de-differentiated in step (a).

61. The method of claim 58 wherein insulin production by said insulin-producing islet like cluster is at least 1% the level produced by native islets.

62. The method of claim 58 wherein said essentially serum-free medium comprises a high glucose concentration.

63. The method of claim 58 wherein step (a) comprises proteolytic digestion of isolated islets to produce a single cell suspension.

64. The method of claim 58 wherein step (b) comprises culture on an extracellular matrix to produce islet-like clusters that produce insulin at a level that is at least 1% that of native islets.

65. The method of claim 58, further comprising the step of placing islet-like cell clusters prepared in step (b) into suspension culture in essentially serum-free medium.

66. A method of treating diabetes, the method comprising the steps of:

a) administering a preparation of insulin-producing cells produced by the method of claim 43 or 58 to a diabetic individual, whereby said diabetes is treated.

67. A method of generating islet progenitor cells, the method comprising:

a) expressing a Snail family or ZEB polypeptide in a pancreatic beta cell from an adult donor, wherein said expression permits said mature pancreatic beta cell to divide, thereby generating an islet progenitor cell.

68. A method of inducing the differentiation of islet progenitor cells, the method comprising contacting said cells with an inhibitor of the activity or expression of a member of the Snail, family or a ZEB polypeptide.

69. The method of claim 68 wherein said inhibitor comprises a siRNA that targets expression of a Snail family member or a ZEB polypeptide.

70. The method of claim 68 wherein said inhibitor comprises a Src kinase inhibitor.

71. The method of claim 70 wherein said Src kinase inhibitor is PP2.

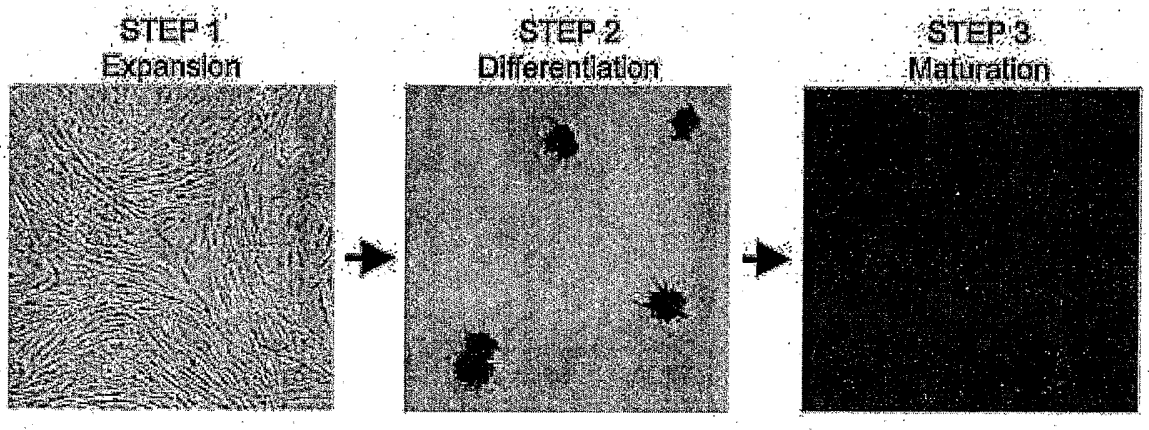


Figure 1

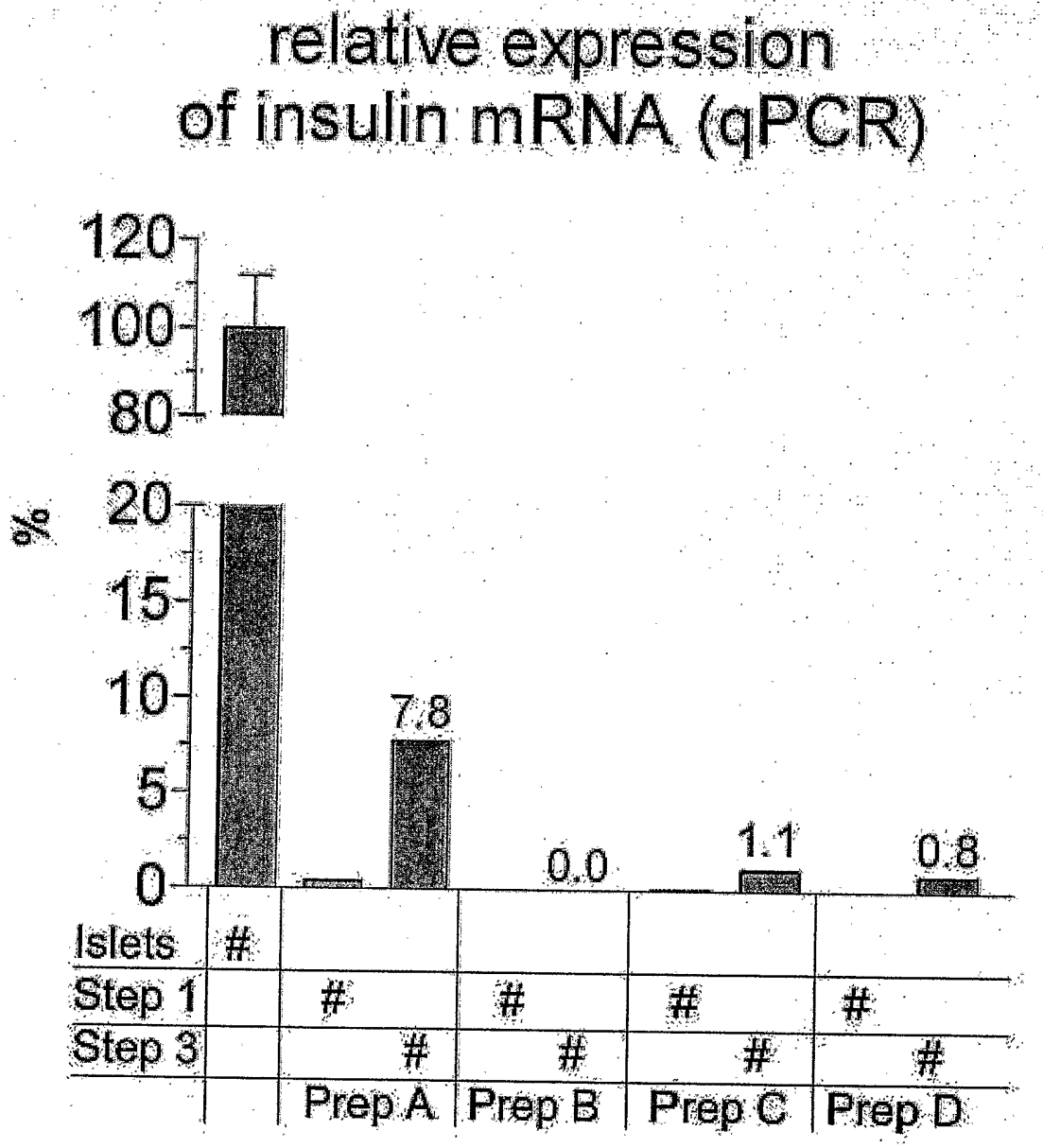
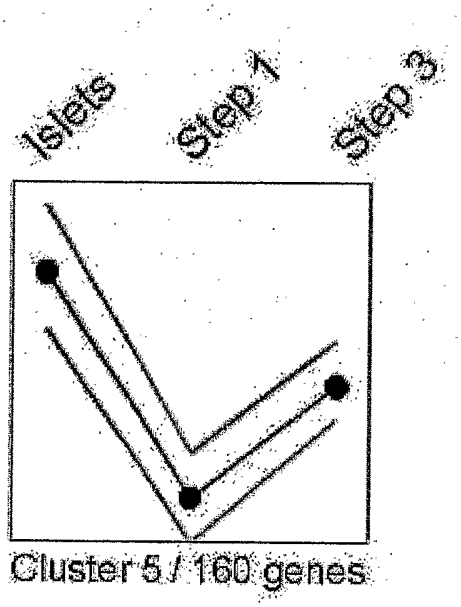

