PROGENITOR CELLS AND METHODS AND USES RELATED THERETO

Background of the Invention

Pluripotent stem cells have generated tremendous interest in the biomedical community. With the realization that stem cells can be isolated from many adult tissues has come the hope that cultures of relatively pure stem cells can be maintained in vitro for use in treating a wide range of conditions. Stem cells, with their capability for self-regeneration in vitro and their ability to produce differentiated cell types, may be useful for replacing the function of aging or failing cells in nearly any organ system. By some estimates, over 100 million Americans suffer from disorders that might be alleviated by tranplantation technologies that utilize stem cells (Perry (2000) *Science* 287:1423). Such illnesses include, for example, cardiovascular diseases, autoimmune diseases, diabetes, osteoporosis, cancers and burns.

Insulin-dependent diabetes mellitus (IDDM) is a good example of a disease that could be cured or ameliorated through the use of stem cells. Insulin-dependent diabetes mellitus is a disease characterized by elevated blood glucose and the absence of the hormone insulin. The cause of the raised sugar levels is insufficient secretion of the hormone insulin by the pancreas. In the absence of this hormone, the body's cells are not able to absorb sugar from the blood stream in normal fashion, and the excess sugar accumulates in the blood. Chronically elevated blood glucose damages tissues and organs. IDDM is treated with insulin injections. The size and timing of insulin injections are influenced by measurements of blood sugar.

There are over 400 million diabetics in the world today. Diabetes is one of the most prevalent chronic diseases in the United States, and a leading cause of death. Estimates based on the 1993 National Health Interview Survey (NHIS) indicate that diabetes has been diagnosed in 1% of the U.S. population age <45 years, 6.2% of those age 45-64 years, and 10.4% of those age >65 years. In other terms, in 1993 an estimated

7.8 million persons in the United States were reported to have this chronic condition. In addition, based on the annual incidence rates for diabetes, it is estimated that about 625,000 new cases of diabetes are diagnosed each year, including 595,000 cases of non-insulin-dependent diabetes mellitus (NIDDM) and 30,000 cases of insulin-dependent diabetes mellitus (IDDM). Persons with diabetes are at risk for major complications, including diabetic ketoacidosis, end-stage renal disease, diabetic retinopathy and amputation. There are also a host of less directly related conditions, such as hypertension, heart disease, peripheral vascular disease and infections, for which persons with diabetes are at substantially increased risk.

While medications such as injectable insulin and oral hypoglycemics allow diabetics to live longer, diabetes remains the third major killer, after heart disease and cancer. Diabetes is also a very disabling disease, because medications do not control blood sugar levels well enough to prevent swinging between high and low blood sugar levels, with resulting damage to the kidneys, eyes, and blood vessels.

Replenishment of functional glucose-sensing, insulin-secreting pancreatic beta cells through islet transplantation has been a longstanding therapeutic target. The limiting factor in this approach is the availability of an islet source that is safe, reproducible, and abundant. Current methodologies use either cadaverous material or porcine islets as transplant substrates (Korbutt et al., 1997). However, significant problems to overcome are the low availability of donor tissue, the variability and low yield of islets obtained via dissociation, and the enzymatic and physical damage that may occur as a result of the isolation process (reviewed by Secchi et al., 1997; Sutherland et al., 1998). In addition are issues of immune rejection and current concerns with xenotransplantation using porcine islets (reviewed by Weir & Bonner-Weir, 1997).

As a further example, stem cells capable of generating blood cells would also be of tremendous value for treatment of several diseases. A number of diseases or conditions result from inappropriate levels or inadequate function of blood platelets. For example, "thrombocytopenias" are the result of an abnormally small number of platelets in the circulating blood. Thombocytopenia can be due to antibody mediated platelet destruction, massive blood transfusions, cardio-pulmonary by-pass or bone marrow

failure from malignant infiltration, aplastic anemia or chemotherapy. "Thrombocythemic" disorders, on the other hand, are the result of a high platelet count. Finally, "thrombocytopathic" blood disorders are characterized by abnormally low or high platelet function, although platelet counts may be normal. Blood platelets are required for the maintenance of normal hemostasis. Platelets initiate blood clot formation and release growth factors that speed the process of wound healing as well as potentially serving other functions. Blood platelets are circulating cells that are crucial for the prevention of bleeding and for blood coagulation. Megakaryocytes are the cellular source of platelets and arise from a common bone marrow precursor cell which gives rise to all hematopoietic cell lineages. Stem cells could be used to generate cells in vitro or could be implanted to provide a stable source of cells capable of producing platelets.

In addition, extensive radiation therapy is used to treat many cancers. The radiation is lethal to the patient's endogenous bone marrow stem cells. Currently, these are replaced by transplantation in a procedure fraught with complications. An abundant supply of hematopoietic stem cells could be used for repeated treatments to replenish the depleted endogenous cells.

Many neural disorders are marked by death of nerve cells. Adult nerve cells regenerate poorly and nerve death often causes irreparable damage to congnitive and sensorimotor functions. There has been some success in treating disorders caused by nerve death with transplants of fetal nerve tissue. Fetal tissue has a greater ability to take up residence in the adult brain and differentiate into the appropriate cell type. However, obtaining sufficient fetal tissue is difficult and presents many ethical problems. Neural stem cells are capable of differentiating into many cell types of the nervous system. Remarkably, some neural stem cells are capable of migrating through the brain and settling in regions of nerve cell death. Such cells may then generate new neural processes to integrate with the endogenous neural network. It is expected that neural stem cells can be used to treat disorders such as Alzheimer's disease, Parkinson's disease, stroke, ischemia, trauma, spinal cord injuries, damage from infectious disease etc.

It is an object of the present invention to provide simple methods for the isolation and propagation of stem cells from virtually any tissue type. Such stem cells can then be used, for example, for direct transplantation or to produce differentiated cells in vitro for transplantation. The invention accordingly provides, for example, pancreatic and hepatic stem cells that may serve as a source for many other, more differentiated cell types such as pancreatic beta cells. Advantages lie in obviating the need for physical dissociation of tissue in order to obtain differentiated cells for various uses, and the potential for greater reproducibility and control of the process. With respect to pancreatic cells, successful achievement requires the differentiation and maturation of glucose-sensing, insulin-secreting beta cells from an expandable precursor population.

Summary of the Invention

The present invention relates to materials and methods for obtaining substantially pure populations of animal stem or progenitor cells. The invention further provides animal stem or progenitor cell populations and cell derivatives thereof, as well as methods of using these cell populations.

In a preferred embodiment the invention provides a method for preparing a substantially pure non-adherent population of progenitor cells which is at least about 50%, but more preferably about 60%, 70%, 80% or most preferably about 90% pure. In certain embodiments, the population of progenitor cells is obtained from an animal tissue, preferably a mammalian organ or other mammalian tissue, which is disrupted by mechanical or enzymatic means so as to yield a cell population which includes at least one progenitor cell. In preferred embodiments, the tissue is human tissue. The animal tissue may be any adult or embryonic tissue including, but not exclusive to: pancreatic tissue, liver tissue, smooth muscle tissue, striated muscle tissue, cardiac muscle tissue, bone tissue, bone marrow tissue, bone spongy tissue, cartilage tissue, liver tissue, pancreatic ductal tissue, spleen tissue, thymus tissue, tonsil tissue, Peyer's patch tissue, lymph nodes tissue, thyroid tissue, epidermis tissue, dermis tissue, subcutaneous tissue, heart tissue, lung tissue, vascular tissue, endothelial tissue, blood cells, bladder tissue, kidney tissue, digestive tract tissue, esophagus tissue, stomach tissue, small intestine

tissue, large intestine tissue, adipose tissue, uterus tissue, testicular tissue, ovarian tissue, prostate tissue, connective tissue, endocrine tissue, mesentery tissue, fetal tissue and umbilical tissue.

In certain embodiments, the tissue is a non-neuronal animal tissue which does not include brain or central nervous system tissue. Preferably the enrichment of stem/progenitor cells from the original cell suspension obtained from the tissue is at least about 100-fold, but more preferably is at least about 1000-fold.

In certain preferred embodiments, the cell suspension derived from this animal tissue is then treated with a growth factor preparation which may include any of a number of different growth factors including epidermal growth factor, transforming growth factor, hepatocyte growth factor, fibroblast growth factor, leukemia inhibitory factor, insulin-like growth factor and platelet-derived growth factor.

The progenitor cell population within the animal cell suspension is then allowed to proliferate in the presence of the growth factor population and takes on a non-adherent, floating characteristic. In certain instances, the progenitor cell population form homotypic cell spheres. The phenotypic characteristics of the progenitor cell population provide both an indication that the cell suspension has become enriched in the stem/progenitor cell population as well as providing certain physical features which may be used to enrich for the stem/progenitor cells.

The invention further provides certain markers, including c-kit, Sca and Nestin, for identifying and/or enriching the population of stem/progenitor cells. The invention still further provides for derivatives of these stem/progenitor populations which can be obtained under proper conditions. The stem/progenitor cell derivatives may express a marker such as Pdx-1, glucagon, or insulin.

The present invention further relates to substantially pure preparations of viable pancreatic progenitor cells, and methods for isolating such cells from essentially any tissue, notably liver, muscle and pancreatic tissue. The present invention further concerns certain uses for such progenitor cells, and their progeny.

In general, the invention features a cellular composition including, as the cellular component, a substantially pure population of viable pancreatic progenitor cells which

progenitor cells are capable of proliferation in a culture medium. In a preferred embodiment, the cellular composition has fewer than about 20%, more preferably fewer than about 10%, most preferably fewer than about 5% of lineage committed cells.

In one embodiment, the progenitor cells of the present invention are characterized by an ability for self-regeneration in a culture medium and differentiation to pancreatic lineages. In a preferred embodiment, the progenitor cells are inducible to differentiate into pancreatic islet cells, e.g., β islet cells, α islet cells, δ islet cells, or ϕ islet cells. Such pancreatic progenitor cells may be characterized in certain circumstances by the expression of one or more of: homeodomain type transcription factors such as STF-1; PAX gene(s) such as PAX6; PTF-1; hXBP-1; HNF genes(s); villin; tyrosine hydroxylase; insulin; glucagon; and/or neuropeptide Y. The pancreatic progenitor cells of the present invention may also be characterized by binding to lectin(s), and preferably to a plant lectin, and more preferably to peanut agglutinin. In certain preferred embodiments, the progenitor cells express PDX1 mRNA and protein, e.g., by FACS sorting, and are capable of differentiation into glucose-responsive insulin secreting cells. In certain preferred embodiments, the progenitor cells express PDX1 and Glut2 mRNA and protein. In certain preferred embodiments, the progenitor cells express PDX1, Glut2 and stain with PNA.

The invention provides multiple methods for obtaining pancreatic progenitor cells. In one embodiment, the cells are obtained by propagation in a non-adherent culture. In another embodiment, the cells are obtained as non-adherent cells (NACs) arising from an adherent culture.

In certain preferred embodiments, the subject pancreatic progenitor cells will have one or more of the following characteristics: (i) able to grow in 2-5 percent fetal calf serum; (ii) able to grow on plastic, e.g., no need to use matrigel; (iii) no statistically significant induction of cells to proliferate or differentiate when treated with TGFβ5 (GenBank accession P16176) at concentrations up to 30 pg/ml.

In yet another embodiment, the invention features a pharmaceutical composition including as the cellular component, a substantially pure population of viable pancreatic progenitor cells, which progenitor cells are capable of proliferation in a culture medium.

In general, the preferred progenitor cells will be of mammalian origin, e.g., cells isolated from a primate such as a human, from a miniature swine, or from a transgenic mammal, or are the cell culture progeny of such cells. In one embodiment, pancreatic ductal tissue is isolated from a patient and subjected to the present method in order to provide a resulting culture of pancreatic progenitor cells (or differentiated cells derived therefrom). Gene replacement or other gene therapy is carried out ex vivo, and the isolated cells are transplanted back into the initial donor patient or into a second host patient.

In another aspect, the invention features a cellular composition comprising, as a cellular population, at least 50% (though more preferably at least 75, 90 or 95%) progenitor cells and capable of self-regeneration in a culture medium.

In yet another aspect, the invention features a cellular composition consisting essentially of, as the cellular population, viable pancreatic progenitor cells capable of self-regeneration in a culture medium and differentiation to pancreatic lineages. For instance, in certain embodiments the progenitor cells are isolated from pancreatic intralobular duct explants, e.g. isolated by biopsy, or are the progeny of such cells.

Several aspects of the invention feature a method for isolating pancreatic progenitor cells from a sample of pancreatic duct. In general, the method provides for a culture system that allows reproducible expansion of pancreatic ductal epithelium while maintaining "stemmedness" and the ability to differentiate into endocrine and exocrine cells. In one embodiment, pancreatic ductal tissue is treated with digestive enzymes to produce a cell suspension. The cell suspension is cultured in a non-adherent culture vessel in the presence of various growth factors. In certain embodiments, propagating cells give rise to spheres of cells that are capable of differentiating into a wide range of cell types. In another instance, pancreatic ductal epithelium is obtained, e.g., by explant or enzymatic digestion, and cultured to confluence. The confluent cell population is contacted with an agent, e.g., a trophic agent such as a growth factor, which causes differentiation of progenitor cells in the cultured population. Subsequently, progenitor cells from the explant that proliferate in response to the agent are isolated, such as by direct mechanical separation of newly emerging buds from the rest of the explant or by

dissolution of all or a portion of the explant and subsequent isolation of the progenitor cell population.

In certain embodiments, the culture is contacted with a cAMP elevating agents, such as 8-(4-chlorophenylthio)-adenosine-3':5'-cyclic-monophosphate (CPT-cAMP) (see, for example, Koike Prog. Neuro-Psychopharmacol. and Biol. Psychiat. 16 95-106 (1992)), CPT-cAMP, forskolin, Na-Butyrate, isobutyl methylxanthine (IBMX) and cholera toxin (see Martin et al. J. Neurobiol. 23 1205-1220 (1992)) and 8-bromo-cAMP, dibutyryl-cAMP and dioctanoyl-cAMP (e.g., see Rydel et al. PNAS 85:1257 (1988)).

In certain embodiments, the culture is contacted with a growth factor, e.g., a mitogenic growth factor, e.g., the growth factor is selected from a group consisting of IGF, TGF, FGF, EGF, HGF, hedgehog or VEGF. In other embodiments, the growth factor is a member of the TGFβ superfamily, preferably of the DVR (dpp and vg1 related) family, e.g., BMP2 and/or BMP7.

In certain embodiments, the culture is contacted with a steroid or corticosteroid such as, for example, hydrocortisone, deoxyhydrocortisone, fludrocortisone, prednisolone, methylprednisolone, prednisone, triamcinolone, dexamethasone, betamethasone and paramethasone. See, generally, The Merck Manual of Diagnosis and Therapy, 15th Ed., pp. 1239-1267 and 2497-2506, Berkow et al., eds., Rahay, N.J., 1987).

In a preferred embodiment, the cultures are contacted with a cAMP elevating agent, a growth factor and a steroid or corticosteroid, e.g., with the DCE cocktail described herein.

In another aspect, the invention features, a method for screening a compound for ability to modulate one of growth, proliferation, and/or differentiation of progenitor cells obtained by the subject method, including: (i) establishing an isolated population of pancreatic progenitor cells; (ii) contacting the population of cells with a test compound; and (iii) detecting one of growth, proliferation, and/or differentiation of the progenitor cells in the population, wherein a statistically significant change in the extent of one of growth, proliferation, and/or differentiation in the presence of the test compound relative to the extent of one of growth, proliferation, and/or differentiation in the absence of the

test compound indicates the ability of the test compound to modulate one of the growth, proliferation, and/or differentiation.

In another aspect, the invention features, a method for treating a disorder characterized by insufficient insulin activity, in a subject, including introducing into the subject a pharmaceutical composition including pancreatic progenitor cells derived by the subject method, or differentiated cells arising therefrom, and a pharmaceutically acceptable carrier. In preferred embodiments, the progenitor cells are derived from a donor source (which may also be the transplant patient), and expanded at least an order of magnitude prior to implantation. As shown in Figure 40, the subject cellular compositions can be used to rescue diabetic mice.

In a preferred embodiment the subject is a mammal, e.g., a primate, e.g, a human.

In another preferred embodiment the disorder is an insulin dependent diabetes, e.g, type I diabetes.

In another aspect the invention provides differentiation media for promoting differentiation of progenitor cells into differentiated pancreatic cell types. In one embodiment, the invention comprises a pancreatic differentiation medium comprising a cAMP elevating agent, PYY and fetal bovine serum. In preferred embodiments the medium comprises forskolin. A particularly preferred pancreatic differentiation medium comprises at least 25 mM forskolin, at least 150 ng/ml PYY and at least 3% fetal bovine serum.

In a further aspect, the invention provides methods for obtaining differentiated pancreatic cell types. In one embodiment, the method for obtaining differentiated pancreatic cell types comprises obtaining a cell suspension from an animal tissue, treating the cell suspension with a growth factor preparation, allowing proliferation of non-adherent cells and contacting said proliferated non-adherent cells with a differentiation medium and an adherent matrix. Under such conditions, non-adherent cells adhere to the matrix and give rise to at least one differentiated pancreatic cell type. In preferred embodiments, progenitor cells are obtained from post-natal human tissue. In preferred embodiments, the growth factor preparation comprises one or more of the

following: epidermal growth factor, basic fibroblast growth factor, hepatocyte growth factor, transforming growth factor alpha, insulin-like growth factor I and insulin-like growth factor II. The methods of the invention permit the formation of both exocrine and endocrine cell types. Exocrine cell types preferably express carboxypeptidase A. Endocrine cell types may include glucagon-expressing cells, insulin-expressing cells and somatostatin expressing cells. Adherent matrices of the method are preferably matrices derived from a cancerous cell line and preferably a sarcoma or a bladder carcinoma. Notably, the methods of the invention permit the formation of insulin producing cells that are capable of producing insulin in a glucose-regulated manner.

In a further embodiment, the invention provides methods of obtaining glucagon-expressing cells, comprising propagating a progenitor cell in the presence of 15% KO-SR; and differentiating the cell by contacting it with an adherent matrix and a differentiation medium. Under such conditions, the progenitor cell gives rise to glucagon expressing cells.

In yet another embodiment, the invention provides a method of obtaining somatostatin-expressing cells, comprising propagating a progenitor cell in the presence of LIF and differentiating the cell by contacting it with an adherent matrix and a differentiation medium. Under these conditions, the progenitor cell gives rise to somatostatin expressing cells.

In yet another embodiment, the pancreatic progenitor cells are induced to differentiate into pancreatic islet cells, e.g., β islet cells, α islet cells, δ islet cells, or ϕ islet cells, subsequent to being introduced into the subject.

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. Patent 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).

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description of Drawings

Figure 1. Isolation of pancreatic ducts. The cartoon illustrates the process by which the ducts under study were obtained. Pancreatic tissue from 2-3 week old rats was dissociated in collagenase solution and ductal material was obtained by handpicking. Clean ducts were placed in culture on plastic. Within 3-5 days a monolayer was obtained, from which non-adherent cells (NACs) were produced upon exposure to an inductive medium. Shown below the cartoon is a series of photographs showing the various stages of duct isolation and purity. The first panel shows the primary digest, and the second panel shows the result of the first round of handpicking. Note that there is still some contaminating exocrine tissue. After the second round of selection, the ducts are free of both islet and exocrine tissue.