relative expression
of insulin mRNA (qPCR)
120
100
80
20
15
10
5
0
%
7.8
0.0
1.1
0.8
Islets
Step 1
Step 3
#
Prep A
Prep B
Prep C
Prep D

Figure 2



Proglucagon
Somatostatin
IAPP
Prohormone Convertase I
Sulfonylurea receptor
NeuroD
ISL-1
Chromogranin A
Chromogranin B
Carboxypeptidase E
Secretory granule protein (7B2)
Secretagogin
HGF-receptor (cMET)

Figure 3

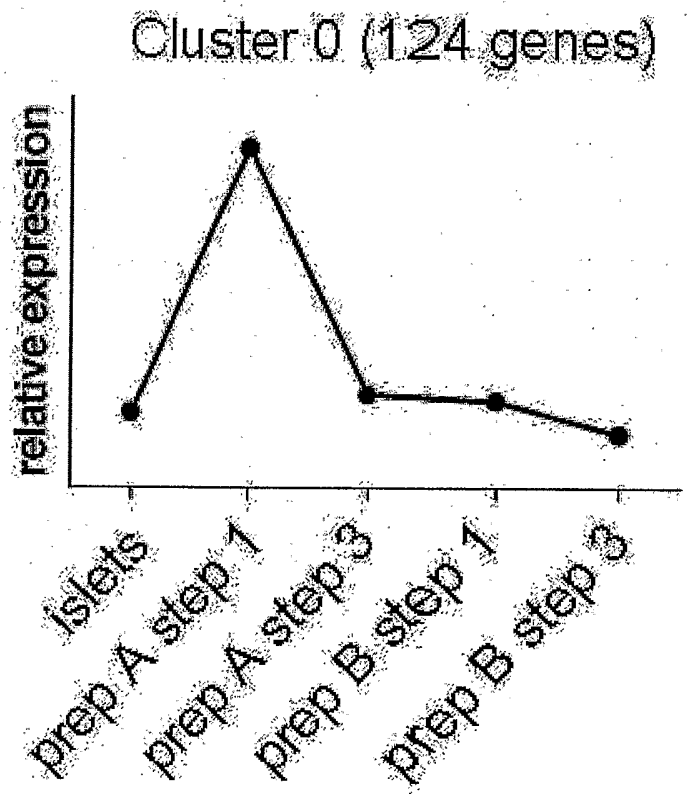
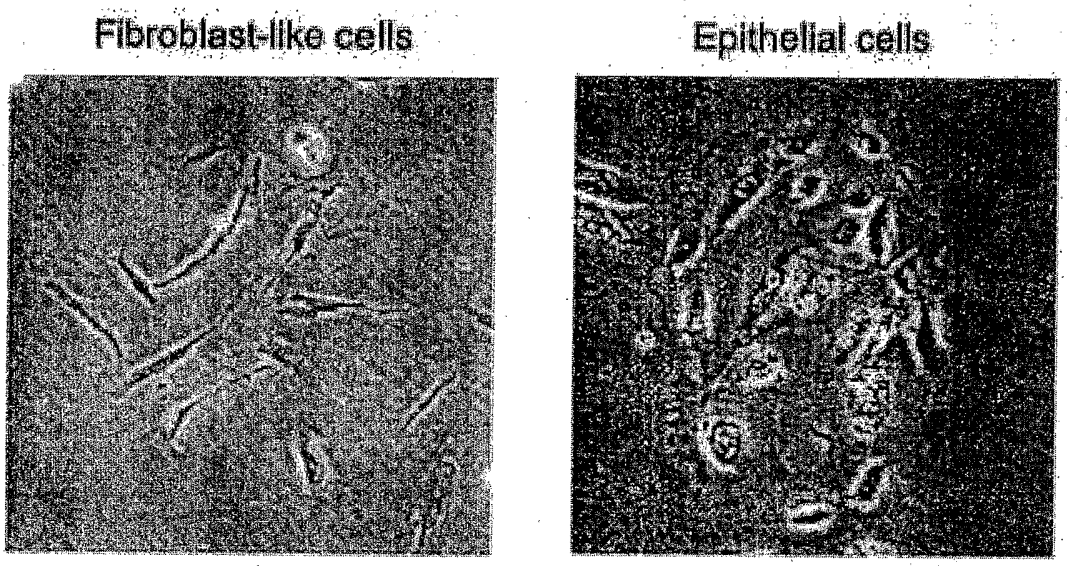

Cluster 0 (124 genes)
relative expression
islets
prep A step 1
prep A step 3
prep B step 1
prep B step 3

Figure 4



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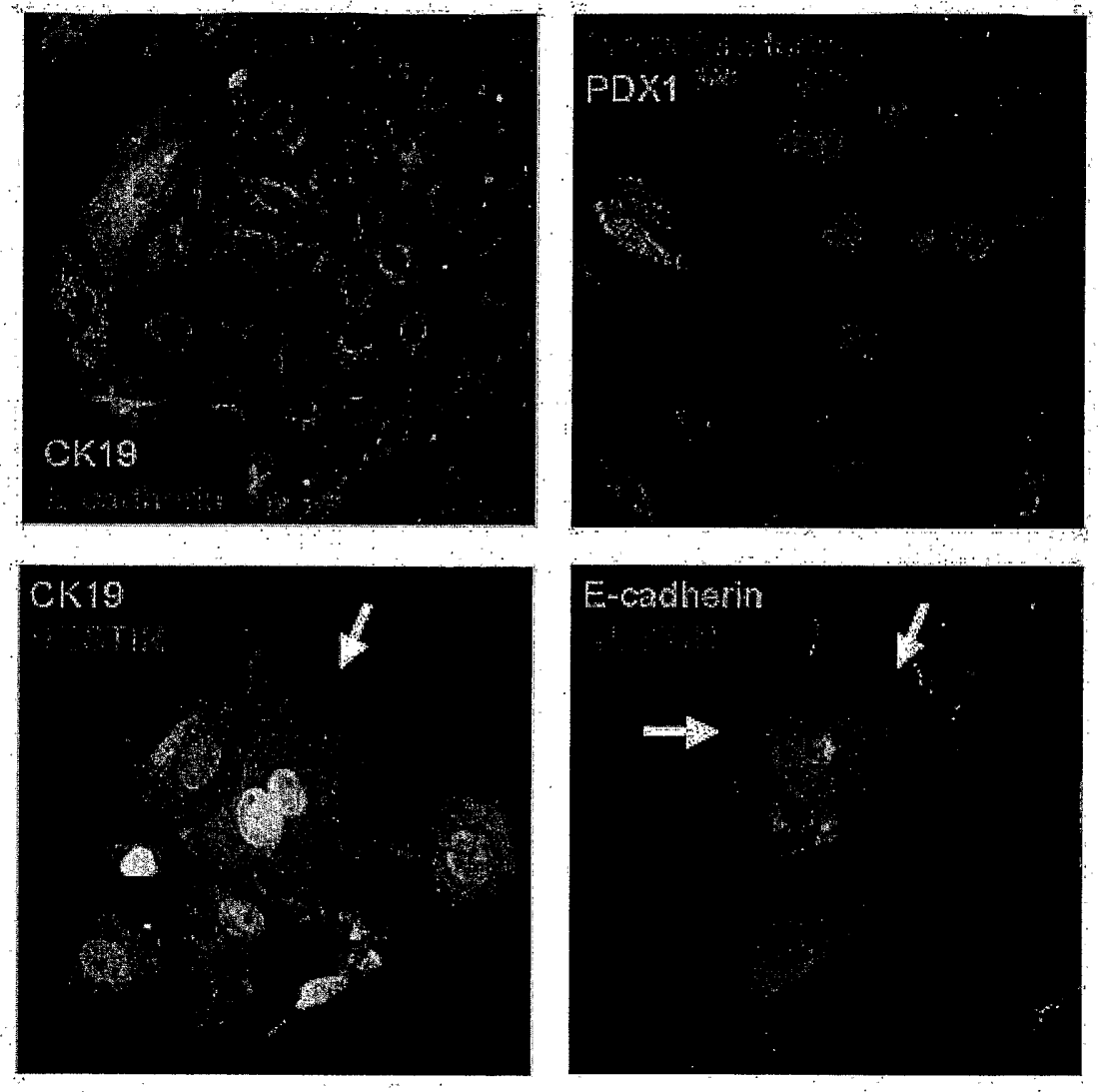