Figure 2. Culture and insulin staining of pancreatic ducts. Depicted is a time series of single ducts in culture from time zero plating (T0) to day five (T5) in culture. The upper panel shows examples of Cy3-labelled immunocytochemical staining for insulin, and the lower panel shows the combined brightfield and fluorescence images. At T0 there are no insulin-expressing cells. Within 24 hours of culture, the duct begins to distend and disintegrate, with cells moving toward the

periphery. Proliferating cells are present primarily in the outgrowing monolayer of mesenchymal cells, although ductal epithelial cells do also incorporate BrdU (not shown). Insulin-positive cells emerged spontaneously from the duct over time in culture, but their overall replication rate is slow (not shown). By T5 some monolayers contained sizable clusters of insulin-positive cells; typically these contained 20 cells or less.

Figure 3. The duct monolayer expresses multiple progenitor cell markers. Monolayers were stained for both insulin (A) and amylase (B). Panel C is a composite showing that some cells express both insulin and amylase. Two morphologically distinct cell types are present, those that are adherent and flat, and cells that are semi-adherent and round. Arrowheads denote* rounded semi-adherent cells that may coexpress both insulin and amylase. Panels D and E show staining for glucagon and PYY, respectively, and Panel F is a composite showing that one of the glucagon-bright cells also expresses PYY. Panel G shows a composite of nuclear PDX-1 (Cy3) and cytoplasmic insulin (FITC) staining. Arrowheads indicate cells that express PDX-1 but not insulin or vice versa.

Figure 4. Factor addition influences the appearance of the non-adherent cell (NAC) type. Hoffman modulation contrast photographs were taken of cultures treated with various factors. Cultures were grown in 5% FBS for five days until a confluent monolayer was obtained; factors were then added for an additional 48 hours and the cultures were photographed. NACs were observed in all conditions. Panel A shows the control culture grown in FBS; panel B, culture treated with DCE (1 μM Dexamethasone, 100 ng/ml Cholera toxin, 10 ng/ml EGF); Panel C, HGF (10 ng/ml), and Panel D, TGFβ1 (10 ng/ml). Arrow 1 in Panel A indicates the adherent and confluent monolayer and the Arrow 2 points to a pair of rounded loosely or non- adherent cells. The HGF and TGFβ1 treated cultures also contained semi- and non-adherent cells. However, the pharmacological cocktail DCE induced, on average, at least 8-fold more NACs than all other conditions tried. BrdU pulsing experiments showed strong proliferation and a confluent monolayer even after 48 hours of DCE exposure,

indicating perhaps asymmetric division as opposed to simple loss of cell adherence. The inset in Panel B illustrates the morphology and granularity of NACs.

Figure 5. Multiple hormone-containing cell types are detected in the NAC population. NACs were collected from DCE-stimulated monolayer cultures and analyzed immunocytochemically for endocrine marker expression. Cells expressing insulin (A, C), PDX-1 (D), glucagon (E), somatostatin (F), and pancreatic polypeptide (G) were all present in the NAC population. Markers were visualized with FITC or Cy3 immunofluorescence and the nuclei counterstained with DAPI (C-G). Panel A shows a 10X objective field magnification of insulin staining. Heterogeneous signal strength was observed; shown here are one brightly stained cell and many dimly stained cells along with negative cells. Panel B shows staining with normal preimmune serum. Note that the dim cells in A are significantly above background, yet contain much less insulin than the bright cell observed. Panel C shows higher magnification (20X objective) of another insulin staining (Cy3) showing dim and negative cells. In this field and others approximately 40-50% of the cells test insulin-positive. Panels D, E, F, and G show PDX-1, glucagon, somatostatin and pancreatic polypeptide staining, respectively (60X objective). Arrows indicate DAPI-stained, hormone-negative cells.

Figure 6. Single cell PCR (SC-PCR) analysis of PDX-1, insulin and glucagon expression in NACs. Forty cells were selected from a random population of NACs and processed for cDNA as described in the Methods section. These cDNAs were then analyzed for insulin (B), glucagon (C), and PDX-1 (D) message. Panel A shows the ethidium bromide staining of the cDNA on a 1.2% agarose gel. The bulk of cDNA product fell within the targeted 500-1000 bp range. Panel B shows that there is variation in the amount of insulin message per cell, with some cells giving much stronger signal than others. 15/40 (37%) of the cells tested positive for insulin mRNA. Of these, one was also positive for glucagon, and both messages were relatively weak compared to the other cells

that expressed insulin only. Panel C shows that 2/40 (5%) of the cells contained glucagon message, a result that correlates well with the immunocytochemistry data. Panel D shows that many of the picked cells contained PDX-1 mRNA. Note that a significant fraction of cells express PDX-1 mRNA only, with no insulin or glucagon.

Figure 7. Insulin content and glucose response of cultured ducts. Freshly isolated ducts (T=0), ducts cultured for one week (T=7), and NACs harvested from the DCE-induced duct culture were all tested for glucose-stimulated insulin secretion (GSIS) and also extracted for total insulin content. The time zero ducts contained no detectable insulin by RIA. In contrast, the cultured ducts did have a discernible increase in insulin content, but no glucose response. In this representative experiment, the isolated NACs showed an insulin content that was 18-fold greater than the DCE-treated monolayer when normalized to a per cell level. In addition, the NACs demonstrated a strong 3-fold GSIS response, well within the 3-5 fold physiological range observed with adult islets. ML = monolayer, n.d. = not detectable.

Figure 8. Glucose-induced calcium currents in NACs. Glucose induces an inward calcium current in the NAC population. Changes in intracellular calcium at the single cell level were monitored using Fluo-3 and confocal microscopy. A, In this representative experiment (n >10) approximately one-third of the population initiated a robust calcium influx in response to glucose administration, and 58% of the cells showed no response to glucose. In every experiment, approximately 6-10% of the cells begin with a high intracellular calcium content that decreases with time; these were judged to be dying cells. 80 cells were analyzed in this experiment. B, The reversibility of the induced calcium current is demonstrated. In this representative experiment (n>6), the glucose-stimulated calcium current could be washed out with Krebs Ringer Phosphate (KRP) solution. A second calcium current could then be stimulated by readministration of 17 mM glucose. Washout of the glucose followed by tolbutamide stimulation, a SUR-linked potassium channel blocker, also stimulated a calcium current, as expected.

Arrows indicate times of administration. A total of 123 cells were analyzed in this experiment. 7-13 % of the cells gave rise to calcium currents in response to the stimulus (shown in red) whereas 45-65 % of the cells showed no response to any of the stimuli (shown in blue). The remaining 35% of cells exhibited varying amplitudes and kinetics in response to challenge, indicating a complex population.

Figure 9. Graph showing induction of differentiation by DCE

Figure 10. Graph showing effect of Forskolin, Dibutyrl cAMP and Na-Butyrate on induction of differentiation.

Figures 11 and 12. Graphs illustrating the effect of secretin on induction of floating progenitor cells.

Figure 13. Graph demonstrating that Vasoactive Intestinal Peptide (VIP) also induces the appearance of floating progenitor cells from duct monolayers.

Figure 14. Graph showing that insulin diminishes secretin-induced differentiation.

Figures 15-17. Micrographs illustrating the phenotype of cells cultured for two weeks after being sorted on the basis of PNA staining.

Figures 18 and 19. Micrographs illustrating the specificity of PNA staining in adult and embryonic pancreas.

Figure 20. Electrophoretic gel showing the results of typical single cell mRNA PCR amplification reactions.

Figure 21. Table illustrating the changes in the gene expression during pancreatic development.

Figure 22. Chart illustrating one embodiment of an array of markers for detecting beta cells and precursors thereof.

Figure 23. Autoradiographs profiling gene expression in adult and embryonic pancreatic tissue, and heart.

Figure 24. Graph demonstrating how quantitative analysis of gene expression can be carried out as part of a determination of the gene expression profile of a cell.

Figure 25. Autoradiographs profiling gene expression in embryonic pancreatic tissue at

different stages and after different stimulus

Figure 26. Graphs illustrating the quantitative analysis of the autoradiagraphs.

Figure 27. Autoradiographs profiling gene expression in the so-called floating progenitor cells.

Figure 28. Graph illustrating the quantitative analysis of the autoradiagraphs of Figure 27.

Figure 29. Table showing the relative levels of expression of certain genes between adult islets and during pancreas development.

Figures 30 -31. Micrographs illustrating the binding of certain lectins to adult rat pancreas.

Figures 32 -39. Micrographs illustrating the binding of certain lectins to adult human pancreas.

Figure 40. Implanted cells from a pancreatic duct-derived culture transiently rescue the diabetic state. A heterogeneous population containing functional beta cells derived from the non-adherent portion of a differentiated pancreatic duct monolayer was implanted into streptozotocin (STZ)-treated diabetic mice. SCID mice injected with STZ became diabetic within 48 hours. Insulin containing pellets were then implanted subcutaneously to stabilize the blood glucose and create a more stable environment for cell implantation. The insulin pellet was designed to expire 7 days post-implantation at T = 11 days (T11). Within 48 hours of pellet implant the fasting blood glucose levels of these animals were reduced from a range of 280-380 mg/dl blood glucose to less than 50 mg/dl. In test groups either cells or adult islets as positive control were then implanted under the renal capsule. One week later (T13) fasting blood glucose was measured and again at days 16, 21, and 28. Black squares represent placebo group (n = 5 mice) and as expected, in the absence of insulin, the blood glucose slowly climbed over time to well over 300 mg/dl. Animals (n = 5) implanted with insulin pellets only and no cell implants also performed as expected, with a transient rescue followed by diabetic rebound after the insulin release tablet had expired (red diamonds). Animals receiving islets (blue triangles, n = 5, 400

islets per animal) showed perfect long term rescue with fasting blood glucose being maintained at approximately 100 mg/dl. The single surviving animal receiving duct-derived cells (green circles, n = 1 of 7) showed a transient rescue of the diabetic state. The single animal demonstrated a 4-5 day lowering of > 150 mg/dl blood glucose before rebounding to pre-implant blood glucose levels.

Figure 41. Micrograph showing non-adherent spheres and attached cells during growth in expansion medium (test medium 3).

Figure 42. Micrographs showing non-adherent spheres during growth on test medium 1, test medium 2, or test medium 3.

Figure 43. Micrographs showing glut2 and Nestin protein expression in cells differentiated from non-adherent progenitor cells. Cells were plated on HTB9 matrix in the presence of PYY and forskolin, then fixed and stained with anti-Glut2 and/or anti-Nestin antibodies.

Figure 44. Micrographs showing PDX-1 and glucagon protein expression in cells differentiated from non-adherent progenitor cells. Cells were plated on HTB9 matrix in the presence of PYY and forskolin, then fixed and stained with anti-PDX-1 and/or anti-glucagon antibodies.

Figure 45. Micrographs showing PDX-1 and insulin protein expression in cells differentiated from non-adherent progenitor cells. Cells were plated on HTB9 matrix in the presence of PYY and forskolin, then fixed and stained with anti-PDX-1 and/or anti-insulin antibodies.

Figure 46A. Duct spheres in growth state and during differentiation were assayed for cell markers. Insulin positive cells increased dramatically during differentiation.

Figure 46B. Duct spheres in growth state and during differentiation were assayed for cell markers. Insulin positive cells increased dramatically during differentiation. Glut2 positive cells were rare prior to propagation of non-adherent cells but were abundant in the duct spheres and remained abundant during differentiation.

Figure 47. Micrographs showing Nestin protein expression in undifferentiated non-adherent progenitor cells. Spheres were dissociated, fixed and stained with anti-Nestin antibodies. Roughly 50% of cells are Nestin positive.

Figure 48. Micrograph showing non-adherent cell clusters in hemesphere culture #1.

Figure 49. Micrograph showing non-adherent cell clusters in hemesphere culture #1.

Figure 50. Micrograph showing non-adherent cell clusters in hemesphere culture #2.

Figure 51. FACS analysis of the starting material for hemesphere culture #1.

Figure 52. FACS analysis after Lin+ depletion of the starting material for hemesphere culture #1.

Figure 53. FACS analysis of hemesphere culture #1 after 14 days in culture.

Figure 54. FACS analysis of hemesphere culture #1 after 20 days in culture.

Figure 55. FACS analysis of the starting material for hemesphere culture #2.

Figure 56. FACS analysis after Lin+ depletion of the starting material for hemesphere culture #2.

Figure 57. FACS analysis of hemesphere culture #2 after 7 days in culture.

Figure 58. FACS analysis of hemesphere culture #2 after 11 days in culture.

Figure 59. Cells from hemesphere culture #1 at 20 days (left panel) and #2 at 13 days (right panel) were cytospun onto glass slides and stained with May-Grunwald Giemsa.

Figure 60. Micrograph showing that primary spheres differentiated for 4 days give rise to a large amount of cells that express both insulin and pdx-1 protein.

Figure 61. Micrograph showing insulin and Pdx-1 protein expression after a longer, 8 day differentiation. More than 50% of the cells are both insulin and pdx-1 positive.

Figure 62. Micrograph showing insulin and Glut2 protein expression after 8 days of differentiation. Interestingly, not all the insulin positive cells are co-expressing glut-2.

Figure 63. A plot showing protein expression of three markers, insulin, Pdx-1 and Glut-2, with their relative level in three different stages: freshly isolated duct cells at T=0, spheres in growth prior to differentiation, and spheres after 8 days differentiation. There are fewer than 3% insulin positive cells at T=0, ~6% insulin positive cells prior to differentiation, however, after 8 days

differentiation, the insulin positive cells went up to 56%.

Figure 64. Micrograph showing glucagon and Pdx-1 protein expression. When 15% KOSR was included in the culture medium in addition to the six growth factors, there were a large amount of glucagon cell expression after 12 days differentiation. Most glucagon expressing cells do not express pdx-1, which is a typical phenotype for a mature cell in the islet.

Figure 65. For the same growth factor condition as described in Fig.64, there are also a substantial amount of insulin expressing cells, that also express pdx-1.

Figure 66. Micrograph of spheres differentiated for 12 days in the presence of LIF and stained for expression somatostatin and Pdx-1 protein. Most Somatostatin expressing cells do not express pdx-1, but there are a few Somatostatin expressing cells that co-express pdx-1, suggesting they might be the precursor cells for mature Somatostatin cells and PP cells.

Figure 67. Graph of glucose-stimulated insulin release by differentiated cells.

Figure 68. Shows the insulin and pdx-1 co-expression cell after 45 days in culture.

Figure 69. Glucagon and Pdx-1 protein expression in cells cultured for 45 days in differentiation conditions. Most glucagon expressing cells do not express pdx-1, however, there are also a few glucagon expressing cells that co-express pdx-1, suggesting the possibility of the early precursor cells for mature β cells and α cells.

Figure 70. Somatostatin expression in cells from the culture shown in Fig. 69.

Figure 71. Shows that the cells from the above culture express the exocrine marker, Carboxypeptidase A (CPA). Further more, these CPA expressing cells appeared in the same cluster of cells that also express insulin, yet, the two proteins were not co-expressed. This strongly suggested that both of these endocrine and exocrine cells are derived from the same progenitor cells.

Figure 72A. Freshly isolated human pancreatic ducts. **B**, ductal cells in culture for 1-day begin to form small aggregates. **C**, ductal cells in culture for three days form spheres which are increasing in size.

Figure 73. Human duct spheres in growth medium.— Duct spheres were grown in serum-free growth medium as described above, and the cells were passaged every 5 days on average. There was an enrichment of CK-7, a duct epithelial marker, over time under this culture condition.

Figure 74. Human duct spheres in differentiation medium for 4 days. Duct spheres were seeded in 96-well plate coated with HTB-9 matrix in differentiation medium containing 5% FBS, 30 uM Forskolin and 200ng/ml PYY in Iscove's Modified Dulbecco's Medium with 25 mM glucose for four days. The cells were fixed with 1% PFA and co-stained for anti-insulin and anti-pdx-1 antibodies.

Figure 75. Human duct spheres in differentiation medium for 11 days. More insulin positive cells appeared after 11 days in culture in differentiation medium on HTB-9 matrix, the same differentiation condition described as above.

Figure 76. Human duct spheres in differentiation medium for 11 days. Cells co-stained with pdx-1 and somatostatin antibodies reveal disntictive somatostatin expressing and pdx-1 expressing cells. Cells expressing these two markers are in the same cell cluster but are not in the same cell. This suggests that these two cell types might derive from the same progenitor cells.

Figure 77. Human duct spheres in vitro differentiation for 11 days. Cells were co-stained with Glut2 and Vimentin antibodies, the later is a marker for fibroblasts. The figure illustrates that Glut2 expressing cells are in located in clusters, and are excluded from Vimentin positive cells.

Best Mode for Carrying Out the Invention

(i) Overview

Certain aspects of the present invention relate to isolated populations of progenitor cells capable of subsequent differentiation to distinct lineages, methods for isolating such cells and therapeutic uses for such cells. Stem cells are thought to exist in most adult and fetal tissues, but the stem cells are rare and difficult to enrich for. The invention is based in part on the surprising discovery that stem cells from many different tissue types all appear to share the common ability to proliferate in the absence of any

attachment (direct or indirect) to a substrate. This property can be used for enrichment of stem cells. Most cell types require a substrate for survival and/or growth. As a result, a suspension of mixed cells obtained from a tissue can be cultured in the absence of an adherent surface with the result that stem cells proliferate to form "spheres" in suspension, while other cells die or at least fail to grow. Continued selective growth of the stem cells results in a substantial enrichment of such non-adherent cells. Such a method can be employed to isolate a purified population of stem cells from any tissue including but not limited to myocardial tissue, lung tissue, kidney tissue, skeletal muscle tissue, and pancreatic tissue.

In one aspect, the invention in part provides novel methods for obtaining stem cells by utilizing their ability to proliferate in the absence of an adherent surface. In another aspect, the invention provides another novel method of isolating pancreatic progenitor cells using a monolayer system that can give rise to non-adherent cells. Stem cells obtained by the various methods of the invention may be used for many purposes.

The existence within the adult pancreas of a progenitor cell that is capable of giving rise to the endocrine islet was proposed long ago (e.g., Bensley, 1911). A number of regeneration models provide early *in vivo* evidence for islet neogenesis in the adult organ (Shaw and Latimer, 1925, Waren and Root 1925). Histological studies indicated a physical attachment between what was assumed to be newly forming islets and the ductal network. From these and more recent work (Bonner-Weir et al. 1993; Gu et al. 1994; Fernandes et al. 1997) developed the now widely held belief that islet progenitor cells derive from a subpopulation of the pancreatic duct epithelium.

The ductal network is one of three functional components of the adult pancreas (the other two being the exocrine acini and the endocrine islets), and is responsible for fluid secretion and delivery of digestive enzymes into the small intestine. Estimates of pancreatic ductal mass in the rat average 11%. The exocrine acini, which produce the digestive enzymes, make up by far the greatest portion of the adult pancreas, accounting for approximately 77-89% of overall tissue mass. The islets contain the insulin-secreting beta cells and are responsible for hormonal regulation of glucose metabolism; they comprise less than 5% of total organ mass (reviewed by Githens, 1988).