Figure 6

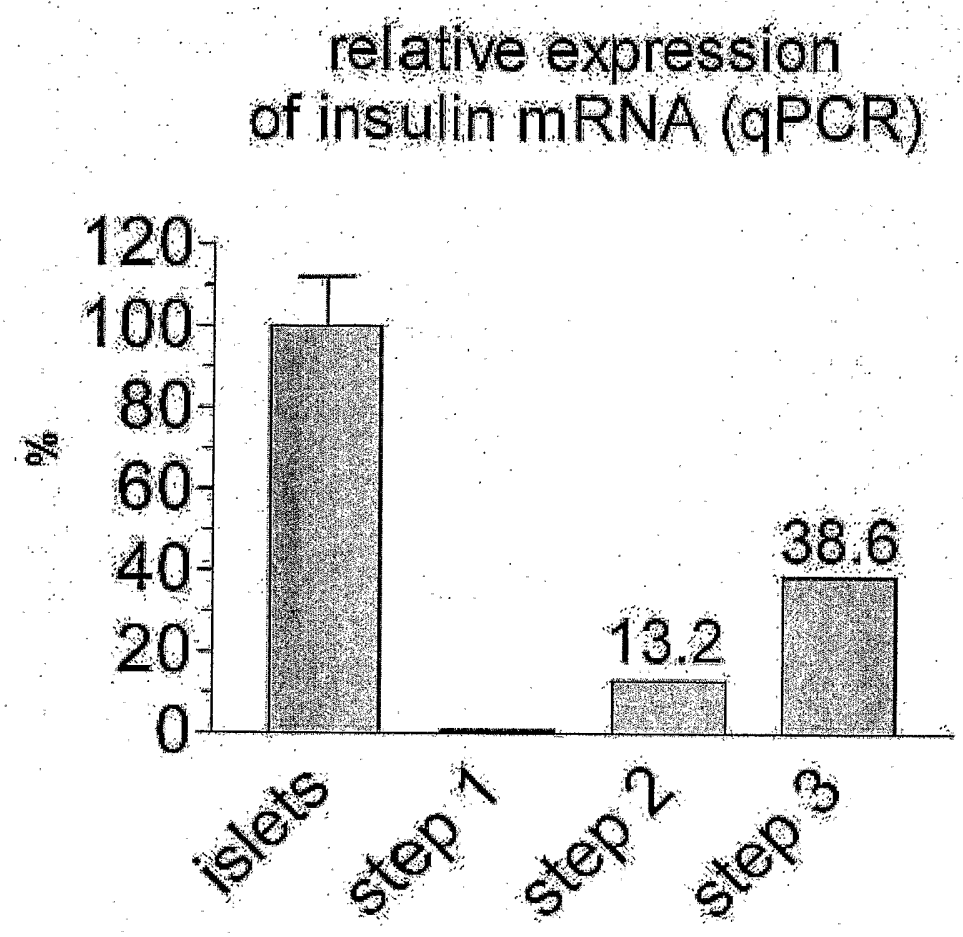

relative expression
of insulin mRNA (qPCR)
%
120
100
80
60
40
20
0
13.2
38.6
islets
step 1
step 2
step 3

Figure 7

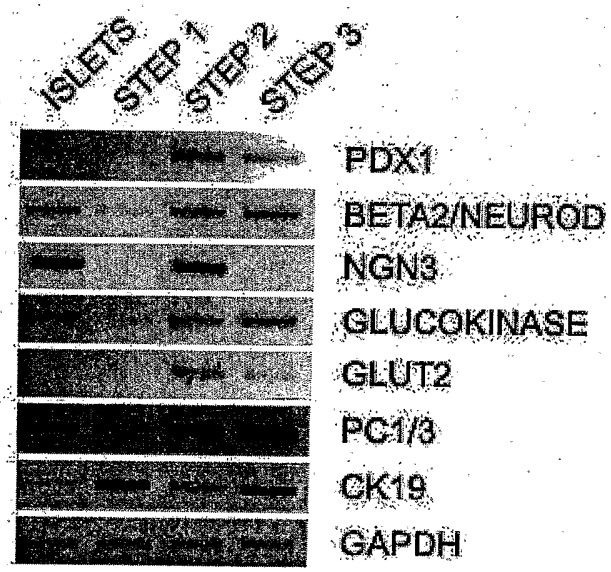

ISLETS
STEP 1
STEP 2
STEP 3
PDX1
BETA2/NEUROD
NGN3
GLUCOKINASE
GLUT2
PC1/3
CK19
GAPDH

Figure 8

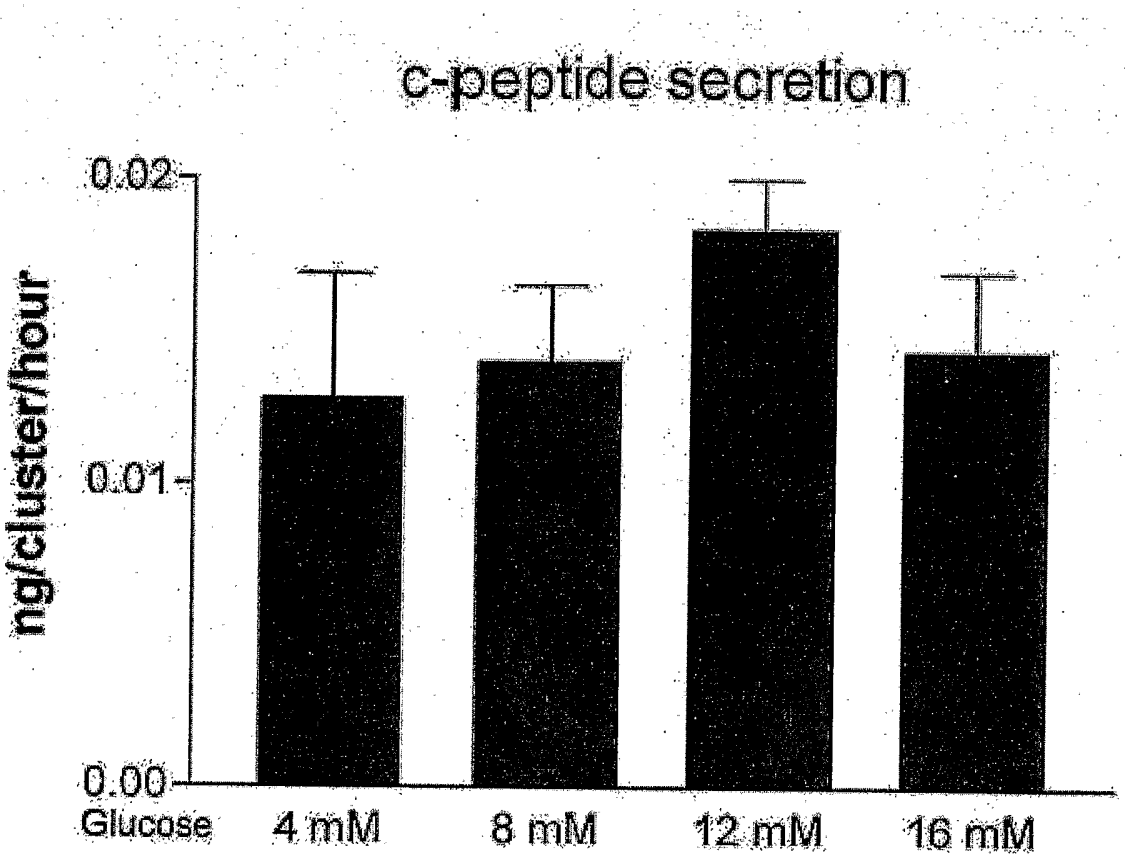

c-peptide secretion
ng/cluster/hour
0.02
0.01
0.00
Glucose
4 mM
8 mM
12 mM
16 mM

Figure 9

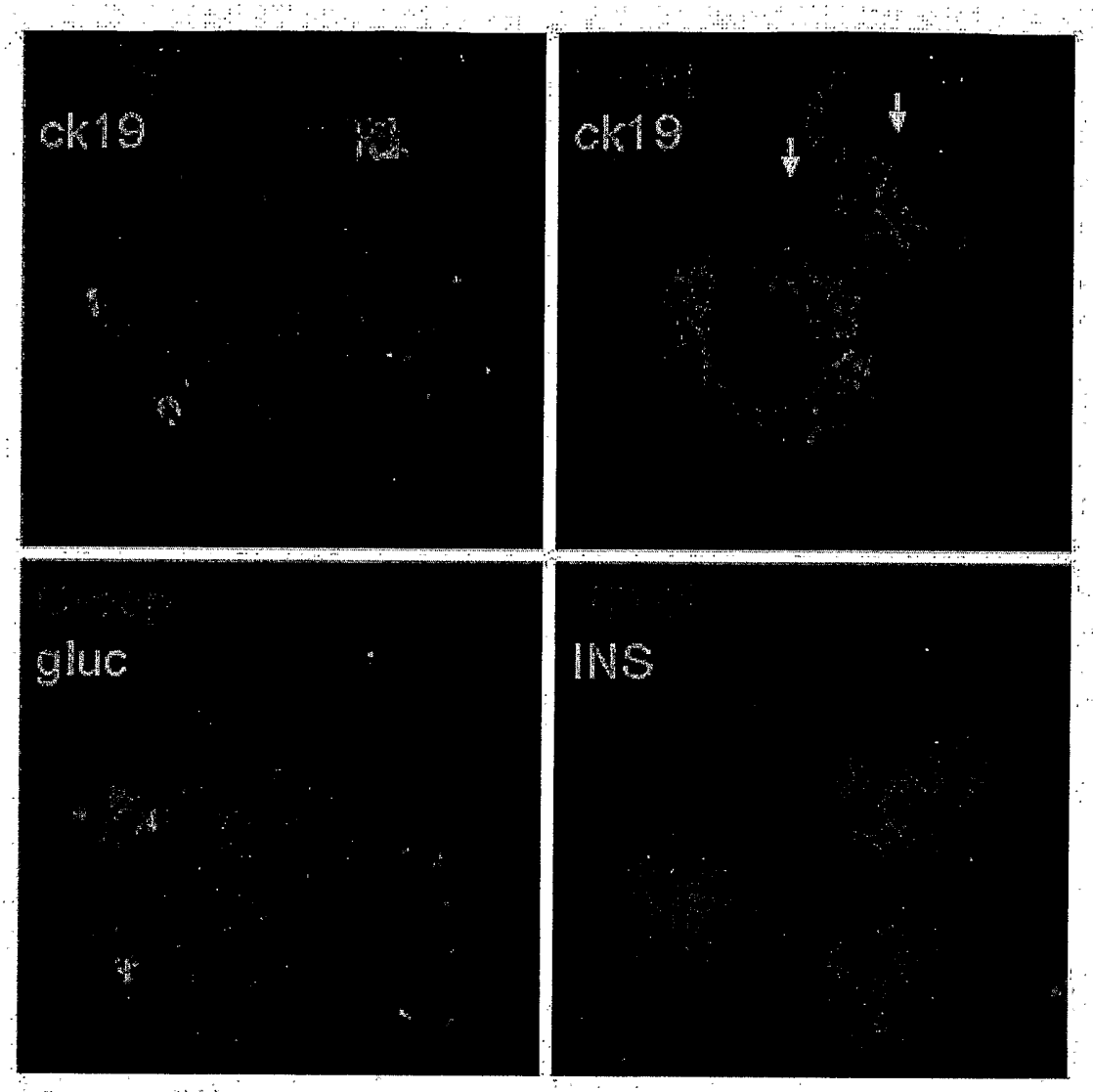


Figure 10

FIGURE 11

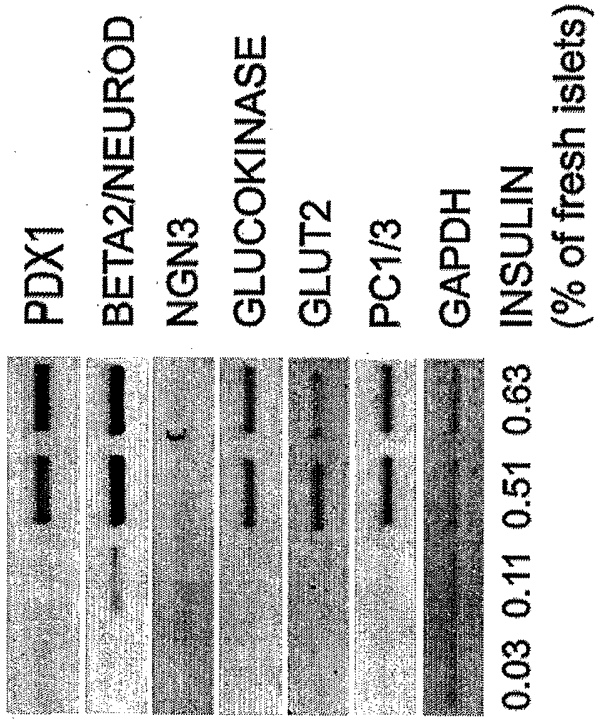

DUCTS
STEP 1
STEP 2
STEP 3
PDX1
BETA2/NEUROD
NGN3
GLUCOKINASE
GLUT2
PC1/3
GAPDH
INSULIN
(% of fresh islets)
0.03 0.11 0.51 0.63