Pancreatic regeneration models such as 90% pancreatectomy (Bonner-Weir et al. 1993, Lampeter et al, 1995), duct ligation (Wang et al. 1995, Rosenberg 1995), and transgenic mice (Gu and Sarvetnick, 1993), all provide further *in vivo* evidence that islet tissue arises *de novo* from duct-associated pancreatic progenitor cells. A common observation in each of these injury models is the rapid appearance of endocrine cells in the proliferating ductal epithelium after experimental insult, and the subsequent appearance over the course of weeks of presumably newly formed islets in the periductular space. In addition, cells that appear to express both insulin and amylase, which mark endocrine and exocrine cells, respectively, have been observed during the regenerative process and it is thought that these cells might represent activated progenitor cells (Melmed, 1979; Cossel, 1984; Gu et al. 1994). The exact origin of these activated cells, whether ductal, acinar, or otherwise, and the mechanism of their activation, from progenitor cells or through selective dedifferentiation, remains to be determined. Despite these uncertainties, these studies underscore the potential islet neogenic capacity of the mature pancreas.

It has been proposed that during pancreatic development, early expression of PDX-1, a transcription factor that regulates expression of insulin and other beta cell components marks progenitor cells capable of giving rise to both the exocrine and endocrine compartments ((Ohlsson et al. 1993; Offield et al. 1996; Ahlgren et al. 1996, and reviewed by Madsen et al. 1996 and Edlund, 1998). Developmental mechanisms may also play a role in adult beta cell neogenesis. Indeed, Fernandes et al. (1997) describe the appearance of PDX-1 expressing cells in adult pancreatic ducts after streptozotocin insult. Another embryonic product is the hormone PYY, whose expression is also postulated to mark committed endocrine progenitor cells (Upchurch et al. 1994). Both of these cell types are found in islets as well as early in pancreatic development, and it is not yet clear if they contribute directly to islet formation. However they provide early markers against which regenerative systems can be analyzed.

(ii) Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The term "adherent matrix" refers to any matrix that promotes adherence of cells in culture (eg. fibronectin, collagen, laminins). Preferably the matrix is derived from the extracellular matrix of a cancerous cell line. Exemplary matrices include Matrigel (Beckton-Dickinson) and HTB9 matrix. Matrigel is derived from a mouse sarcoma cell line. HTB9 is derived from a bladder cell carcinoma line (US Patent 5,874,306).

As used herein the term "animal" refers to mammals, preferably mammals such as humans. Likewise, a "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal.

As used herein, the term "cellular composition" refers to a preparation of cells, which preparation may include, in addition to the cells, non-cellular components such as cell culture media, e.g. proteins, amino acids, nucleic acids, nucleotides, co-enzyme, anti-oxidants, metals and the like. Furthermore, the cellular composition can have components which do not affect the growth or viability of the cellular component, but which are used to provide the cells in a particular format, e.g., as polymeric matrix for encapsulation or a pharmaceutical preparation.

The term "culture medium" is recognized in the art, and refers generally to any substance or preparation used for the cultivation of living cells. Accordingly, a "tissue culture" refers to the maintenance or growth of tissue, e.g., explants of organ primordia or of an adult organ *in vitro* so as to preserve its architecture and function. A "cell culture" refers to a growth of cells *in vitro*; although the cells proliferate they do not organize into tissue *per se*.

Tissue and cell culture preparations of the subject micro-organ explants and amplified progenitor cell populations can take on a variety of formats. For instance, a "suspension culture" refers to a culture in which cells multiply while suspended in a suitable medium. Likewise, a "continuous flow culture" refers to the cultivation of cells or explants in a continuous flow of fresh medium to maintain cell growth, e.g. viability. The term "conditioned media" refers to the supernatant, e.g. free of the cultured cells/tissue, resulting after a period of time in contact with the cultured cells such that

the media has been altered to include certain paracrine and/or autocrine factors produced by the cells and secreted into the culture.

"Differentiation" in the present context 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.

The term "ED_{50}" means the dose of a drug which produces 50% of its maximum response or effect.

An "effective amount" of, e.g., a cAMP regulator, with respect to the subject method, refers to an amount of a cAMP elevating agent which, when added to pancreatic cell cultures, brings about a change in the rate of cell proliferation and/or the state of differentiation of a cell.

The term "explant" refers to a portion of an organ taken from the body and grown in an artificial medium.

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

"Hematopoietic stem cells" (HSCs) as used herein are stem cells that can give rise to cells of at least one of the major hematopoietic lineages in addition to producing daughter cells of equivalent potential. Three major lineages of blood cells include the lymphoid lineage, eg. B-cells and T-cells, the myeloid lineage, eg. monocytes, granulocytes and megakaryocytes, and the erythroid lineage, eg. red blood cells. Certain HSCs are capable of giving rise to many other cell types including brain cells. "Multipotent" or "pluripotent" HSCs are HSCs that can give rise to at least three of the major hematopoietic lineages.

The term "lineage committed cell" refers to a progenitor cell that has been induced to differentiate into a specific cell type, e.g., a pancreatic cell.

The term "liver" refers to the large, dark-red gland in the upper part of the abdomen on the right side, just beneath the diaphragm. Its manifold functions include

storage and filtration of blood, conversion of sugars into glycogen, and many other metabolic activities. It also supplies bile to intestine. In adult vertebrates, this function is a minor one, but the liver originally arose as a digestive gland in lower chordates. Throughout the liver, a network of tiny tubules collects bile--a solution of salts, bilirubin (made when hemoglobin from red blood cells is broken down in liver), and fatty acids. Bile accumulates in the gall bladder, which empties into the small intestine by way of a duct. Bile has two functions in the intestine. First, it acts as a detergent, breaking fat into small globules that can be attacked by digestive enzymes. Second, and more important, bile salts aid in the absorption of lipids from the intestine. Removal of the gall bladder sometimes causes difficulty with lipid absorption.

The term "organ" refers to two or more adjacent layers of tissue, which layers of tissue maintain some form of cell-cell and/or cell-matrix interaction to form a microarchitecture.

The term "primary culture" denotes a mixed cell population of cells that permits interaction of many different cell types isolated from a tissue. The word "primary" takes its usual meaning in the art of tissue culture. For example, a primary culture of pancreatic duct cells may allow the interaction between mesenchymal and epithelial cells.

The term "progenitor cell" is used synonymously with "stem cell". Both terms refer to an undifferentiated cell which is capable of proliferation and giving rise to more progenitor cells having the ability to generate a large number of mother cells that can in turn give rise to differentiated, or differentiable daughter cells. In a preferred embodiment, the term progenitor or stem cell refers to a generalized mother cell whose descendants (progeny) specialize, often in different directions, by differentiation, e.g., by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and tissues. Cellular differentiation is a complex process typically occurring through many cell divisions. A differentiated cell may derive from a multipotent cell which itself is derived from a multipotent cell, and so on. While each of these multipotent cells may be considered stem cells, the range of cell types each can give rise to may vary considerably. Some differentiated cells also have the capacity to

give rise to cells of greater developmental potential. Such capacity may be natural or may be induced artificially upon treatment with various factors.

"Proliferation" indicates an increase in cell number.

The term "tissue" refers to a group or layer of similarly specialized cells which together perform certain special functions.

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 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.

The endocrine portion of the pancreas is composed of the islets of Langerhans. The islets of Langerhans appear as rounded clusters of cells embedded within the exocrine pancreas. Four different types of cells- α, β, δ, and ϕ-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.

The term "pancreatic duct" includes the accessory pancreatic duct, dorsal pancreatic duct, main pancreatic duct and ventral pancreatic duct. Serous glands have extensions of the lumen between adjacent secretory cells, and these are called intercellular canaliculi. The term "interlobular ducts" refers to intercalated ducts and striated ducts found within lobules of secretory units in the pancreas. The "intercalated ducts" refers to the first duct segment draining a secretory acinus or tubule. Intercalated ducts often have carbonic anhydrase activity, such that bicarbonate ion may be added to the secretions at this level. "Striated ducts" are the largest of the intralobular duct components and are capable of modifying the ionic composition of secretions.

The term "pancreatic progenitor cell" refers to a cell which can differentiate into a cell of pancreatic lineage, e.g. a cell which can produce a hormone or enzyme normally produced by a pancreatic cell. For instance, a pancreatic progenitor cell may be caused to differentiate, at least partially, into α, β, δ, or ϕ islet cells, or a cell of exocrine fate. The pancreatic progenitor cells of the invention can also be cultured prior to administration to a subject under conditions which promote cell proliferation and differentiation. These conditions include culturing the cells to allow proliferation and confluence *in vitro* at which time the cells can be made to form pseudo islet-like aggregates or clusters and secrete insulin, glucagon, and somatostatin.

The term "substantially pure", with respect to progenitor cells, refers to a population of progenitor cells that is at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to progenitor cells making up a total cell population. Recast, the term "substantially pure" refers to a population of progenitor cells of the present invention that contain fewer than about 20%, more preferably fewer than about 10%, most preferably fewer than about 5%, of lineage committed cells in the original unamplified and isolated population prior to subsequent culturing and amplification.

(iii) Exemplary Embodiments

Certain terms being set out above, it is noted that one aspect of the present invention features a method for enriching for stem cells, and differentiated progeny thereof, from a range of tissue types. It is anticipated that the trait of nonadherency may

be used to isolate stem cells from essentially any tissue including, but not limited to the following tissue types: smooth muscle, striated muscle, cardiac muscle, bones (including marrow and spongy bone), cartilage, liver, pancreas (including ductal tissue), spleen, thymus, tonsils, Peyer's patches, lymph nodes, thyroid, epidermis, dermis, subcutaneous tissue, heart, lung, vascular tissue (including smooth muscle and endothelium), blood cells, bladder, kidney, digestive tract (including esophagus, stomach, small intestine, large intestine), adipose tissue, uterus, testis, ovaries, prostate, connective tissue, endocrine tissue, mesentery tissue, fetal tissue, umbilical tissue.

Various techniques may be employed to obtain suspensions of cells (both differentiated and undifferentiated) from tissues. Preferred isolation procedures are ones that result in as little cell death as possible. For example, the cells can be removed from the explant sample by mechanical means, e.g., mechanically sheared off with a pipette. In other instances, it will be possible to dissociate the progenitor cells from the entire explant, or sub-portion thereof, e.g., by enzymatic digestion of the explant, followed by isolation of the activated progenitor cell population based on specific cellular markers, e.g., using affinity separation techniques or fluorescence activated cell sorting (FACS). Cells may be obtained from liquid samples, such as blood, by centrifugation.

In general, the tissue is prepared using any suitable method, such as by gently teasing apart the excised tissue or by digestion of excised tissue with collagenase (for example, collagenase A), via, to illustrate, perfusion through a duct or simple incubation of, for example, teased tissue in a collagenase-containing buffer of suitable pH and tonic strength. The prepared tissue may then, optionally, be concentrated using suitable methods and materials, such as centrifugation through Ficol gradients for concentration (and partial purification). The concentrated tissue then is resuspended into any suitable vessel, such as tissue culture glassware or plasticware. In certain embodiments, the tissue is allowed to form a confluent monolayer culture, from which (non-adherent cells) NACs are formed. In other preferred embodiments, the cell suspension is placed in a non-adherent culture container and non-adherent spheres of progenitor cells are formed.

One salient feature of the subject method is that the starting material can be adult or fetal tissue or tissue from any developmental stage. Moreover, the method can be

practiced with relatively small amounts of starting material. Accordingly, small samples of tissue from a donor can be obtained without sacrificing or seriously injuring the donor. The progenitor cells of the present invention can be amplified, and subsequently isolated from a tissue sample.

In certain embodiments, the culture may be contacted with a growth factor or a composition comprising a growth factor, e.g., a mitogenic growth factor, e.g., the growth factor is selected from a group consisting of IGF-I, IGF-II, LIF, TGFα, TGFβ, bFGF, aFGF, EGF, PDGF, HGF, hedgehog or VEGF. In other embodiments, the growth factor is a member of the TGFβ superfamily, preferably of the DVR (dpp and vg1 related) family, e.g., BMP2 and/or BMP7.

In certain embodiments, the culture is contacted with a cAMP elevating agents, such as 8-(4-chlorophenylthio)-adenosine-3':5'-cyclic-monophosphate (CPT-cAMP) (see, for example, Koike Prog. Neuro-Psychopharmacol. and Biol. Psychiat. 16 95-106 (1992)), CPT-cAMP, forskolin, Na-Butyrate, isobutyl methylxanthine (IBMX) and cholera toxin (see Martin et al. J. Neurobiol. 23 1205-1220 (1992)) and 8-bromo-cAMP, dibutyryl-cAMP and dioctanoyl-cAMP (e.g., see Rydel et al. PNAS 85:1257 (1988)).

In certain embodiments, the culture is contacted with a steroid or corticosteroid such as, for example, hydrocortisone, deoxyhydrocortisone, fludrocortisone, prednisolone, methylprednisolone, prednisone, triamcinolone, dexamethasone, betamethasone and paramethasone. See, generally, The Merck Manual of Diagnosis and Therapy, 15th Ed., pp. 1239-1267 and 2497-2506, Berkow et al., eds., Rahay, N.J., 1987).

In a preferred embodiment, the cultures are contacted with a cAMP elevating agent, a growth factor and a steroid or corticosteroid, e.g., with the DCE cocktail described herein.

Alternatively, or in addition, treatment with cAMP upregulating agents can be used as described above to induce differentiation. The cell products of such a method can include insulin-producing cells, and more preferably, glucose-responsive insulin-producing cells.

There are a large number of tissue culture media that exist for culturing tissue from animals. Some of these are complex and some are simple. While it is expected that the ductal epithelial explants may grow in complex media, it will generally be preferred that the explants be maintained in a simple medium, such as Dulbecco's Minimal Essential Media (DMEM), in order to effect more precise control over the activation of certain progenitor populations in the explant. In a preferred embodiment, pancreatic ductal epithelium is cultured in Isocove's modified MEM cell culture medium with 5% FBS. Moreover, the explants can be maintained in the absence of sera for extended periods of time. In preferred embodiments of the invention, the growth factors or other mitogenic agents are not included in the primary media for maintenance of the cultures *in vitro*, but are used subsequently to cause proliferation of distinct populations of progenitor cells. See the appended examples.

In a preferred embodiment, stem cells are enriched because of their ability to grow in the absence of adherence, either direct or indirect, to a culture surface. In other words, one aspect of the invention requires that the stem cells are obtained from a culture wherein said stem cells are free floating and are not in fixed or semi-fixed contact with a surface of the culture vessel or with cells or materials that are in contact

In an alternative embodiment, progenitor cells can be isolated from pancreatic ducts using a monolayer intermediate stage. Pancreatic ducts in their entirety were chosen as a source of stem cells because it was considered that the duct fragment inclusive of its component epithelium and mesenchyme might be the basic biological unit that contains endocrine progenitor cell activity. The dependence of pancreatic epithelium on its surrounding mesenchyme for survival and growth during development was first demonstrated by the early work of Goloslow and Grobstein (1962) and others (Wessels and Cohen, 1967). More recent work using gene knockout technology demonstrated that the loss of dorsal pancreatic mesenchyme correlated with the loss of the developing pancreatic epithelium (Ahlgren, et al. 1997) and consequently the absence of pancreas formation from the dorsal bud. Respecification of pancreatic mesenchyme identity to smooth muscle by sonic hedgehog protein also resulted in deranged pancreatic epithelial outgrowth (Apelqvist et al. 1997). From these results in

the developing pancreas, it was hypothesized that the relationship between mesenchyme and epithelium may continue to be functionally important in the adult pancreas, particularly with respect to islet neogenesis and regrowth.

Freshly isolated duct fragments are comprised of a single epithelial layer surrounded by mesenchymal stroma and contain few, if any, differentiated endocrine cells. After the ducts are grown in culture, we observed the presence of multiple endocrine cell types and also potential progenitor cells coexpressing markers such as insulin and amylase, which might contribute to the formation of differentiated beta cells. In addition, we observed the emergence of a nonadherent cell type previously undescribed in pancreatic cell culture. The number and properties of this novel cell type are affected by the addition of various factors, one combination of which reproducibly leads to the formation of functional, glucose-responsive beta-like cells. Our data thus suggest the presence and induction of pancreatic progenitor cell activities in this duct culture system, which now makes possible the *in vitro* study of beta cell neogenesis and also provides a first step in the process of producing beta cells for the treatment of insulin-dependent diabetes.

This shows for the first time that functional beta-like cells can be obtained via *in vitro* duct culture, suggests the presence and activation of a progenitor cell from pancreatic ducts, and provides a system for the isolation and manipulation of those cells. In one preferred embodiment, the subject method can be used to produce islet-like cell clusters ("ICC") containing a high percentage of β-epithelial cells with increased insulin production.

Moreover, as demonstrated in the appended examples, the subject cellular compositions can be used to rescue diabetic mice.

Accordingly, in one embodiment, the invention provides a method for isolating pancreatic progenitor cells. In general, the method includes the steps of obtaining pancreatic ductal cells; culturing the pancreatic cells in a suitable nutrient medium; isolating a population of progenitor cells from said culture. In preferred embodiments, the ductal epithelial cells are obtained from intralobular ducts. For instance, the pancreatic ductal epithelial cells can be obtained by enzymatic digestion or other

mechanical separation of ductal fragments. The pancreatic ductal cells are grown to confluence, e.g., preferably in a monolayer. Viable, non-adherent cells can be isolated from the culture, optionally after treatment of the culture with an agent(s) that induce proliferation/differentiation of pancreatic progenitor cells from the adherent epithelial cells. As described below, the non-adherent cell population is enriched for pancreatic progenitor cells.

Another aspect of the present invention relates to our finding that cAMP elevating agents can be used to promote proliferation and differentiation of pancreatic progenitor cells. In this regard, the invention relates to the use of a cAMP elevating agents to induce ex vivo the proliferation and differentiation of pancreatic cells prior to their transplantation into a diabetic subject. In yet other embodiments, the invention contemplates the *in vivo* administration of cAMP agonists to patients which have been transplanted with pancreatic tissue, as well as to patients which have a need for improved pancreatic performance, or are at risk for developing functional deficits in the organ, especially of glucose-dependent insulin secretion, e.g., the subject method can be used prophylactically.

Another salient feature of certain embodiments of the subject method concerns the use of defined culture conditions for isolating and propagating discrete pancreatic progenitor cell populations.

For instance, as described below, the progenitor source ductal tissue explants preferably are digested or otherwise teased apart, thereby providing purified ductal properties, which in turn are placed in the culture medium and grown. The pancreatic duct preparations are permitted to expand and form a monolayer of cells in culture, e.g., a ductal epithelial monolayer. In preferred embodiments, the majority (e.g., >25 percent, more preferably >10%) of the cells are vimentin-positive, non-endocrine and proliferative. Viable non-adherent cells (NACs) can be isolated from the culture of otherwise adherent pancreatic cells. As described below, these NAC preparations are enriched for pancreatic progenitor cells.

We have also discovered that ductal cells of the subject cultures, and the NACs arising in the culture, can be purified based on binding to lectins. For example,

fluorescently labeled lectins can be used to, e.g., facilitate FACS or other cell sorting. In other embodiments, the lectin can be derivatized for immobilization, e.g., on a solid surface such as a filter or bead, and used for affinity purification of the cells. Exemplary lectins for these purposes include the fluorescent lectins, peroxidase conjugated lectins and biotinylated lectins marketed by Vector Laboratories, Inc. of Burlingame, CA. In preferred embodiments, the lectin is a plant lectin, and more preferably to a peanut agglutinin. In other embodiments, the lectin is selected from the group consisting of Aleuria Aurantia Lectin (AAL); Amaranthus Caudatus Lectin (ACL, ACA); Bauhinia Purpurea Lectin (BPL, BPA); Concanavalin A (Con A); Succinylated Concanavalin A (Con A); Datura Stramonium Lectin (DSL); Dolichos Biflorus Agglutinin (DBA); Erythrina Cristagalli Lectin (ECL, ECA); Euonymus Europaeus Lectin (EEL); Galanthus Nivalis Lectin (GNL); Griffonia (Bandeiraea) Simplicifolia Lectin I (GSL I, BSL I); Isolectin-B4; Griffonia (Bandeiraea) Simplicifolia Lectin II (GSL II, BSL II); Hippeastrum Hybrid Lectin (HHL, AL); Lens Culinaris Agglutinin (LCA, LcH); Lotus Tetragonolobus Lectin (LTL); Lycopersicon Esculentum (Tomato) Lectin (LEL, TL); Maackia Amurensis Lectin I (MAL I); Maackia Amurensis Lectin II (MAL II); Maclura Pomifera Lectin (MPL); Narcissus Pseudonarcissus Lectin (NPL, NPA, DL); Peanut Agglutinin (PNA); Phaseolus Vulgaris Agglutinin (PHA); Pisum Sativum (PSA); Psophocarpus Tetragonolobus Lectin I (PTL I, WBA I); Psophocarpus Tetragonolobus Lectin II (PTL II, WBA II); Ricinus Communis Agglutinin I (RCA I, RCA120); Ricinus Communis Agglutinin II (RCA II, RCA60, ricin); Sambucus Nigra (EBL, SNA); Solanum Tuberosum (Potato) Lectin (STL, PL); Sophora Japonica Agglutinin (SJA); Soybean Agglutinin (SBA); Ulex Europaeus Agglutinin I (UEA I); Ulex Europaeus Aggluutinin II (UEA II); Vicia Villosa Lectin (VVA, VVL); Wheat Germ Agglutinin (WGA); Succinylated Wheat Germ Agglutinin; and Wisteria Floribunda Lectin (WFA, WFL).

In certain embodiments, the dissociated monolayers derived from the ductal explants can be sorted by presence of A2B5 epitope, e.g., the ability to be bound by the A2B5 monoclonal antibody (Eisenbarth et al., (1979) PNAS 76:4913), or other glycolipids present on astrocytes, such as gangliosides like GM3 or GD3.

Moreover, we have unexpectedly found that a combination of growing such cells as monolayers, with treatment of such cultures with cAMP elevating agents produces an increased induction of NACs, e.g., pancreatic progenitor cells. Accordingly, when the ductal cells have grown to confluence (cells covering the surface of a culture plate), the cells can be treated with a cAMP elevating agent in order to cause differentiation of certain cells in the culture into non-adherent progenitor cells, and subsequently into insulin-producing or other endocrine or exocrine cells. Accordingly, carefully defined conditions can be acquired in the culture so as selectively activate discrete populations of cells in the tissue explant. The progenitor and differentiated cells of the present invention can be amplified, and subsequently isolated from the culture.

In general, in such an expanded culture procedure a commercial-sized bioreactor, such as the OPTICAL TM culture system, Model 5300E (Charles River Labs.; Wilmington, Mass.), or the CELLMAX TM QUAD cell culture system (Cellco, Inc.; Germantown, Md.), is seeded with a primary culture of human pancreatic cells. The bioreactor is perfused with a suitable, complete growth medium supplemented with an appropriately effective concentration of mitogens, and as appropriate, cAMP elevating agents. The β-epithelial cell-containing islet-like clusters can then be harvested. Cells may be cryopreserved prior to use as described, for example, by Beattie et al., Transplantation 56: 1340 (1993).

The cultures may be maintained in any suitable culture vessel, such as a 12 or 24 well microplate, and may be maintained under typical culture conditions for cells isolated from the same animal, e.g., such as 37°C in 5% CO_2. The cultures may be shaken for improved aeration, the speed of shaking being, for example, 12 rpm.

In order to isolate progenitor cells from the ductal cultures, it will generally be desirable to contact the explant with an agent which causes proliferation of one or more populations of progenitor cells in the explant. For instance, a mitogen, e.g., a substance that induces mitosis and cell transformation, can be used to detect a progenitor cell population in the explant, and where desirable, to cause the amplification of that population. To illustrate, a purified or semi-purifed preparation of a growth factor can be applied to the culture. Induction of progenitor cells which respond to the applied

growth factor can be detected by proliferation of the progenitor cells. However, as described below, amplification of the population need not occur to a large extent in order to use certain techniques for isolating the responsive population.

In yet other embodiments, the ductal explants and/or amplified progenitor cells can be cultured on feeder layers, e.g., layers of feeder cells which secrete inductive factors or polymeric layers containing inductive factors. For example, a matrigel layer can be used to induce hematopoietic progenitor cell expansion, as described in the appended examples. Matrigel (Collaborative Research, Inc., Bedford, Mass.) is a complex mixture of matrix and associated materials derived as an extract of murine basement membrane proteins, consisting predominantly of laminin, collagen IV, heparin sulfate proteoglycan, and nidogen and entactin was prepared from the EHS tumor as described Kleinman et al, "Basement Membrane Complexes with Biological Activity", Biochemistry, Vol. 25 (1986), pages 312-318. Other such matrixes can be provided, such as Humatrix. Likewise, natural and recombinantly engineered cells can be provided as feeder layers to the instant cultures.

As described in further detail below, it is contemplated that the subject methods can be carried out using cyclic AMP (cAMP) agonists to induce differentiation of the cultures cells of endocrine or exocrine phenotypes. In yet other embodiments, the invention contemplates the *in vivo* administration of cAMP agonists to patients which have been transplanted with pancreatic tissue, as well as to patients which have a need for improved pancreatic performance, especially of glucose-dependent insulin secretion.

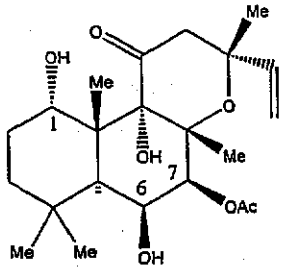
In light of the present disclosure, it will be apparent to those in the art that a variety of different small molecules can be readily identified, for example, by routine drug screening assays, which upregulate cAMP-dependent activities. For example, the subject method can be carried out using compounds which may activate adenylate cyclase including forskolin (FK), cholera toxin (CT), pertussis toxin (PT), prostaglandins (e.g., PGE-1 and PGE-2), colforsin and β-adrenergic receptor agonists. β -Adrenergic receptor agonists (sometimes referred to herein as "β-adrenergic agonists") include albuterol, bambuterol, bitolterol, carbuterol, clenbuterol, clorprenaline, denopamine, dioxethedrine, dopexamine, ephedrine, epinephrine, etafedrine,

ethylnorepinephrine, fenoterol, formoterol, hexoprenaline, ibopamine, isoetharine, isoproterenol, mabuterol, metaproterenol, methoxyphenamine, oxyfedrine, pirbuterol, prenalterol, procaterol, protokylol, reproterol, rimiterol, ritodrine, soterenol, salmeterol, terbutaline, tretoquinol, tulobuterol, and xamoterol.

Compounds which may inhibit cAMP phosphodiesterase(s), and thereby increase the half-life of cAMP, are also useful in the subject method. Such compounds include amrinone, milrinone, xanthine, methylxanthine, anagrelide, cilostamide, medorinone, indolidan, rolipram, 3-isobutyl-1-methylxanthine (IBMX), chelerythrine, cilostazol, glucocorticoids, griseolic acid, etazolate, caffeine, indomethacin, theophylline, papverine, methyl isobutylxanthine (MIX), and fenoxamine.

Certain analogs of cAMP, e.g., which are agonists of cAMP, can also be used. Exemplary cAMP analogs which may be useful in the present method include dibutyryl-cAMP (db-cAMP), (8-(4)-chlorophenylthio)-cAMP (cpt-cAMP), 8-[(4-bromo-2,3-dioxobutyl)thio]-cAMP, 2-[(4-bromo-2,3-dioxobutyl)thio]-cAMP, 8-bromo-cAMP, dioctanoyl-cAMP, Sp-adenosine 3':5'-cyclic phosphorothioate, 8-piperidino-cAMP, N^6-phenyl-cAMP, 8-methylamino-cAMP, 8-(6-aminohexyl)amino-cAMP, 2'-deoxy-cAMP, N^6,2'-O-dibutryl-cAMP, N^6,2'-O-disuccinyl-cAMP, N^6-monobutyryl-cAMP, 2'-O-monobutyryl-cAMP, 2'-O-monobutryl-8-bromo-cAMP, N^6-monobutryl-2'-deoxy-cAMP, and 2'-O-monosuccinyl-cAMP.

Above-listed compounds useful in the subject methods may be modified to increase the bioavailability, activity, or other pharmacologically relevant property of the compound. For example, forskolin has the formula:



Forskolin

Modifications of forskolin which have been found to increase the hydrophilic character of forskolin without severely attenuating the desired biological activity include acylation of the hydroxyls at C6 and/or C7 (after removal of the acetyl group) with hydrophilic acyl groups. In compounds wherein C6 is acylated with a hydrophilic acyl group, C7 may optionally be deacetylated. Suitable hydrophilic acyl groups include groups having the structure $-(CO)(CH_2)_nX$, wherein X is OH or NR_2; R is hydrogen, a C_1-C_4 alkyl group, or two Rs taken together form a ring comprising 3-8 atoms, preferably 5-7 atoms, which may include heteroatoms (e.g., piperazine or morpholine rings); and n is an integer from 1-6, preferably from 1-4, even more preferably from 1-2. Other suitable hydrophilic acyl groups include hydrophilic amino acids or derivatives thereof, such as aspartic acid, glutamic acid, asparagine, glutamine, serine, threonine, tyrosine, etc., including amino acids having a heterocyclic side chain. Forskolin, or other compounds listed above, modified by other possible hydrophilic acyl side chains known to those of skill in the art may be readily synthesized and tested for activity in the present method.

Similarly, variants or derivatives of any of the above-listed compounds may be effective as cAMP agonists in the subject method. Those skilled in the art will readily be able to synthesize and test such derivatives for suitable activity.

In certain embodiments, the subject cAMP agonists can be chosen on the basis of their selectivity for cAMP activation.

In certain embodiments, it may be advantageous to administer two or more of the above cAMP agonists, preferably of different types. For example, use of an adenylate cyclase agonist in conjunction with a cAMP phosphodiesterase antagonist may have an advantageous or synergistic effect.

In certain preferred embodiments, the subject agents raise effective cAMP levels with an ED_{50} of 1 mM or less, more preferably of 1 μM or less, and even more preferably of 1 nM or less.

In certain embodiments of the subject method, it will be desirable to monitor the growth state of cells in the culture, e.g., cell proliferation, differentiation and/or cell death. Methods of measuring cell proliferation are well known in the art and most

commonly include determining the DNA synthesis characteristic of cell replication. There are numerous methods in the art for measuring DNA synthesis, any of which may be used according to the invention. In an embodiment of the invention, DNA synthesis has been determined using a radioactive label (^{3}H-thymidine) or labeled nucleotide analogues (BrdU) for detection by immunofluorescence.

However, in addition to measuring DNA synthesis, morphological changes can be, and preferably will be, relied on as the basis for isolating responsive progenitor cell populations. For instance, as described in the appended examples, we have observed that certain growth factors cause amplification of progenitor cells in ductal explants so as to form structures that can be easily detected by the naked eye or microscopy. In an exemplary embodiment, those progenitor cells which respond to growth factors by proliferation and subsequent formation of outgrowths from the explant, e.g., buds or blebs, can be easily detected. In another illustrative embodiment, other structural changes, e.g., changes in optical density of proliferating cells, can be detected via contrast microscopy.

To further illustrate, ICCs can be incubated with bromodeoxyuridine ("BrdU"), fixed in formaldehyde, embedded in paraffin and sectioned. Sections can be stained for insulin using an immunoalkaline phosphatase technique described, for example, by Erber et al., Am. J. Clin. Path. 88: 43 (1987), using polyclonal guinea pig anti-porcine insulin (Chemicon; El Sequndo, Calif.) as the primary antibody.

Cell nuclei that have incorporated BrdU during DNA synthesis can be identified using mouse monoclonal anti-BrdU (Dako; Carpintaria, Calif.), detected with the immuno-peroxide technique of Sternberger et al., J. Histochem., Cytochem. 18: 315 (1970), followed by hematoxylin counterstaining.

Epithelial cells can be identified on separate sections using a mouse monoclonal anti-epithelial antigen antibody (Ber-EP4, Dako, above) as the primary antibody.

Surface areas of insulin-positive and epithelial cells, calculated as percent of the total ICC area, can be quantified with a computerized image analyzer (American Innovision; San Diego, Calif.). The same method can be used for the determination of

the BrdU labeling index. Cells positive for both insulin and BrdU may also be recorded in separate sections of the same samples after double staining of the two antigens.

Mean cell size can be calculated by the ratio of total ICC area to the number of nuclei.

Mean beta -cell size can be estimated by measuring the surface area of individual insulin-positive cells.

A sufficient number of ICC sections (at least 15) and nuclei (at least 1000) should be analyzed for each sample to correct for biological and experimental variability of the samples.

To further illustrate, the examples below demonstrate that ductal explants contain growth factor responsive progenitor cell types. It is further demonstrated that different growth factors can induce/amplify distinct populations of progenitor cells within the ductal tissue explant to proliferate. This indicates the presence of specific growth factor receptors on the surface of distinct progenitor cell populations. This is important because the expression of these receptors mark the progenitor cell populations of interest. Monoclonal antibodies are particularly useful for identifying markers (surface membrane proteins, e.g., receptors) associated with particular cell lineages and/or stages of differentiation. Procedures for separation of the subject progenitor cell may include magnetic separation, using antibody coated magnetic beads, affinity chromatography, and "panning" with antibody attached to a solid matrix, e.g., plate, or other convenient technique. Techniques providing accurate separation include fluorescence activated cell sorting, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

Conveniently, the antibodies may be conjugated with markers, such as magnetic beads, which allow for direct separation, biotin, which can be removed with avidin or streptavidin bound to a support, fluorochromes, which can be used with a fluorescence activated cell sorter, or the like, to allow for ease of separation of the particular cell type. Any technique may be employed which is not unduly detrimental to the viability of the cells.

In an illustrative embodiment, some of the antibodies for growth factor receptors that exist on the subject progenitor cells are commercially available (e.g., antibodies for EGF receptors, FGF receptors and/or TGF receptors), and for other growth factor receptors, antibodies can be made by methods well known to one skilled in the art. In addition to using antibodies to isolate progenitor cells of interest, one skilled in the art can also use the growth factors themselves to label the cells, for example, to permit "panning" processes.

Upon isolation, the progenitor cells of the present invention can be further characterized in the following manner: responsiveness to growth factors, specific gene expression, antigenic markers on the surface of such cells, and/or basic morphology.

For example, extent of growth factor responsivity, e.g., the concentration range of growth factor to which they will respond to, the maximal and minimal responses, and to what other growth factors and conditions to which they might respond, can be used to characterize the subject progenitor cells.

Furthermore, the isolated progenitor cells can be characterized by the expression of genes known to mark the developing (i.e., stem or progenitor) cells for the pancreas.

Once isolated and characterized, the subject progenitor cells can be cultured under conditions which allow further differentiation into specific cell lineages. This can be achieved through a paradigm of induction that can be developed. For example, the subject progenitor cells can be recombined with the corresponding embryonic tissue to see if the embryonic tissue can instruct the adult cells to codevelop and codifferentiate. Alternatively, the progenitor cells can be contacted with one or more growth or differentiation factors which can induce differentiation of the cells. For instance, the cells can be treated with an agent such as Forskolin, Di-butyrl cAMP, Na-Butyrate, dexamethasone or cholera toxin, or a growth factor such as TGFβ or a DVR sub-family member. It has been observed that a media comprising a cAMP elevating agent and PYY promotes differentiation into various pancreatic cell types. Preferred media comprise fetal bovine serum, of at least 1%, 2%, 3%, 4% or at least about 5% concentration. An adherent matrix may also assist in promoting differentiation.

Preferred adherent matrices include HTB9, derived from a bladder cancer cell line, and Matrigel, derived from a mouse sarcoma.

In a further embodiment, the invention provides the observation that treatment of progenitor cells during propagation with different growth factors can then cause the cells to give rise to different differentiated cell types after exposure to differentiation conditions. For example, LIF administered in the growth medium promotes formation of somatostatin-expressing cells upon differentiation. As another example, KO-SR administered in the growth medium promotes formation of glucagon-expressing cells. KO-SR is Knockout™ serum replacement provided by Gibco BRL. KO-SR is designed to replace FBS and is a defined mixture of ingredients further described in Goldsborough, M., Tilkins, M. L., Price, P., Lobo-Alfonso, J., Morrison, J., Stevens, M., Meneses, J., Pederson, R., Koller, B., and Latour, A. (1998) Focus® 20, 8.

In an illustrative embodiment, the hepatocyte nuclear factor (HNF) transcription factor family, e.g., HNF1-4, are known to be expressed in various cell types at various times during pancreas development. For example, the progenitor cell may express one or more HNF protein such as HNF1α, HNF1β, HNF3β, HNF3γ, and/or HNF4. The glucose transporter Glut2 is also a marker for both early pancreatic cells. Certain of the "forkhead" transcription factors, such as fkh-1 or the like, are understood to be markers in early gut tissue.

In another illustrative embodiment, homeodomain type transcription factors such as STF-1 (also known as IPF-1, IDX-1 or PDX) have recently been shown to mark different populations of the developing pancreas. Some LIM genes have also been shown to regulate insulin gene expression and would also be markers for protodifferentiated β islet cells. Likewise, certain of the PAX genes, such as PAX6, are expressed during pancreas formation and may be used to characterize certain pancreatic progenitor cell populations. Other markers of pancreatic progenitor cells include the pancreas specific transcription factor PTF-1, and hXBP-1 and the like. Moreover, certain of the HNF proteins are expressed during early pancreas development and may be used as markers for pancreatic progenitor cells.

Progenitor cells giving rise to pancreatic cells may also express markers such as villin and/or tyrosine hydroxylase, as well as secrete such factors as insulin, glucagon and/or neuropeptide Y.