FIGURE 12

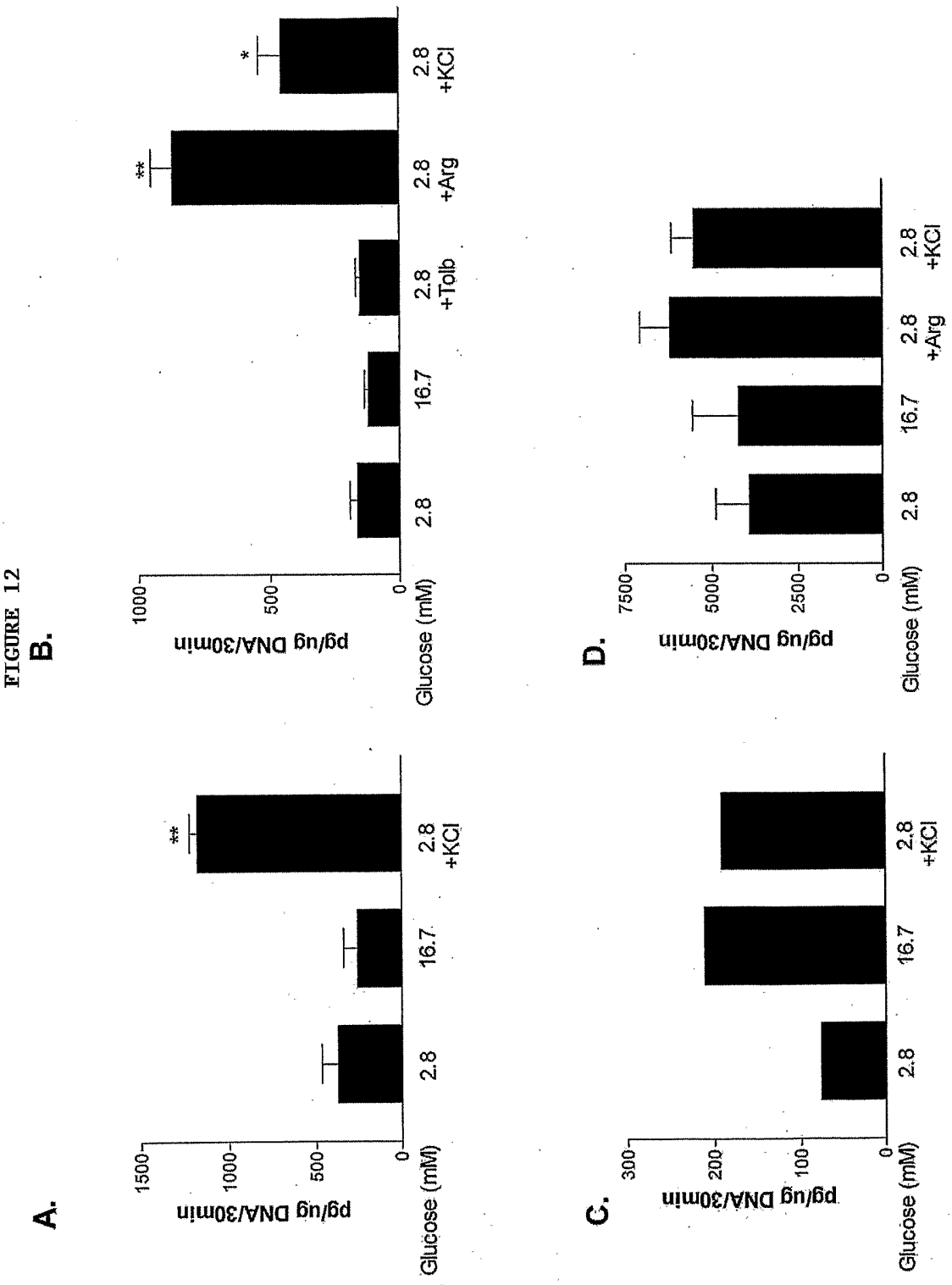
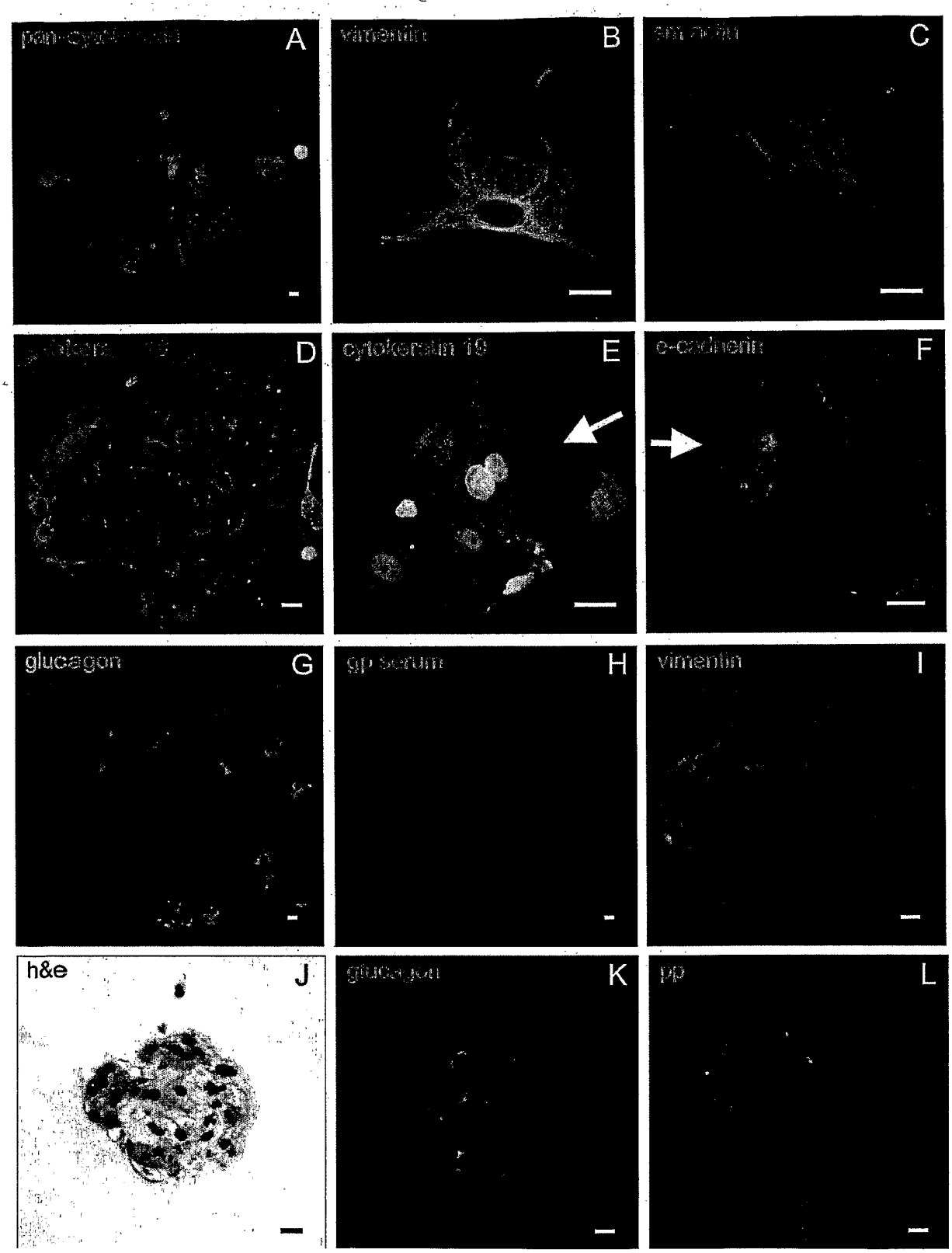

A.
B.
C.
D.
pg/ug DNA/30min
Glucose (mM)
2.8
16.7
2.8 +Tolb
2.8 +Arg
2.8 +KCl
**
*
0
500
1000
1500
2500
5000
7500
100
200
300

FIGURE 13


A
B
C
D
cytokeratin 19
E
e-cadherin
F
glucagon
G
gp serum
H
vimentin
I
h&e
J
K
pp
L

SEQUENCE LISTING

<110> The General Hospital Corporation

Habener, Joel F.
Lechner, Andreas
Rukstalis, J. Michael

<120> Expansion and Differentiation of Islet Progenitor Cells

<130> 17633/2058

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<223> Synthetic oligonucleotide PCR primer for human Glut-2.

<400> 46
gcagctgctc aactaatcac 20

<210> 47

<211> 20

<212> DNA

<213> Artificial sequence

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<223> Synthetic oligonucleotide PCR primer for human Glut-2.

<400> 47
tcagcagcac aagtcccact 20

<210> 48

<211> 30

<212> DNA

<213> Homo sapiens

<400> 48
acgggcattc ttattagtca gattattggt 30

<210> 49

<211> 16

<212> DNA

<213> Artificial sequence

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<223> Synthetic oligonucleotide PCR primer for human insulin.

<400> 49
aggcttcttc tacaca 16

<210> 50

<211> 16

<212> DNA

<213> Artificial sequence

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<223> Synthetic oligonucleotide PCR primer for human insulin.

<400> 50
caggctgcct gcacca 16

<210> 51

<211> 16

<212> DNA

<213> Homo sapiens

<400> 51
aggcagagga cctgca 16

<210> 52

<211> 6

<212> DNA

<213> Artificial sequence

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<223> E-box sequence

<400> 52
caggtg 6

<210> 53

<211> 6

<212> DNA

<213> Artificial sequence

<220>

<223> E-box sequence.

<400> 53
cacctg 6

<210> 54

<211> 21

<212> PRT

<213> Artificial sequence

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<223> Snail Zinc finger I consensus.

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<223> X is any amino acid.

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<222> (7)..(7)

<223> X is any amino acid

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<223> X is any amino acid

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<223> X is any amino acid

<400> 54

Xaa Xaa Cys Xaa Xaa Cys Xaa Lys Xaa Tyr Xaa Thr Xaa Xaa Leu Xaa
1 5 10 15

Xaa His Xaa Xaa Xaa
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<210> 55

<211> 23

<212> PRT

<213> Artificial sequence

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<223> Snail zinc finger domain II consensus.

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<222> (1)..(2)

<223> X is any amino acid.

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<223> X is any amino acid.

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<222> (20)..(21)

<223> X is any amino acid.

<400> 55

Xaa Xaa Cys Xaa Xaa Cys Xaa Lys Xaa Tyr Xaa Ser Xaa Xaa Ala Leu
1 5 10 15

Xaa Met His Xaa Xaa Thr His
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<210> 56

<211> 23

<212> PRT

<213> Artificial sequence

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<223> Snail zinc finger domain III consensus.

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<222> (1)..(2)

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<223> X is any amino acid

<400> 56

Xaa Xaa Cys Xaa Xaa Cys Gly Lys Xaa Phe Ser Arg Pro Trp Leu Leu
1 5 10 15

Gln Gly His Xaa Arg Xaa His
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<210> 57

<211> 23

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<213> Artificial sequence

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<223> Snail zinc finger domain IV consensus.

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<222> (2)..(2)

<223> X is any amino acid.