Other markers which can be scored in the NACs include: Rab3A (Zahraoui et al. (1989) J. Biol. Chem. 12:394; Baldini et al. (1995) PNAS 92:4284); vesicle-associated membrane protein 2 (VAMP2, Fujita-Yoshigaki et al. (1996) J. Biol. Chem. 271:13130; and Nielsen et al. (1995) J Clin Invest 96:1834); amylin, and/or A2B5 (Eisenbarth et al., (1979) PNAS 76:4913)

In other embodiments, the subject cultures of small ductal epithelial cells, as well as possibly the pancreatic progenitor cells arising therefrom, are characterized by binding to lectin(s), and preferably to a plant lectin, and more preferably to peanut agglutinin. In a preferred embodiment, the lectin is peanut agglutinin. In other embodiments, the lectin is selected from the group consisting of Aleuria Aurantia Lectin (AAL); Amaranthus Caudatus Lectin (ACL, ACA); Bauhinia Purpurea Lectin (BPL, BPA); Concanavalin A (Con A); Succinylated Concanavalin A (Con A); Datura Stramonium Lectin (DSL); Dolichos Biflorus Agglutinin (DBA); Erythrina Cristagalli Lectin (ECL, ECA); Euonymus Europaeus Lectin (EEL); Galanthus Nivalis Lectin (GNL); Griffonia (Bandeiraea) Simplicifolia Lectin I (GSL I, BSL I); Isolectin-B4; Griffonia (Bandeiraea) Simplicifolia Lectin II (GSL II, BSL II); Hippeastrum Hybrid Lectin (HHL, AL); Lens Culinaris Agglutinin (LCA, LcH); Lotus Tetragonolobus Lectin (LTL); Lycopersicon Esculentum (Tomato) Lectin (LEL, TL); Maackia Amurensis Lectin I (MAL I); Maackia Amurensis Lectin II (MAL II); Maclura Pomifera Lectin (MPL); Narcissus Pseudonarcissus Lectin (NPL, NPA, DL); Peanut Agglutinin (PNA); Phaseolus Vulgaris Agglutinin (PHA); Pisum Sativum (PSA); Psophocarpus Tetragonolobus Lectin I (PTL I, WBA I); Psophocarpus Tetragonolobus Lectin II (PTL II, WBA II); Ricinus Communis Agglutinin I (RCA I, RCA120); Ricinus Communis Agglutinin II (RCA II, RCA60, ricin); Sambucus Nigra (EBL, SNA); Solanum Tuberosum (Potato) Lectin (STL, PL); Sophora Japonica Agglutinin (SJA); Soybean Agglutinin (SBA); Ulex Europaeus Agglutinin I (UEA I); Ulex Europaeus Aggluutinin

II (UEA II); Vicia Villosa Lectin (VVA, VVL); Wheat Germ Agglutinin (WGA); Succinylated Wheat Germ Agglutinin; and Wisteria Floribunda Lectin (WFA, WFL).

For instance, as shown in the attached figures, various components of human pancreas can be marked by different lectins. DSL marks inter- and intralobular ducts. LCA appears to mark mesenchyme. ECL marks intralobular ducts without marking larger ducts. Succinylated-Wheat Germ Agglutinin marks a subset of main duct cells and is quite restricted compared to WGA.

In another example, mammalian blood cells can be used to obtain stem cells as per the methods provided herein. Stem cells derived from blood can provide for an extraordinarily diverse range of cell types. Three major lineages of blood cells include the lymphoid lineage, eg. B-cells and T-cells, the myeloid lineage, eg. monocytes, granulocytes and megakaryocytes, and the erythroid lineage, eg. red blood cells. Hematopoietic stem cells (HSCs) are cells that can give rise to cells of at least two of the above lineages in addition to producing daughter cells of equivalent multipotency. In preferred embodiments, the HSCs can give rise to three major blood cell lineages.

HSCs may be isolated from suspensions of a variety of tissue types. Bone marrow cells are a good source of HSCs. Bone marrow cells may be obtained from a source of bone marrow, e.g., iliac crests, tibiae, femora, spine, or other bone cavities. Other sources of human hematopoietic stem cells include embryonic yolk sac, fetal liver, fetal and adult spleen, blood, including adult peripheral blood and umbilical cord blood.

HSCs can be identified both by the types of cells they give rise to and by various cytological markers. HSCs often extrude certain dyes, such as Hoechst 33324 and Rhodamine 123 (Bhatia et al. (1998) *Nature Med.* 4:1038). Such dye staining properties can be used to identify HSCs among other cells of the circulatory system. Antibodies that react with certain cell markers can also be used to identify and purify HSCs. For example, mAb AC133 is thought to specifically bind to HSCs (Miraglia et al. (1997) *Blood* 90:5013). The Thy-1 molecule is a highly conserved protein present in the brain and hematopoietic system of rat, mouse and man. The Thy-1 molecule has been identified on rat, mouse and human HSCs and can be useful in identifying HSCs (U.S.

Patent No. 5,914,108). Many HSCs are CD34+ and/or CD38+ as well (U.S. Patent No. 5,840,580). A population of HSCs will often have some variation in cell surface markers and a positive identification may be made on the basis of the presence of at least two of the above cytological markers.

HSCs can also be distinguished from other more differentiated cell types by the absence of certain markers. CD3, CD7, CD8, CD10, CD14, CD15, CD19, CD20 and CD33 are all typically absent from HSCs. The absence of several of the above markers adds confidence to the identification of HSCs. Morphology may also help distinguish an HSC, as described above.

It is understood that HSCs may be identified by an aggregation of multiple traits, such as morphology, the presence of certain markers and the absence of other markers. A positive identification does not typically require detection of all of the above markers.

The culturing of HSCs to give rise to differentiated stem cells can be achieved in many ways. For example, cells may be cultured in a defined, enriched medium such as Iscove's Modified Dulbecco's Medium (IMDM), generally composed of salts, amino acids, vitamins, antibiotics and fetal calf serum. Cultures supplemented with hydrocortisone tend to give rise to myeloid cells, while cultures lacking cortisone tend to give rise to B lymphocytes. To demonstrate that HSCs can develop in cells of the erythroid lineage, various conventional methods can be used. For example culturing on methylcellulose culture can stimulate formation of erythroid cells. (U.S. Patent Nos. 5,840,580 and 5,914,108; Metcalf (1977) In: Recent Results in Cancer Research 61. Springer-Verlag Berlin, pp. 1-227).

In a further example, stem cells may be isolated from liver tissue. Such cells may be identified by certain markers. Such cells will typically express Sca-1and c-kit, but will not express Lin.

In another preferred embodiment, the subject progenitor cells can be implanted into one of a number of regeneration models used in the art, e.g., a host animal which has undergone partial pancreatectomy or streptozocin treatment or radiation killing of bone marrow cells.

Accordingly, another aspect of the present invention pertains to the progeny of

the subject progenitor cells, e.g. those cells which have been derived from the cells of the initial explant culture. Such progeny can include subsequent generations of progenitor cells, as well as lineage committed cells generated by inducing differentiation of the subject progenitor cells after their isolation from the explant, e.g., induced *in vitro*.

Another aspect of the invention relates to cellular compositions enriched for progenitor cells, or the progeny thereof. In certain embodiments, the cells will be provided as part of a pharmaceutical preparation, e.g., a sterile, free of the presence of unwanted virus, bacteria and other (human) pathogens, as well as pyrogen-free preparation. That is, for human administration, the subject cell preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

In certain embodiments, such cellular compositions can be used for transplantation into animals, preferably mammals, and even more preferably humans. The cells can be autologous, allogeneic or xenogeneic with respect to the transplantation host. In one aspect, the present invention relates to transplantation of fetal or mature pancreatic cells to treat Type 1 diabetes mellitus.

Yet another aspect of the present invention concerns cellular compositions which include, as a cellular component, substantially pure preparations of the subject progenitor cells, or the progeny thereof. Cellular compositions of the present invention include not only substantially pure populations of the progenitor cells, but can also include cell culture components, e.g., culture media including amino acids, metals, coenzyme factors, as well as small populations of non-progenitor cells, e.g, some of which may arise by subsequent differentiation of isolated progenitor cells of the invention. Furthermore, other non-cellular components include those which render the cellular component suitable for support under particular circumstances, e.g., implantation, e.g., continuous culture.

As common methods of administering the progenitor cells of the present invention to subjects, particularly human subjects, which are described in detail herein, include injection or implantation of the cells into target sites in the subjects, the cells of

the invention can be inserted into a delivery device which facilitates introduction by, injection or implantation, of the cells into the subjects. Such delivery devices include tubes, e.g., catheters, for injecting cells and fluids into the body of a recipient subject. In a preferred embodiment, the tubes additionally have a needle, e.g., a syringe, through which the cells of the invention can be introduced into the subject at a desired location. The progenitor cells of the invention can be inserted into such a delivery device, e.g., a syringe, in different forms. For example, the cells can be suspended in a solution or embedded in a support matrix when contained in such a delivery device. As used herein, the term "solution" includes a pharmaceutically acceptable carrier or diluent in which the cells of the invention remain viable. Pharmaceutically acceptable carriers and diluents include saline, aqueous buffer solutions, solvents and/or dispersion media. The use of such carriers and diluents is well known in the art. The solution is preferably sterile and fluid to the extent that easy syringability exists. Preferably, the solution is stable under the conditions of manufacture and storage and preserved against the contaminating action of microorganisms such as bacteria and fungi through the use of, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. Solutions of the invention can be prepared by incorporating progenitor cells as described herein in a pharmaceutically acceptable carrier or diluent and, as required, other ingredients enumerated above, followed by filtered sterilization.

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. Patent No. 4,298,002 and U.S. Patent 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.

The present invention also provides substantially pure progenitor cells which can be used therapeutically for treatment of various disorders associated with insufficient functioning of the pancreas.

To illustrate, the subject progenitor cells can be used in the treatment or prophylaxis of a variety of pancreatic disorders, both exocrine and endocrine. For instance, the progenitor cells can be used to produce populations of differentiated pancreatic cells for repair subsequent to partial pancreatectomy, e.g., excision of a portion of the pancreas. Likewise, such cell populations can be used to regenerate or replace pancreatic tissue loss due to, pancreatolysis, e.g., destruction of pancreatic tissue, such as pancreatitis, e.g., a condition due to autolysis of pancreatic tissue caused by escape of enzymes into the substance.

In an exemplary embodiment, the subject progenitor cells can be provided for patients suffering from any insulin-deficiency disorder. For instance, each year, over 728,000 new cases of diabetes are diagnosed and 150,000 Americans die from the disease and its complications; the total yearly cost in the United States is over 20 billion dollars (Langer et al. (1993) *Science* 260:920-926). Diabetes is characterized by pancreatic islet destruction or dysfunction leading to loss of glucose control. Diabetes mellitus is a metabolic disorder defined by the presence of chronically elevated levels of blood glucose (hyperglycemia). Insulin-dependent (Type 1) diabetes mellitus ("IDDM") results from an autoimmune-mediated destruction of the pancreatic β-cells with consequent loss of insulin production, which results in hyperglycemia. Type 1 diabetics require insulin replacement therapy to ensure survival. Non-insulin-dependent (Type 2) diabetes mellitus ("NIDDM") is initially characterized by hyperglycemia in the presence of higher-than-normal levels of plasma insulin (hyperinsulinemia). In Type 2 diabetes, tissue processes which control carbohydrate metabolism are believed to have decreased sensitivity to insulin. Progression of the Type 2 diabetic state is associated with increasing concentrations of blood glucose, and coupled with a relative decrease in the rate of glucose-induced insulin secretion.

The primary aim of treatment in both forms of diabetes mellitus is the same, namely, the reduction of blood glucose levels to as near normal as possible. Treatment

of Type 1 diabetes involves administration of replacement doses of insulin. In contrast, treatment of Type 2 diabetes frequently does not require administration of insulin. For example, initial therapy of Type 2 diabetes may be based on diet and lifestyle changes augmented by therapy with oral hypoglycemic agents such as sulfonylurea. Insulin therapy may be required, however, especially in the later stages of the disease, to produce control of hyperglycemia in an attempt to minimize complications of the disease, which may arise from islet exhaustion.

More recently, tissue-engineering approaches to treatment have focused on transplanting healthy pancreatic islets, usually encapsulated in a membrane to avoid immune rejection. Three general approaches have been tested in animal models. In the first, a tubular membrane is coiled in a housing that contained islets. The membrane is connected to a polymer graph that in turn connects the device to blood vessels. By manipulation of the membrane permeability, so as to allow free diffusion of glucose and insulin back and forth through the membrane, yet block passage of antibodies and lymphocytes, normoglycemia was maintained in pancreatectomized animals treated with this device (Sullivan et al. (1991) *Science* 252:718).

In a second approach, hollow fibers containing islet cells were immobilized in the polysaccharide alginate. When the device was place intraperitoneally in diabetic animals, blood glucose levels were lowered and good tissue compatibility was observed (Lacey et al. (1991) *Science* 254:1782).

Finally, cells have been placed in microcapsules composed of alginate or polyacrylates. In some cases, animals treated with these microcapsules maintained normoglycemia for over two years (Lim et al. (1980) *Science* 210:908; O'Shea et al. (1984) *Biochim. Biochys. Acta.* 840:133; Sugamori et al. (1989) *Trans. Am. Soc. Artif. Intern. Organs* 35:791; Levesque et al. (1992) *Endocrinology* 130:644; and Lim et al. (1992) *Transplantation* 53:1180). However, all of these transplantation strategies require a large, reliable source of donor islets.

The progenitor cells of the invention can be used for treatment of diabetes because they have the ability to differentiate into cells of pancreatic lineage, e.g., β islet cells. The progenitor cells of the invention can be cultured *in vitro* under conditions

which can further induce these cells to differentiate into mature pancreatic cells, or they can undergo differentiation in vivo once introduced into a subject. Many methods for encapsulating cells are known in the art. For example, a source of β islet cells producing insulin is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the β islet cells (Aebischer et al. U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) *Expt. Neurobiol.* 110:39-44; Jaeger et al. (1990) *Prog. Brain Res.* 82:41-46; and Aebischer et al. (1991) *J. Biomech. Eng.* 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the β islet cells (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) *Trans. Am. Artif. Intern. Organs* 35:791-799; Sefton et al. (1987) *Biotehnol. Bioeng.* 29:1135-1143; and Aebischer et al. (1991) *Biomaterials* 12:50-55).

In certain embodiments, the cells of the invention may also be used for the treatment of, for example, diseases of the liver, or disorders of insufficient production of blood cells, such as thrombocytopenias, anemias or for transplanting into radiation therapy patients.

In certain embodiments, the cells of the invention may also be used for the treatment of myocardial damage following, for example, heart attack or viral infection.

In certain embodiments, the cells of the invention may also be used to treat conditions of skeletal muscle degeneration or damage, for example, muscular dystrophy.

Moreover, in addition to providing a source of implantable cells, either in the form of the progenitor cell population of the differentiated progeny thereof, the subject cells can be used to produce cultures of pancreatic cells for production and purification of secreted factors. For instance, cultured cells can be provided as a source of insulin. Likewise, exocrine cultures can be provided as a source for pancreatin.

Yet another aspect of the present invention provides methods for screening various compounds for their ability to modulate growth, proliferation or differentiation of distinct progenitor cell populations from pancreatic ductal epithelial culture. In an illustrative embodiment, the subject progenitor cells, and their progeny, can be used to screen various compounds or natural products. Such explants can be maintained in

minimal culture media for extended periods of time (e.g., for 7-21 days or longer) and can be contacted with any compound, e.g., small molecule or natural product, e.g., growth factor, to determine the effect of such compound on one of cellular growth, proliferation or differentiation of progenitor cells in the explant. Detection and quantification of growth, proliferation or differentiation of these cells in response to a given compound provides a means for determining the compound's efficacy at inducing one of the growth, proliferation or differentiation in a given ductal explant. Methods of measuring cell proliferation are well known in the art and most commonly include determining DNA synthesis characteristic of cell replication. There are numerous methods in the art for measuring DNA synthesis, any of which may be used according to the invention. In an embodiment of the invention, DNA synthesis has been determined using a radioactive label (^{3}H-thymidine) or labeled nucleotide analogues (BrdU) for detection by immunofluorescence. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the compound. A control assay can also be performed to provide a baseline for comparison. Identification of the progenitor cell population(s) amplified in response to a given test agent can be carried out according to such phenotyping as described above.

(iv) Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1: Isolation of Pancreatic Progenitor Cells

METHODS

Duct isolation and culture

Pancreata from two litters of 2 week old Sprague-Dawley rat pups were isolated and placed into 10ml of (1U/ml in DMEM) Collagenase A (Boehringer-Mannheim, St. Louis) and digested for 40min at 37°C in a shaking water bath at 150-175rpm. The digest was vortexed briefly and washed once with Ca^{++}/Mg^{++}-free HBSS (Gibco BRL, Grand Island, NY). The pellet was resuspended in HBSS and filtered through a 500μm

mesh (Costar Corning, Cambridge, MA) and washed again. The pellet was resuspended to 50ml in HBSS; 10ml was transferred to a 10 cm culture plate (Costar Corning) and placed under a dissecting scope. Individual duct fragments were selected by aspiration with a micropipette and transferred to a plate containing medium with serum. The fragments were selected from this plate a second time, transferred to a fresh tube of medium and serum, and washed before being plated. Fragments were cultured on plastic in Iscove's modified DMEM (Gibco-BRL) containing 5% FBS (Gibco-BRL), glutamine and 1% Pen/Strep (Gibco-BRL). For the study of individual duct fragments, ducts were grown on 8 well chamber slides (Lab-Tek, Naperville IL). For generating monolayers or NACs, duct fragments were plated to four 4-well plates (Nunc) per prep. Induction of NACs was achieved by a medium change after confluence (generally day 5 in culture) to Iscove's DMEM supplemented with 5% FBS, glutamine, Pen/Strep and Dexamethasone (1μM, Sigma), Cholera Toxin (100ng/ml, Sigma), and EGF (10ng/ml, Gibco). NACs were harvested after 48 hours.

Immunocytochemistry

Cultures, ducts, and non-adherent cells were fixed in 1% paraformaldehyde and permeabilized in PBS containing 0.3% TritonX-100 (PBST). Nonspecific binding sites were blocked by preincubation in a blocking buffer consisting of 5% normal donkey serum (Jackson ImmunoResearch) and 1% BSA (Sigma) in PBST. All antibodies were diluted in this blocking buffer. Incubations with primary antibody were carried out overnight at 4°C in a humidified chamber. Primary antibodies used were: guinea pig anti-insulin (Linco, 1:2000); mouse anti-insulin/proinsulin (Biodesign, directly conjugated to biotin using a labeling kit from Boehringer Mannheim); guinea pig anti-glucagon (Linco, 1:2500), mouse anti-somatostatin (Biomeda, 1:50); rabbit anti-pancreatic polypeptide (Zymed, 1:50); rabbit anti-amylase (Sigma, 1:1500), and rabbit anti-PDX-1 (gift of Christopher Wright, Vanderbilt, 1:2000). Secondary antibodies and tertiary reagents were: FITC-conjugated donkey anti-guinea pig IgG (Jackson ImmunoResearch, 1:200); Cy3-conjugated donkey anti-guinea pig, rabbit, or mouse IgG (Jackson, 1:1000); biotin-conjugated donkey anti-rabbit or mouse IgG (Jackson, 1:500); AvidinD-FITC (Vector Labs, 1:1000); streptavidin-Cy3 (Jackson, 1:1000). Cells were

scored on a Nikon Eclipse E800 epifluorescent or Nikon Diaphot 300 inverted fluorescent/phase photomicroscope.

Single cell cDNA amplification and PCR analysis

cDNAs from single cells were amplified according to Brady et al. (1993) and Dulac and Axel (1995). Single NACs were randomly picked and transferred into PCR tubes containing ice-cold lysis buffer. The first strand cDNA synthesis and subsequent PCR amplification were performed exactly as described (Dulac and Axel, 1995) except that the PCR reactions were performed in a total volume of 50 µl instead of 100 µl. The amplified cDNAs were electrophoresed on a 1% agarose gel and the size of DNA fragments ranged from 0.5 - 1 kb as expected. The aliquots of individual cDNAs were then analyzed for marker genes by PCR using specific PCR primers. The PCR reactions were run for 35 cycles each at 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min. Amplimer sequences were:

ATGTCGTCCAGGCCGCTCTGGACAAAATATGAATTCT$_{24}$; insulin: 5' primer, CACAACTGGAGCTGGGTGGAG; 3' primer, CAAAGGCTTTATTCATTGCAGAGG; PDX-1: 5' primer, GACCGCAGGCTGAGGGTGAG; 3' primer, CAGAGGTCTGCCAGCATCTCG; glucagon: 5' primer, TCCCAGAAGAAGTCGCCATTG; 3' primer, TTCATTCCGCAGAGATGTTGTG; beta-actin: 5' primer, AAG TCC CTC ACC CTC CCA AAA G; 3' primer, AAC ACC TCA AAC CAC TCC CAG G.

Insulin release assay

Insulin release was measured under static incubation conditions using NACs, isolated ducts, monolayer cells, or batches of 10 islets. Cells or islets were preincubated in Krebs Ringer Phosphate buffer (KRP) containing 3 mM Glucose (Sigma) and 0.2% BSA (Sigma) for 30 minutes at 37°C. Supernatant was collected and the cells washed once before further incubation in 17 mM glucose for 1 hour at 37°C. This supernatant was then collected and all samples were kept at -20°C until the insulin-specific radioimmunoassay was performed using a RIA kit for rat C-peptide from Linco

Research (St. Charles, MO). For insulin content measurements, cells were extracted in acid-ethanol and sonicated prior to assay.

Calcium Imaging

NACs were immobilized in 0.7% low melting agarose in Hanks buffer (GIBCO) and dye-loaded for 1 hour at room temperature with 5 μM Fluo-3 acetoxy-methyl (AM) ester (Molecular Probes) in standard Krebs Ringer Phosphate (KRP) buffer that additionally contained 0.1% pluronic acid (Molecular Probes) and 1% dimethyl sulfoxide (Sigma). Cells were then washed to remove excess dye and placed onto a heated microscope stage (Olympus) and maintained at 32°C. Fluo-3 fluorescence intensity was used as an indicator of intracellular calcium concentration and was measured with a confocal laser-scanning microscope (Olympus). The excitation wavelength was set to 488 nm (argon ion laser) and a 40X water lens was used. The same parameters for laser scanning were set for each experiment, including confocal aperture and laser intensity. The laser scanning was performed as an XYT series with an interval of 10 seconds between each scan in order to resolve glucose-induced changes in intracellular calcium. The imaging files were stored and subsequently analyzed with FLUOVIEW software (Olympus). Cells of interest were circled and the mean intensity of the circled areas was plotted over time.

RESULTS

Isolation, characterization, and culture of pancreatic ducts

In order to establish a defined *in vitro* culture system, a population of interlobular ducts was isolated and characterized. Adult tissue from 1-2 month old animals was initially used to establish the described culture system, but was eventually replaced with tissue from 2-3 week old rats which provide a more consistent and greater yield of clean ducts. Pancreatic tissue was harvested and subjected to collagenase digestion (Githens & Whelan, 1983; Githens et al. 1989). Digested tissue was then handpicked in multiple iterations until a pure population of ducts was obtained (Figure 1). A typical experiment provided up to 200-300 fairly uniform duct fragments per 20 animals. Staining for tubulin beta III and acetylated -LDL-DiI showed that the selected duct population was free of neurons and blood vessels (not shown).

Characterization of the starting material was performed by analyzing single ducts for expression of insulin, PDX-1, PYY, and amylase proteins. Table 1 shows that the majority of the handpicked ducts at time zero were free of these endocrine and exocrine markers. Greater than 92% of all ducts contained no insulin-immunopositive cells at the start of culture, and a similar proportion of all ducts had no immunohistochemically detectable PDX-1. Of those ducts that tested positive, almost all had only 1-2 cells that were insulin immunopositive. Analysis of dissociated ducts also showed that less than 0.05% of time zero duct cells were immunopositive for insulin and PDX-1 protein (not shown). Similarly, PYY-expressing cells were rare, constituting no more than 0.015% of the counted cells. Amylase-positive cells constituted 0.02% of the initial population and probably represented exocrine carryover since they occurred in rare clusters. The number of cells expressing either PDX-1 or the endocrine markers insulin or PYY totaled much less than 0.1% of the cells (Table 1), corroborating the immunohistochemical observations on sections of adult pancreas that these cells are rare in mature ducts (data not shown). Since the average duct fragment contained 3450 ± 1860 cells (n=10 determinations), and the average duct yield was 225 fragments, the initial number of insulin positive cells at the start of culture ranged from 80-400 per approximately 800,000 cells (Table 1).

Culturing was performed by placing single duct fragments within a 1 cm^2 well (Figure 2) or multiple fragments into 4-well plates (1.9 cm^2/well). Various substrates were tested (Matrigel, collagen, hydrogels) but the cleanest and most interesting results were obtained by simply plating onto charged plastic. Iscove's Modified Dulbeccos Media (IMDM) containing 5% fetal calf serum (FCS) was added to each well and the ducts cultured over 5 days. The top panel in Figure 2 shows insulin staining in a time series of cultured ducts and the bottom panel the corresponding bright field images. An analysis of single duct populations indicated that whereas at time zero 8% of the duct fragments were positive for insulin (Table 1), in as short a time as 24 hours, the number of ducts containing insulin-positive cells had increased to 13% and to 17% by day two. These positives appeared most frequently as single cells or small foci of 2-4 cells (Figure 2). By day five of individual culture 23-25% of the wells contained insulin-

positive cells on the monolayer, with little change thereafter (through day seven, 46/185 single duct cultures). One implication of these results is that non-insulin positive ducts become insulin-positive through culture.

Increasing the number of duct fragments per well resulted in a more rapid outgrowth and confluence of the monolayer generally within 5 days, suggesting that some cross-feeding takes place. We have standardized our platings to 16 wells (1.9 cm^2) per duct preparation as a balance between time and cell yield. At seven days of culture in FBS(T7), the monolayer contains an average of 25±20 (range 0-51) insulin-positive cells per well (0.02% of total cells) for a maximum expansion of 5-fold over the starting material. In this system, the majority of cells are vimentin-positive, non-endocrine and proliferative (as indicated by BrdU uptake), and probably arise from the stromal cells surrounding the epithelial layer of duct cells. The insulin-immunoreactive cells are capable of BrdU uptake but this occurs rarely (not shown). The bulk of BrdU uptake is by vimentin-positive fibroblasts. The slow growth rate of the early appearing insulin-positive cells on the monolayer agrees with observations from other investigators demonstrating that beta cells replicate very infrequently *in vitro* and *in* vivo (reviewed by Sjoholm, 1996; Nielsen et al. 1999). Further culture beyond seven days did not significantly increase either the number of wells containing insulin-positive cells or the number of insulin-positive cells.

In addition to an increase in the number of cells expressing insulin, we also observed that some wells in FBS culture contained a number of amylase-positive cells, often these amylase-positive cells coexpressed insulin (Figure 3A-C). Cells expressing both insulin and amylase have been documented to appear during pancreatic regeneration and are thought to be activated progenitor cells (Melmed, 1979, Gu et al. 1994). In addition, these cultures contained a rounded and semi-adherent cell type, many of which appear to express both markers although the insulin is faint.

Cells that express PYY and/or glucagon are observed in our duct cultures (Figure 3D-F). The coexpression of PYY and glucagon during early pancreatic development has been postulated to mark endocrine progenitor cell progression (Upchurch et al. 1994). In contrast to insulin, the number of PYY-immunopositive cells

did not change during culture (Table 2). Of cells that were insulin-positive on the monolayer, most but not all coexpressed PDX-1. Cells that expressed PDX-1 but did not yet express insulin and vice versa were also observed in culture (Figure 3G, arrows). Thus cells representing various stages of differentiation and different developmental lineages appear in our cultures.

Appearance of a non-adherent cell type

The number of insulin-expressing cells did not increase significantly with culture beyond 5 days due to their slow replication rate and perhaps cell death. Factors were added to T5 cultures to determine if an increase in the number of insulin-positive cells could be induced. A number of factors that affect either epithelial cells or pancreatic development were tested: dexamethasone, cholera toxin, EGF, TGFα, PDGFα, HGF, TGFβ1, IL-1α, GLP-1, glucagon, gastrin, GIP, PYY, NPY, and PP were added to T5 cultures for 2 further days of incubation. When tested alone most of the factors did not significantly boost the number of observed insulin-positive cells (not shown). However, a cocktail of DCE, dexamethasone, cholera toxin and EGF, significantly increased the number of insulin-expressing cells on the monolayer (an average increase of 2-3 fold over n = 8 experiments, Table 1). In addition, the presence of DCE significantly enhanced the appearance of a population of non-adherent cells (NACs) over the course of 48 hours (Figure 4). NACs were observed even in control cultures (Figure 4A) as well as in growth factor-treated cultures (Figures 4C,D), but none of these conditions led to the level of induction seen with DCE. In the examples shown, HGF and TGFβ1 were tested for effects on the monolayer. HGF has been shown to stimulate the growth of fetal islets (Otonkoski et al. 1994) and TGFβ1 has been shown to inhibit the appearance of endocrine cells in *in vitro* pancreatic culture (Sanvito et al. 1994). In our system, HGF and TGFβ1 had only slight effects on culture phenotype or NAC production.

NACs appear spontaneously in confluent monolayer cultures. Both the number and rate of appearance of NACs are significantly increased (often > 8-fold) by the addition of the DCE cocktail (Figure 4B). These cells are characteristically phase-bright, possess a secretory appearance with high granularity, and generally range in size

from 20-50 μm (Figure 4 inset). True NACs most often appear as large round cells freely floating at the surface of the monolayer. Many others are often loosely attached and apparently in the process of emerging. An increase in NACs can be seen by 24 hours post-DCE addition, but appears maximal at 48 hours. Repeated dosing of DCE into the cultures gave rise to successive waves of NAC formation but with successively fewer numbers (not shown).

DCE has previously been shown to promote the growth and function of primary purified pancreatic epithelial cultures (Githens et al. 1987, 1989), but no NACS or endocrine cell types were reported. Perhaps the effect of DCE is indirect; working on the stromal component of our mixed cell culture system to induce the differentiation of beta and other islet cell types. Subsequent testing in our cultures showed that neither dexamethasone nor EGF alone had significant effects on NAC generation in comparison to controls, but that the majority of the activity was associated with the cAMP-elevating effects of the cholera toxin. In fact, many cAMP agonists also had this effect (data not shown; to be described elsewhere). The presence of dexamethasone and EGF appeared to enhance the effect of CT. The size and granularity of the cells within the NAC population varied markedly, but vital dye staining showed greater than 99% of the NACs to be viable.

In terms of the number of ducts that were responsive to DCE, over 95% of the DCE-treated wells (n>10 experiments, single duct per well) gave rise to at least a 2-fold increase in NACs over control wells. Each treated well in a normal culture (8-16 ducts per well) yielded 3,000-18,000 NACs after 48 hours in DCE (n = 9 experiments) with an average yield of about 7000 per well or approximately 1 x 10^5 NAC cells per prep. BrdU incorporation experiments demonstrated that one of the effects of DCE is to stimulate cell division. Pulsing at the end of the 48 hours showed 4-fold more BrdU-positive cells in the DCE-treated monolayers than in controls, indicating a long-lasting stimulation of proliferation (not shown). When pulsed at the beginning of DCE addition, 10% of the NACs recovered at 48 hours were BrdU-positive, indicating that these cells may derive from a DCE-responsive, cycling cell. DCE does not appear simply to stimulate the loss of cell adhesion.

Hormone expression in NACs

Analysis of the monolayer demonstrated that approximately 0.02% of the cells in FBS culture expressed insulin protein (see Table 1), with DCE addition increasing that number 2-3 fold to give an average of 59±52 (range 5-196) insulin-positive cells per well. In addition to analysis of the monolayer, NACs were analyzed for expression of insulin and other endocrine markers. Since NACs are free-floating in the media, they were collected by aspiration at 48 hours post DCE addition, when their appearance was maximal. All four islet endocrine cell types could be detected immunocytochemically in this population. Figure 5 shows NAC immunostaining for insulin, PDX-1, glucagon, somatostatin, and pancreatic polypeptide. As shown in Figure 5A, insulin labeling demonstrated a continuum of fluorescence intensity with approximately 4-5% of the cells consistently bright (range 2.5-13%, n = 6 measurements), and the majority of positive cells (>30-40% of the total cell population) exhibiting low levels of immunofluorescence (A, C) that were still above background (B). This number of low-insulin-expressing cells was confirmed by FACS analysis (data to be described elsewhere). Cells expressing PDX-1, glucagon, somatostatin, and pancreatic polypeptide were also present in the population (D, E, F and G respectively), with glucagon-positive cells being the next most frequent (6%), followed by somatostatin (3%) and pancreatic polypeptide cells the rarest (2%). The NAC population thus constitutes an enrichment from a cultured monolayer of the full set of islet endocrine cell types.

Transcriptional profiling

As a further determination of the number of insulin-expressing cells within the NAC population, we also performed semi-quantitative single-cell PCR (Brady et al. 1993, Dulac & Axel, 1995) to detect insulin mRNA in randomly selected individual NACs. Figure 6 shows that 15 of 40 or >35% of the cells analyzed contained insulin mRNA. Panel A demonstrates that cDNA was amplified from each single cell sample. Panel B shows that the insulin message varied in intensity among the positive cells. This variation in signal intensity, similar to that observed by immunocytochemistry, was also observed and verified by array hybridization analysis (data not shown). Two of the

40 selected cells contained glucagon message (Panel C) with one of them also containing insulin and PDX-1 message. Panel D shows that greater than 80% (35/40) of the cells analyzed contained PDX-1 message. Only one of the insulin-positive cells did not express PDX-1, whereas there were many cells that were PDX-1 positive with no detectable insulin or glucagon message. The absolute and relative numbers of insulin and glucagon expressing cells are in good agreement with that observed by immunocytochemistry, and in the case of insulin, with flow analysis as well. Interestingly, there were a number of NACs that did not express any of the three markers. The identities of these cells is currently unknown. Array hybridization of the 40 cDNAs with a labeled probe to the ribosomal component S6 (RPS6) demonstrated its presence in all samples (not shown). Transcriptional profiling confirms the immunocytochemistry results that many of the NACs express PDX-1, that approximately 40% of the cells are both insulin mRNA and protein positive, albeit at varying levels, and that the majority of NACs appear to have a beta cell phenotype. In addition, there is a significant fraction of cells that are PDX-1 positive but insulin-negative, which might indicate progenitor cell status. SC-PCR of monolayer cells showed 23/23 actin-positive, 0/23 insulin positive cells (not shown), demonstrating the relative enrichment of endocrine phenotypes in the NAC population.

Insulin content and glucose-stimulated insulin secretion

A hallmark of functional beta cells is their ability to secrete insulin in response to elevated glucose levels. In order to determine whether there are functional beta cells within the duct cultures, we performed static insulin release assays on both the monolayer and the NAC populations to determine their responses to glucose challenge. We also measured total insulin content by RIA in order to determine the relative increase in insulin expression due to culture. Figure 7 shows the glucose-induced insulin release in isolated time zero ducts, DCE-cultured monolayer, and harvested NACs. The NAC population demonstrated a 3-fold increase in secreted insulin in response to elevated glucose. In contrast, the cells contained in the monolayer exhibited little glucose response and secreted far less insulin in either high or low glucose conditions. Analysis of the time zero ducts (n=3) showed no glucose-stimulated insulin

secretion and acid-ethanol extraction of the ducts showed no detectable insulin within the level of sensitivity (approx. 100 pg/ml) of the RIA. Extraction for total insulin showed a content of 1.34 ng of insulin per 50,000 cells on the monolayer, and 25.0 ng of insulin per 50,000 NAC cells. In comparison, a normal rat islet of approximately 1000 cells typically contains about 20 ng of insulin (data not shown). Furthermore, when NACs recovered from cultures exposed to DCE for 24, 48, or 72 hours were compared, only the NACs from the 48-hour cultures demonstrated a reliable glucose-stimulated insulin response. The data thus show a large increase in the amount of insulin produced through duct culture and that the insulin can be productively released in response to glucose, demonstrating the presence of functional beta cells. These studies once again demonstrate that NACs represent an enriched population of cells with an endocrine phenotype. Current studies are focused on a further understanding of the variables leading to increased cell number, insulin content and/or functionality.

Demonstration of a glucose-stimulated reversible calcium current

A key question to address is how many of the insulin-containing cells generated in culture possess a functional glucose response. One way to determine this is to measure the number of cells capable of generating an inward calcium current in response to glucose administration. Insulin secretion is known to be mediated by an inward calcium current linked to glucose metabolism (Kalkhoff & Siegesmund, 1981, Wang & McDaniel, 1990). To assess the presence and number of functional beta cells within the culture, we measured cytosolic calcium influx in response to glucose using the calcium-dependent fluorescent dye Fluo-3. The results of a representative experiment ($n > 10$) using NACs is shown in Figure 8. In this example 33% of the sampled cells showed strong amplitude and kinetics of calcium current induction in response to elevated glucose. Measurements of cells still attached to the monolayer failed to detect glucose-induced changes in intracellular calcium.

The amplitude and kinetics of the glucose-induced calcium current that we observe in our duct culture-derived beta cells are similar with those documented to occur in islet-derived beta cells (Asada et al. 1998; Schuit, 1996). Cell-to-cell variations in the amplitude and kinetics of glucose induced calcium currents have been interpreted

as evidence of heterogeneity in beta cell physiology. Single beta cells studied in isolation have been shown to have altered insulin secretion rates and glucose sensitivity in comparison to intact islets (Halban et al. 1982; Bosco et al. 1989), and individual beta cells have been shown to have markedly different rates of insulin synthesis (Moitoso de Vargas et al. 1997), all of which are observed in our culture. The amplitude variation in calcium currents might also be explained by differences in Fluo-3 dye loading.