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<222> (20)..(20)

<223> X is any amino acid.

<400> 57

Phe Xaa Cys Xaa His Cys Xaa Xaa Ala Phe Ala Asp Arg Ser Asn Leu
1 5 10 15

Arg Ala His Xaa Gln Thr His
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<210> 58

<211> 23

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<213> Artificial sequence

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<223> Snail zinc finger domain V consensus.

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<400> 58

Xaa Xaa Cys Xaa Xaa Cys Xaa Xaa Xaa Phe Xaa Xaa Xaa Ser Xaa Leu
1 5 10 15

Xaa Lys His Xaa Xaa Xaa Xaa
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<210> 59

<211> 9

<212> PRT

<213> Homo sapiens

<400> 59

Met Pro Arg Ser Phe Leu Val Lys Lys
1 5

<210> 60

<211> 9

<212> PRT

<213> Homo sapiens

<400> 60

Met Pro Arg Ser Phe Leu Val Arg Lys
1 5

<210> 61

<211> 9

<212> PRT

<213> Mus musculus

<400> 61

Met Pro Arg Ser Phe Leu Val Lys Thr
1 5

<210> 62

<211> 9

<212> PRT

<213> Branchiostoma floridae

<400> 62

Met Pro Arg Ser Phe Leu Ile Lys Lys
1 5

<210> 63

<211> 9

<212> PRT

<213> Patella vulgata

<400> 63

Met Pro Arg Ala Phe Leu Ile Lys Lys
1 5

<210> 64

<211> 9

<212> PRT

<213> Drosophila melanogaster

<400> 64

Met Pro Arg Cys Leu Ile Ala Lys Lys
1 5

<210> 65

<211> 5

<212> PRT

<213> Artificial sequence

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<400> 65

Pro Leu Asp Leu Ser
1 5

<210> 66

<211> 5

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<223> CtBP binding sequence.

<400> 66

Pro Leu Asn Leu Ser
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<210> 67

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human insulin.

<400> 67
ccgcagcctt tgtgaaccaa 20

<210> 68

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo primer for human insulin.

<400> 68
ctcgttcccc gcacactagg 20

<210> 69

<211> 24

<212> DNA

<213> Homo sapiens

<400> 69
agcttccacc aggtgtgagc cgca 24

<210> 70

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human GAPDH.

<400> 70
ggacctgacc tgccgtctag 20

<210> 71

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human GAPDH.

<400> 71
tagcccagga tgcccttgag 20

<210> 72

<211> 24

<212> DNA

<213> Homo sapiens

<400> 72
cctccgacgc ctgcttcacc acct 24

<210> 73

<211> 24

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human cytokeratin 19.

<400> 73
gatcactaca acaatttgtc tgcc 24

<210> 74

<211> 21

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human cytokeratin 19.

<400> 74
cccttccttc ccatccctct a 21

<210> 75

<211> 24

<212> DNA

<213> Homo sapiens

<400> 75
ccaaggtcct ctgaggcagc aggc 24

<210> 76

<211> 24

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human vimentin.

<400> 76
tgtccaaatc gatgtggatg ttc 24

<210> 77

<211> 23

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human vimentin.

<400> 77
ttgtaccatt cttctgcctc ctg 23

<210> 78

<211> 22

<212> DNA

<213> Homo sapiens

<400> 78
ctgacctcac ggctgccctg cg 22

<210> 79

<211> 24

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human nestin.

<400> 79
ggcagacatc attggtgtta atgg 24

<210> 80

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligon PCR primer for human nestin.

<400> 80

ccttgcacca cttcctcaga 20

<210> 81

<211> 24

<212> DNA

<213> Homo sapiens

<400> 81
tgctccagcc cgttcatcac tccc 24

<210> 82

<211> 26

<212> DNA

<213> Artificial sequence

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<223> Synthetic oligo PCR primer for human GAPDH.

<400> 82
tgaaggtcgg agtcaacgga tttggt 26

<210> 83

<211> 24

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human GAPDH.

<400> 83
catgtgggcc atgaggtcca ccac 24

<210> 84

<211> 21

<212> DNA

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<220>

<223> Synthetic oligo PCR primer for human PDX-1.

<400> 84
gcgcaccttc accaccacct c 21

<210> 85

<211> 17

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human PDX-1.

<400> 85
cccgccaccc ccgacag 17

<210> 86

<211> 24

<212> DNA

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<223> Synthetic oligo PCR primer for human Beta2/neuroD.

<400> 86
ggaggcccca gggttatgag acta 24

<210> 87

<211> 25

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human Beta2/neuroD.

<400> 87
caggttggtg gtgggttggg ataag 25

<210> 88

<211> 22

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for Ngn3.

<400> 88
cccacggccc tcgctgctca tc 22

<210> 89

<211> 23

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for Ngn3.

<400> 89
tcacagaaaa tctgagaaag cca 23

<210> 90

<211> 21

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for Ngn3.

<400> 90
tgcgccggta gaaaggatga c 21

<210> 91

<211> 24

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for Ngn3.

<400> 91
gctcgcggtc gttggccttc tttc 24

<210> 92

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human Glut 2.

<400> 92
ggtgtgcgag ccatccttca 20

<210> 93

<211> 19

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human Glut-2.

<400> 93
tcgccctgcc ttctccaca 19

<210> 94

<211> 20

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human glucokinase.

<400> 94
ccggcgagct ggacgagttc 20

<210> 95

<211> 19

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human glucokinase.

<400> 95
ccagccccgc cgagcacat 19

<210> 96

<211> 25

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for pc1/3.

<400> 96
tttgattttg cccactccta acact 25

<210> 97

<211> 24

<212> DNA

<213> Artificial sequence

<220>

<223> Synthetic oligo PCR primer for human pc 1/3.

<400> 97
gctctgcaaa caccctctac acaa 24

<210> 98

<211> 6

<212> DNA

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<223> Consensus E-box sequence.

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<400> 98
canntg 6