The characteristic calcium current profile was not induced by 2-deoxyglucose, a non-metabolized glucose analog (Niki et al. 1974, 1993; Malaisse 1979). It was completely inhibited by diazoxide, a high affinity inhibitor of the SUR-linked potassium channel (Thomas et al. 1996) that is necessary for calcium-induced insulin secretion (Henquin et al. 1982; Trube et al. 1986), and was also inhibited by EGTA (Wollheim & Sharp 1981; Wang & McDaniel, 1990), which sequesters extracellular calcium (not shown). It could however be activated by tolbutamide, a high-affinity activator of the SUR-linked potassium channel, which is used specifically to stimulate insulin secretion in diabetics (Sato et al. 1999; Melander, 1998). The reversibility of this stimulated calcium current is demonstrated in Figure 8B. In these experiments (n = 3) 10% of the cells could respond reversibly to glucose and be stimulated finally by the insulin secretagogue tolbutamide. Fifty-five percent of the cells did not respond to either stimulus, and the remaining 35% of the cells responded either to glucose but not tolbutamide, or to tolbutamide but not glucose, indicating a heterogeneous and complex population. We conclude that, despite having heterogeneous insulin expression levels, 10-40% of the NACs could respond to glucose and thus behave like functional beta cells.

DISCUSSION

We describe here an *in vitro* culture system that allows the study of functional beta cell formation from a purified pancreatic duct population. Culture of such ducts resulted both in an increase in insulin-positive cells over time and an increase in the total number of duct fragments that became insulin-positive. This latter result indicates that cells capable of expressing insulin may become activated during culture. In addition to these results, we describe the appearance of an interesting population of non-

adherent cells that arises during culture, and whose numbers and emergence can be directly regulated by addition of factors and agents.

These non-adherent cells, which we refer to as NACs, are heterogeneous in size, granularity, and marker expression. Immunocytochemical analysis shows that all four islet endocrine markers can be detected within this population. Our analysis also shows that these cells appear in ratios similar to their ratios in the adult pancreatic islet, with the insulin expressing cells being the most numerous, followed by glucagon, somatostatin, and pancreatic polypeptide expressing cells. Because of their relevance to human disease, assessing the number and functionality of the insulin-expressing cells has been our major focus.

The handpicked duct material contained very few insulin-positive cells at the start of culture. Analysis of the number of insulin-positive cells at the start and endpoint of culture demonstrated an increase of up to 500-fold, primarily in the new non-adherent cell population. Since the insulin-positive cells observed early in culture rarely incorporated BrdU, we propose that the majority of the observed beta-like cells arose from an expanding precursor within the duct.

The mechanism of DCE stimulation of insulin expression and NAC formation is not known. One of the components, dexamethasone, is a glucocorticoid analog known to have multiple effects on pancreas, including stimulation of fetal islet differentiation (Korsgren et al. 1993), stimulation of pancreatic tumor cell growth (Brons et al. 1984), and upregulation of exocrine marker expression (Rall et al. 1977; Van Nest et al. 1983), but surprisingly also suppresses insulin expression in mature mouse islets (Lambillotte et al. 1997). Glucocorticoids have also been shown to upregulate EGF receptor expression in some cell types such as hepatocytes (Gladhaug et al. 1989). EGF is an important mitogen and regulator of gastric and pancreatic epithelium (Meittinen 1997), and in fact has been shown to stimulate epithelial-like outgrowths in cultured pancreatic ducts (Heimann & Githens 1991). Agents such as cholera toxin which raise intracellular cAMP levels have been shown to stimulate epithelial cell properties (Rindler et al. 1979), and cholera toxin in combination with EGF has been shown to induce cyst formation in pancreatic duct and islet cultures (Heimann & Githens, 1991; Yuan et al.

1996). Interestingly, Heimann and Githens (1991) used this combination of DCE to identify and purify ductal epithelium from fibroblasts through the stimulation of cyst formation in collagen or agarose-embedded cultures. In our hands, continuous culture of ducts in DCE also leads to monolayer formation, but without NAC formation. It may be that the choices of culture architecture (embedded versus flat monolayer) and the timing of factor addition are responsible for these differences, and these are currently being investigated.

Perhaps due to the early and possibly immature nature of the generated cells, or the fact that the cells have not yet formed the electrical contacts required for full islet function, the level of hormone expression found within individual monolayer or NAC cells was much lower than in adult beta cells. Our insulin extraction studies indicated that the average NAC cell contains 20-50 fold less insulin than a comparable adult rat beta cell . It may be that only the insulin bright cells (highly expressing insulin) possess a glucose response and that the dim cells (expressing a lower level of insulin) represent less mature, non-glucose responsive pre-beta cells. Nonetheless, a much larger proportion of cells could be demonstrated to possess a glucose-stimulated insulin secretion response in the NAC population than in a randomly selected population of monolayer cells. Thus, the NAC population herein identified represents an enriched population of cells with an endocrine phenotype. It is likely that our culture system is missing the trophic influences necessary for stimulating cell-cell contact, full hormone expression and complete beta cell maturation. A number of factors have been shown to increase fetal beta cell insulin expression and enhance insulin secretion (Otonkoski et al., 1993, 1994; Huotari et al., 1998; Sorenson & Brelje, 1997) and these are currently being tested in our system.

The system we describe herein allows for the first time the *in vitro* study of regenerative and neogenic events that until now have only been described *in vivo*. We show that the system can be manipulated to give a range of cell identities, that a significant increase in insulin-positive cells can be obtained and that within this population of cells, beta cell-like function can be detected. Our studies indicate that much of the regenerative and stem cell activities ascribed to the pancreatic duct system

by *in vivo* manipulations can be recapitulated through *in vitro* culture. This system now makes possible a systematic search for those cells responsible for these activities as well as the identification of factors that influence their numbers, hormone content, and functionality. In addition, this system constitutes the first step towards achieving the goal of a controlled and defined process to create functional beta cells through a naturally occurring, non-cell-engineered process as a therapeutic pathway for the treatment of insulin-dependent diabetes.

Example 2: Induction of Pancreatic Progenitor Cell Differentiation

The monolayer can be grown in the presence of EGF (10ng/ml) or TGF-α (10ng/ml) to enhance growth. Induction of differentiation is believed to be cAMP dependent. Agents which induce an increase in intracellular cAMP levels are anticipated to induce differentiation.

The cocktail DCE (1 μM Dexamethasone, 100 ng/ml Cholera toxin, 10 ng/ml EGF) induces an increase in the number of insulin positive cells in the cultured duct monolayers. Figure 9 shows the comparison of monolayers treated with DCE + 5% FCS versus 5%FCS alone. Ducts were cultured for five days and then treated for an additional 48 hours. Note that there is an approximate 5-fold increase in the total number of insulin positive cells in the culture in response to DCE treatment. The total number of cells in the culture also increases by approximately 20%. The bars represent the average of quadruplicate wells.

Dexamethasone, Cholera toxin, Forskolin, Dibutyrl cAMP and Na-Butyrate have all been tested and found to induce differentiation. Figure 10 shows that Forskolin, Dibutyrl cAMP and Na-Butyrate can substitute for DCE in inducing the appearance of floating progenitor cells. Briefly, monolayers of ductal fragments induced after 5 days culture with the cAMP agonists forskolin and dibutyryl cAMP as well as the fetal islet differentiating agent sodium butyrate. Both low and high concentrations of each factor were applied to the duct monolayer. After 48 hours, the resultant NACS were collected and counted. Treatments are shown on the x-axis and number of floating progenitor cells is shown on the y-axis. Each bar is the added total of duplicate wells.

We have also observed that secretin can induce differentiation of the monolayer and the appearance of pancreatic progenitor cells. In Figure 11, after 5 days of culture, secretin was added to the monolayers in a dose range of 1-100 nM. The number of floating, non-adherent progenitor cells was determined after 48 hours of treatment. Each bar represents the total of two combined 1.9 cm^2 wells.

The duct cultures used in Figure 12 were cultured as described above. After 48 hours in varying secretin doses, both the number of insulin expressing cells on the monolayer and the total number of floating progenitor cells were counted and scored. Scoring of insulin was done by immunocytochemistry. Note that there is a dose dependent increase in the number of floating progenitor cells. The number of insulin positive cells in the monolayer also increases with secretin dose and the apparent decrease in insulin positive cells at the 50nm dose is anomalous. Each point represents the average of duplicate wells. The left hand y-axis denotes the total number of obtained floating progenitor cells, and the right hand y-axis denotes the number of insulin positive cells per well. Secretin dose is shown on the x-axis.

Figure 13 demonstrates that Vasoactive Intestinal Peptide (VIP) also differentiates duct monolayers by inducing the appearance of floating progenitor cells. After five days in culture, VIP was added to the cultures and the number of induced floating progenitor cells was determined after 48 hours of treatment. C=control and is 5%FCS. The optimal dosage in this experiment was 50ng/ml of VIP, which induced a >3-fold increase in the number of floating progenitor cells versus control. The y-axis denotes the number of such cells (x 100). Each bar is the total of two pooled wells.

We also observed that the presence of insulin diminishes secretin-induced differentiation. See Figure 14. Floating progenitor cells were induced with secretin (100nM). Simultaneous addition of insulin (10ng/ml) with secretin diminished the overall induction of floating progenitor cells. Each bar represents the total of two pooled duplicate wells and the number is expressed on the y-axis as number cells (x 100).

Example 3: Isolation of Pancreatic Progenitor Cells using Lectin Cell-surface Marker

We also set out to identify cell-type specific markers which could be used to isolate/purify pancreatic progenitor cells, or the pancreatic/ductal epithelial which gives rise to such progenitor cells. Among the various candidate reagents we tested, we discovered that certain lectins preferentially bound to, and therefor facilitate the isolation of, duct epithelial cells ultimately able to produce pancreatic progenitor cells.

Arachis hypogaea (Peanut Agglutinin, PNA) is a plant lectin that binds to specific carbohydrate groups on cell surfaces. PNA binds to galactosyl (β-1,3) N-acetyl galactosamine. It was initially selected for study as a beta cell marker (ref: Heald KA, Hail CA, Hurst RP, Kane N, Downing R, Diabetes Res 1991 May;17(1):1-6, Separation of beta-cells from dispersed porcine pancreas by selective lectin binding); however, in our hands, PNA did NOT label islet cells from rat, but DID label duct epithelial cells.

PNA (*Arachis hypogaea*, Peanut Agglutinin) was obtained from Vector Laboratories, FITC-conjugated (Cat.# FL-1071) and used at 1:250-500 dilution.

Paraffin sections of adult human pancreas were obtained from Carolina Biological Supply.

Protocol for histochemistry: Paraffin sections of adult human pancreas, adult rat pancreas, embryonic rat pancreas, or cryosections of adult mouse pancreas were used. Alternatively, Time 0 duct or cultured duct preparations from 2 week old rat pancreas were examined, with or without paraformaldehyde fixation. PNA-FITC was used usually at 1:250 dilution in PBS or DMEM/HEPES medium and incubated for 1hr to overnight at 4°C, then washed and mounted under VectaShield mounting medium containing DAPI. Cells stained without prior fixation were post-fixed before addition of mounting medium.

Protocol for FACS: PNA-FITC was diluted 1:250 in sterile wash buffer (Ca^{++} Mg^{++}-free PBS containing 1% FBS). Dispersed live cells (approx. $2x10^6$ cells) were spun down and resuspended in 100 µl of lectin and incubated for 30-45 min at 4°C. Cells were then washed twice with sterile wash buffer, resuspended in 2ml of Iscove's

modified DMEM containing 5% FBS, Pen/Strep, 1mM glutamine, and held on ice until run on the FACSVantage. Standard FACS procedures were used.

FACS sorted cells were collected into tubes or delivered directly into multiwell culture plates containing complete Iscove's medium (see above). Cell density at seeding, surface substrates, and culture times were varied. Some cultures were re-analyzed by FACS, and some were analyzed by histochemistry.

2 week rat duct, Percoll prep

↓

Dissociation to single cell

↓

Incubation of live single cells with PNA-FITC

↓

FACS and recovery of PNA+ and PNA- cells

↓

Culture of sorted cells (currently 2 weeks)

↓

Analysis for differentiated cell types:
Histochemistry, LDL uptake, etc.

↓

Testing of factors that cause (e.g.,) proliferation,
retention of PNA+ character, or
differentiation to islet subtypes

↓

In vitro growth &/or differentiation

↓

Expansion,
implantation into animals for rescue

Using PNA, we made the following observations:

(i) PNA as a marker for Epithelial Cells. PNA marks the single layer of epithelial cells in the pancreatic duct. It does NOT mark islet cells in rat, in contrast to the report in the literature of beta cell marking in porcine islets. It does not mark blood vessels or stromal cells, by immunohistochemistry. PNA marks the epithelial cells of pancreatic duct in adult animals as well as the epithelial sheet in embryogenesis (shown at stages e15, e16 and e18 in rat).

(ii) PNA labels cell surfaces.

(iii) PNA labels live, unfixed, unpermeabilized cells.

(iv) PNA does not mark the major pancreatic duct (common bile duct, CBD). PNA marks primarily the medium-sized interlobular ducts and many of the larger intralobular ducts.

(v) PNA is a suitable reagent for Fluorescence Activated Cell Sorting. PNA allows a viable cell sorting and recovery by FACS (Becton Dickinson FACSVantage); PNA-positive cells can be sorted directly into multiwell plates. We have applied PNA labeling to RIN, islet cells, T0 duct, and cultured duct monolayers. Approximately 5-15% of a T0 duct prep is PNA-positive by FACS analysis. The percentage does not seem to change very much with culture (over 4 days in FBS). PNA-positive sorted cells are 76-94+% pure upon reanalysis, depending on the selectivity of the sort (events per drop, sort selection mode, etc.). The PNA-negative population is 99+% negative.

(vi) PNA-sorted cells have favorable Growth Characteristics. Cells are viable but non-adherent after sorting. Dispersed duct cells take a minimum of 7 days to adhere to substrate and begin to grow. In contrast, whole single ducts sit and begin to spread after 24 hr. Cell viability is dependent on plating density in the absence of culture additives. An unsorted population of cells (PreSort) proliferates readily; PNA-positive sorts are slowest. The implication is that other cell types besides the PNA-positive cells are required for maintaining healthy outgrowth, as well as certain cell characteristics (below). PNA-positive cells in culture do not remain PNA-positive (cells restained with PNA-FITC).

A distinct population of cuboidal endothelial-like cells is prominent; also flatter, larger, more fibroblastic cells are present. The former exhibit uptake of DiI-conjugated acetylated-LDL, a characteristic of endothelial cells, while the larger flatter cells do not.

The unsorted and PNA-negative populations grow into cultures of very mixed phenotype and morphologies. A very small percentage of cells in these cultures are dil-Ac-LDL-positive (i.e., endothelial-like).

PNA-positive cells in culture are NOT insulin or PDX-1 positive. Many cells in the mixed PreSort population are positive for both of these beta cell markers. By and large, the strong PDX-positive cells are weak or negative for PNA. Strongly PNA-positive cells are not PDX-positive. This suggests a progression from one cell type to another.

A small number of cells in the PNA-negative cell cultures are positive for Insulin and PDX-1. This suggests either that a small number of PDX+ cells (that were PNA-negative) were recovered, or that the presence of the other cell types has activated PDX-1 expression, perhaps from a small carryover of PNA-positive progenitor cells.

PDX-1 expression is much higher (more numerous and relatively brighter) than Insulin expression in these cultured duct preps. Since PDX-1 protein is a regulator of Insulin expression, this finding also suggests a progression, from PDX-1 positive to Insulin-positive.

Glucagon-positive cells outnumber PYY-positive cells, although both are rare in all sort fractions. Previous work has indicated that PYY-positive cells precede appearance of Glucagon cells; thus these results would suggest progenitor cells have already progressed beyond this point.

Based on these findings, we conclude that the ability of PNA to selectively detect pancreatic duct epithelial cells may permit the recovery of a population of cells containing an islet progenitor cell type. These cells in themselves appear to be insufficient to survive and differentiate; that is, other cells or factors may potentiate proliferation and differentiation. Nonetheless, PNA-selection represents a large step forward in being able to perform recombination experiments to identify the components necessary to grow pancreatic islets.

Figures 15-17 illustrate the phenotype of cells which have been cultured for two weeks after being sorted on the basis of PNA staining.

Figures 18 and 19 illustrate the specificity of PNA in adult and embryonic pancreas.

Figures 30 and 31 illustrate the binding of other lectins to adult rat pancreas.

Figures 32-39 illustrate the specificity of binding of lectins to adult human pancreas.

Example 4: Identification of genotype of pancreatic progenitor cells

In order to improve our technique for isolating pancreatic progenitor cells, we have designed a protocol for determining the identity of a pancreatic beta cell, or its precursor, in terms of its gene expression profile.

In general, the method applies single cell cDNA amplification to gene expression analysis. In such a manner, the gene expression "fingerprint" for a cell at a particular stage of development can be obtained by arrayed hybridization.

Briefly, single cells are isolated, e.g., from pancreatic tissue, The cDNA from each cell are amplified by the single cell PCR developed by Brail et al. (1999) Mutat Res 406: 45-54, and labelled with ^{32}P. The cDNAs are then selected for existence of particular messages, e.g., insulin and PDX1.

cDNAs of known pancreatic markers are generated by PCR and arrayed on nylon membranes. The resulting assays are used to hybridize with the labelled single cell cDNAs. The autoradiograph images of the array can be used to define and identify the gene expression profile for an individual cell.

Figures 20-29 further illustrate the protocol. Figure 20 shows the results of typical single cell mRNA PCR amplification reactions. Figure 21 illustrates the changes in the gene expression during pancreatic development, as determined by the subject method. Figure 22 illustrates one embodiment of an array of markers for detecting beta cells and precursors thereof.

Figure 23 shows typical autoradiographs profiling gene expression in adult and embryonic pancreatic tissue, and heart. Figure 24 demonstrates how quantitative analysis of gene expression can be carried out as part of a determination of the gene

expression profile of a cell. Likewise, Figure 25 shows autoradiographs profiling gene expression in embryonic pancreatic tissue at different stages and after different stimulus; Figure 26 illustrates the quantitative analysis of the autoradiagraphs.

Figure 27 shows autoradiographs profiling gene expression in the so-called floating progenitor cells described in the examples above; and Figure 28 illustrates the quantitative analysis of the autoradiagraphs of Figure 27. Using the subject method, we have demonstrated that our pancreatic progenitor cells express PNA mRNA but do not express PDX1 mRNA when they are first isolated. As the cells differentiate to insulin-secreting cells (insulin$^+$), they become PNA$^+$, PDX1$^+$. The earlier progenitor cells, in addition to being PNA$^+$, PDX1$^-$, are also insulin$^-$, PYY$^-$, glucagon$^-$ and cytokeratin$^+$.

Figure 29 shows the relative levels of expression of certain genes between adult islets and during pancreas development.

Example 5: Implanted cells from a pancreatic duct-derived culture transiently rescue the diabetic state.

SCID/icrl mice were obtained from Taconic. Average weight per mouse was 25g. Streptozotocin (STZ) was purchased from Sigma and made into a 30 mg/ml solution in 20 mM sodium citrate buffer, pH 4.5. Each animal received STZ at a dose of 200 mg/kg and fed ad libitum prior to fasting blood glucose (food removed the previous evening) on the morning of the 3rd day post injection. Diabetic animals were those found to have blood glucose in excess of 200 mg/dl with the average centering around 300 mg/dl. Insulin pellets (Innovative Research of America) that released 1.2 U of porcine insulin per day for 7 days were then implanted via trochar subcutaneously over the scapula. After monitoring for recovery from the diabetic state (blood glucose = 100 mg/dl), either cells derived from pancreatic duct culture, or isolated adult pancreatic islets were then implanted under the renal capsule using standard surgical procedures. Either 500,000 or 10^6 cells (non-adherent cell (NAC) fraction of duct culture) were implanted into mice. All operated mice survived in placebo, islet, and insulin pellet only groups. 6/7 cell implanted mice died 48-72 hours post implantation. For all groups fasting blood glucose was measured at standard intervals by tail bleed.

As shown in Figure 40, a heterogeneous population containing functional beta cells derived from the non-adherent portion of a differentiated pancreatic duct monolayer was implanted into streptozotocin (STZ)-treated diabetic mice. SCID mice injected with STZ became diabetic within 48 hours. Insulin containing pellets were then implanted subcutaneously to stabilize the blood glucose and create a more stable environment for cell implantation. The insulin pellet was designed to expire 7 days post-implantation at T = 11 days (T11). Within 48 hours of pellet implant the fasting blood glucose of these animals were reduced from a range of 280-380 mg/dl blood glucose to less than 50 mg/dl. In test groups either cells or adult islets as positive control were then implanted under the renal capsule. One week later (T13) fasting blood glucose was measured and again at days 16, 21, and 28. Black squares represent placebo group (n = 5 mice) and as expected, in the absence of insulin, the blood glucose slowly climbed over time to well over 300 mg/dl. Animals (n = 5) implanted with insulin pellets only and no cell implants also performed as expected, with a transient rescue followed by diabetic rebound after the insulin release tablet had expired (red diamonds). Animals receiving islets (blue triangles, n = 5, 400 islets per animal) showed perfect long term rescue with fasting blood glucose being maintained at approximately 100 mg/dl. The single surviving animal receiving duct-derived cells (green circles, n = 1 of 7) showed a transient rescue of the diabetic state. The single animal demonstrated a 4-5 day lowering of > 150 mg/dl blood glucose before rebounding to pre-implant blood glucose levels.

Example 6: Pancreatic Duct Sphere Culture

Methods:

Isolation of pancreatic ducts

P14 rat pancreas were collected and digested with Collagenase type A (1U/ml) in a 37 degree C water bath with vigorous shaking for 40 minutes. The digested tissues were washed with HBSS, filtered through a 500 micron mesh (Co-Star Netwell), and taken into HBSS. The tissue suspensions were then layered on top of 35% Percoll over 45% Percoll gradient (at a 1:1:1 volume ratio) and centrifuged at 1970 rpm for 10 minutes. The ducts were collected at the interface between the top layer and the percoll

gradient and washed with HBSS containing 2% BSA. The islets were carefully picked out from the duct suspension under the dissecting microscope.

Dissociation of the duct cells

The duct suspensions in HBSS were centrifuged at 1000 rpm for 5 minutes. The duct pellets were taken into dissociation enzyme cocktail containing 1.33 mg/ml trypsin, 0.7 mg.ml hyaluronidase, 0.2 mg/ml kynerenic acid, and 200 U/ml DNAse in HBSS with Ca^{2+}/Mg^{2+} and were incubated at 37 degrees C for 4 minutes followed by 3 minutes incubation at room temperature. The digestions were stopped by the addition of ¼ volume of 4% BSA in HBSS. The dissociated duct cells were passed through a 70 micron nylon mesh and centrifuged at 1450 rpm for 5 minutes. The cell pellets were washed again with 4% BSA in HBSS with Ca^{2+}/Mg^{2+}. The dissociated single duct cells were resuspended in the culture media and counted with Trypan blue for viable cells.

Duct sphere culture for expansion

The dissociated duct cells were seeded at the density of 6 million cells/P100 Petri dish in the culture media containing 10 ng/ml bFGF, 20 ng/ml EGF, 10 ng/ml HGF, 8 ng/ml TGFα, 30 ng/ml IGF-I and IGF-II, 2% B27 supplements (Gibco/BRL), 8 mM HEMES, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in DMEM-F12 medium. The growth factors were added to the medium every 48 hours after the initial plating of the cells. The sphere passages were made by obtaining the spheres in suspension and dissociating the spheres with 0.016% trypsin at 37 degrees C for 3 minutes and the digestions were stopped by ¼ volume of 4% BSA. The medium above is the optimal medium for growth. Other media were tested with varying effects. Test Media 1 is a KO-Basal medium with 15% KO-SR plus TGFα, HGF, IGF-I and IGF-II. Test Media 2 is a Liver Basal medium with 15% KO-SR plus TGFα, HGF, IGF-I and IGF-II. Test Media 3 is an N-2 based DMEM/F12 Basal medium with EGF, bFGF and LIF. Cells could be grown rapidly but appeared less healthy in Test Medium 2. Cells grew more slowly but appeared healthier in Test Medium 3. (see Figure 42).

Partial in vitro differentiation of the duct spheres

The spheres were gently collected from the culture medium into a 15 ml tube and

the spheres were allowed to settle by gravity. The spheres were then seeded into 96-well plates containing HTB9 matrix in the differentiation medium containing 30 micromolar forskolin, 100 ng/ml PYY, 5% FBS in Iscove's Modified Dulbecco's Medium with 25 mM glucose. The cells formed a monolayer on the matrix and under went differentiation for 4 to 7 days, and were then stopped for differentiation by washing with PBS and fixed with 1% PFA.

Results:

Duct cells cultured in medium containing growth factors such as EGF, bFGF and LIF in non-tissue culture (non-adherent) plates formed spheres over a period of two days after initial seeding. A few cells became attached to the bottom of the plate, but the spheres were found in suspension, not adhered to the plate or to other cells adhered to the plate (Figure 41). The spheres were propagated for 24 days by passaging every five days on average. Only non-adhered cells were passaged.

After plating of cells on HTB9 matrix, most cells migrated out of the spheres and formed a monolayer within 24 hours of plating. The cells were fixed by 1% PFA and costained with anti-Glut2 and anti-nestin antibodies. Glut2 expressing cells are in clusters and they are excluded from nestin expressing cells, suggesting that nestin cells are undifferentiated cells in the population. Nestin is typically a marker for relatively undifferentiated cells. Glut2 is a marker for cells tending towards becoming pancreatic β cells. There were about 26 Glut2 clusters in a 96 well plate. (Figure 43).

Cells differentiated from the spheres also expressed Pdx-1 protein and glucagon protein. There were many colonies of Pdx-1 expressing cells on the plates and interspersed in these colonies were glucagon-expressing cells. The glucagon positive cells were not Pdx-1 positive. Given that a colony of cells on the plate are likely to have arisen from a common set of progenitor cells, it appears that a single group of progenitor cells is able to give rise to both glucagons expressing and Pdx-1 expressing cells. There were about 24-26 Pdx-1 positive colonies per 96 well plate and each colony contained 15-20 cells on average. (Figure 44).

Co-staining for Pdx-1 protein and insulin protein showed, as above, a large number of Pdx-1 expressing colonies. Insulin expressing cells were tightly associated

with the Pdx-1 expressing colonies. About 16% of Pdx-1 positive cells were also insulin positive, and some insulin-positive cells did not express Pdx-1. This strongly suggests that Pdx-1 and insulin positive cells were derived from the same set of progenitor cells. (Figure 45).

Duct spheres in growth state (undifferentiated) were assayed for cell marker expression. Spheres were dissociated and put onto cytospin slides. The cells were stained with anti-nestin antibodies. 50% of cells express nestin protein -- a far greater proportion of nestin-positive cells than after differentiation. This again suggests that nestin-positive cells are undifferentiated. Cells from undifferentiated spheres were further co-stained with either Pdx-1/insulin or Glut-2/nestin. Positive cells were counted under a fluorescent microscope. Although there were Pdx-1 expressing cells, the ratio of positive cell/total cell population was lower with spheres in growth state (approx. 2%) than in cells from spheres after differentiation (approx. 5-8%). (Figures 46A, 46B, 47, and Table 3).

Example 7: Hematopoietic Cell Sphere ("Hemesphere") Culture

A. Isolation of Lin- fetal liver cells

Fetal livers from E13.0 mouse embryos were isolated and titurated to obtain a single cell suspension. Cells were incubated with a cocktail of biotin conjugated antibodies specific for the following hematopoietic lineage markers: Ter-119, Mac-1, Gr-1, B220, CD4, CD8a, CD8b.2. After incubation with Streptavidin-MACS magnetic microbeads the labeled cells were passed through a MACS depletion column. The flow through fraction contained the Lin- cell population. All cell fractions were FACS analyzed for the presence of Sca-1, c-kit and Lin markers at the time of isolation (see below).

B. Culture of Lin- E 13.0 fetal liver cells

4-8 x 10^6 Lin- fetal liver cells were resuspended in 10 ml of hemesphere culture media (DMEM/F12, 2 mM L-Glutamine, 8 mM Hepes buffer, 100 U/ml Penicillin/Streptomycin, 1:50 dilution of B-27, 20 ng/ml EGF, 10 ng/ml bFGF). Two separate culture conditions were established; Hemesphere Culture #1 was supplemented with 100 ng/ml mSCF, and Hemesphere Culture #2 was supplemented with 100 ng/ml mSCF, 1000U/ml mLIF, 50 ng/ml mFlt3 Ligand. The cells were plated in their

respective media on 10 cm non-adherent plastic Petri dishes. Cells were incubated for 1 week during which time cell aggregates or clusters appeared in the media. Cell death was also apparent in the cultures. After one week the cells were trypsinized to single cells and re-seeded in fresh media. Each time the culture was passaged by trypsinization new cell clusters formed. In both culture conditions the cell clusters contained only healthy, viable cells.

C. Remarks on the Cultures

Hemesphere Culture #1 (SCF added): The cell clusters contained only healthy, viable cells. There were two populations of single cells evident in the cultures; healthy, viable cells and dead cells. The cell clusters grew as loose aggregates in suspension (see Fig. 48 and 49). Although the clusters could become quite large, the cells within them remained healthy.

Hemesphere Culture #2 (SCF, LIF, Flet3-Ligand added): The cell clusters contained only healthy, viable cells. There were three populations of single cells evident in the cultures; a few healthy, viable cells, many irregularly shaped cells and many dead cells (Fig. 50). This culture showed much higher cell proliferation rate than Culture #1. Cell clusters did not become large aggregates as in Culture #1.

FACS Analysis of Cultures

The mouse hematopoietic cells with the cell surface antigen profile Sca-1+, c-kit+, Lin- have been demonstrated previously to be highly enriched in long-term repopulating stem cells, both from fetal liver and from bone marrow (Ref. 1, Review).

Hemesphere Culture #1

The population of Sca+, c-kit+, Lin- cells in the starting population of isolated E13.0 fetal liver before Lin depletion was 0.06% (in the stem cell enriched gate, R2) (Fig. 51, lower right, gate G5). After Lin depletion it was increased to 0.39% (Fig. 52, lower right, gate G5). Less than 1% Lin+ cells remained in the Lin- population (not shown). After 14 days in culture 90% of the cells remained Lin-. Of those Lin- cells, 79% are also Sca+ and c-kit+ (top left dot plot, Fig. 53). The cell population was very homogeneous, and contained a few dead cells, as shown by the FSC and SSC plots (Fig. 53, right panel). After 20 days in culture the cell population remained homogeneous.

The Sca+, c-kit+, Lin- population comprised 67% of the viable cells in the culture (Fig. 54).

Hemesphere Culture #2

The population of Sca+, c-kit+, Lin- cells in the starting population of isolated E13.0 fetal liver before Lin depletion was 0.27% (in the stem cell enriched gate, R2) (Fig. 55, lower right, gate G5). After Lin depletion it was increased to 1.99% (Fig. 56, lower right, gate G5). Approximately 1-2% Lin+ cells remained in the Lin- population (not shown). After 7 days in culture 7.35% of the cells remained Lin-. Of those Lin- cells, 10.78% are also Sca+ and c-kit+ (Fig. 57). This low percentage is because the majority of the Lin- population does not express Sca-1 or c-kit. Also, the majority of the cells in the total population were dead, as judged by propidium iodide staining (not shown). The dead cells are localized to the large diagonal population of cells located outside the R1 gate on the FSC and SSC plot (Fig. 57, right panel). After 11 days in culture the Sca+, c-kit+, Lin- population comprised 28.28% of all the viable cells in the culture (Fig. 58, lower right, gate G5). Virtually all of the viable cells were now expressing Sca-1. There were still many dead cells in the culture, as shown by the FSC and SSC plot.

Histological Analysis of Cells in Hemesphere Culture

Cells from each hemesphere culture were cytospun on to glass slides and stained with May-Grunwald Giemsa (Fig. 59). Cells from Hemesphere Culture #1 (cultured for 20 days) are shown in the left panel. Cells from Hemesphere Culture #2 (cultured for 13 days) are shown in the right panel. Cell clusters from both cultures show cells that contain mostly nucleus, with little cytoplasm. This is consistent with an immature cell type. Many granules and vacuoles are also seen. The cells on the periphery of the cluster (in Culture #1) appear more macrophage-like. The immature phenotype and granular appearance of the cells are similar to that obtained from in vitro AGM culture (Ref. 2), and from fetal liver cells grown on an AGM-derived endothelial cell line (Ref. 3).

Example 8: Rat Pancreatic Duct Sphere Culture and Differentiation

Part 1. The primary spheres, that are cultured from dissociated ducts, are very potent

stem cells or progenitor cells for islet endocrine cells differentiation.

Ducts are dissociated into single cells by the dissociation enzyme cocktail containing 1.33 mg/ml Trypsin, 0.7 mg/ml Hyaluronidase, 0.2 mg/ml Kynerenic Acid, and 200 U/ml DNase and plated into sphere culture media (culture media containing 10 ng/ml bFGF, 20 ng/ml EGF, 40 ng/ml HGF, 8 ng/ml TGF α, 30 ng/ml IGF-I and IGF-II, 2% B27 supplements (Gibco/BRL), 8 mM HEMES, 2 mM Glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin in DMEM-F12 medium). After two days in culture, the primary spheres were taken onto HTB-9 matrix in the differentiation media (5% FBS in Iscove Media containing 200ng/ml PYY and 30 uM Forskolin) for 4 days differentiation. The cells are fixed by 1% PFA followed by IHC analysis.

Primary spheres differentiated for 4 days gave rise to large amounts of cells that express both insulin and pdx-1 proteins. The percentage of insulin expressing cells in the total cell population is much higher than that of previous sphere culture methods, which started from non-dissociated ducts. (Fig.60).

Longer differentiation time points show better in vitro differentiation results. When the undifferentiated sphere went into differentiation conditions for 8 days, the level of insulin expression greatly increased compared with that of spheres differentiated for only 4 days, as detected by immunohistochemistry. Figure 61 shows that more than 50% of cells express both insulin and pdx-1. (Fig.61).

In addition to insulin expressing cells, there are also many glut-2 expressing cells. Glut-2 is a glucose transporter expressed in mature islet β cells that may also be a marker for β cell precursors. Interestingly, not all the insulin expressing cells co-express glut-2 protein. It may suggest a possibility that these glut-2 negative, insulin positive cells are newly derived β cells from their progenitor cells, and that they are not fully matured β cells. (Fig.62).

A semi-quantitative plot showing expression of three markers, insulin, pdx-1 and glut-2, with their relative level in three different stages: freshly isolated duct cells at T=0, spheres in growth prior to differentiation, and spheres after 8 days differentiation. There are less than 3% insulin expressing cells at T=0, ~6% insulin expressing cells

prior to differentiation, however, after 8 days differentiation, the insulin expressing cells went up to 56%. (Fig.63).

Part 2. Different growth factors in the growth medium affect the endocrine cell lineage obtained upon differentiation, although the cells were given the same differentiation condition.

When 15% KOSR was included in the growth culture medium in addition to the six growth factors, there were many glucagon-expressing cells after 12 days differentiation. Most glucagon cells do not express pdx-1, which is a typical phenotype for mature α cells in the islet. (Fig.64).

For the same growth factor condition as described in Fig.64, there are also substantial numbers of insulin expressing cells that also express pdx-1. (Fig. 65).

When LIF was included in the growth culture medium (as opposed to the differentiation medium) in addition to the other six growth factors, there were many fewer insulin expressing cells upon differentiation; however, the relative amount of Somatostatin expressing cells was greatly increased. Most Somatostatin expressing cells do not express pdx-1 (typical of δ cells), but a few Somatostatin expressing cells co-express pdx-1, suggesting they might be the precursor cells for mature Somatostatin cells and PP cells (ϕ cells), according to some endocrine lineage models. (Fig. 66).

Part 3. Ductal derived, insulin positive cells are glucose responsive.

Glucose stimulated insulin release in the spheres described in Fig 64 and 66. (Fig. 67). This demonstrates that the differentiated insulin positive cells are capable of carrying out the glucose regulatory activities of endogenous pancreatic β cells.

Part 4. Duct cells in sphere culture condition maintain multi- potentiality for both endocrine and exocrine lineage, after 45 days in culture.

Even after 45 days in culture, insulin and pdx-1 are co-expressed in many cells, suggesting that most cells remain undifferentiated. (Fig. 68).

Fig. 69 shows glucagon expression in cells; most glucagon cells do not express pdx-1, however, there are a few cells which co-express glucagon and pdx-1, suggesting the possibility of the early precursor cells for mature β cells and α cells. Interestingly,

there were large clusters that were almost 90% pdx-1 positive cells. It strongly suggested that there might be a synergistic event occurring that allows the cells to become more pdx-1 positive during the long-term sphere culture. Fig. 70 shows the Somatostatin expression in cells from that same culture.

Fig. 71 shows that cells from the above culture express an exocrine marker, Carboxypeptidase A (CPA). Furthermore, these CPA cells appeared in the same clusters as cells that express insulin, yet, there were no cells expressing both CPA and insulin. This strongly suggests that these endocrine and exocrine cells are derived from the same progenitor cells. Therefore, these cells have a broad developmental capability, ranging from exocrine to endocrine cells and including a number of different endocrine cell types.

Example 9: Human Pancreatic Duct Sphere Culture

Methods:

1. Isolation of human pancreatic ducts.

Human pancreatic tissue (Donor Age: 5 - 60 years) was obtained from National Diabetes Research Institute (NDRI). The pancreas was infused with UW medium, and shipped on wet ice in RPMI medium. Tissue was received for dissociation within 20 hours of donor removal. Upon arrival, the pancreas was placed in ice cold HBSS and dissected free of excess fat, lymphatic tissue, the small intestine and spleen. The pancreas was cut into small pieces, (~0.5 cm^3) and collected in ice-cold 1X HBSS (without calcium and magnesium). The pancreatic tissue was then centrifuged at 1500 rpm and the pellet resuspended in ice-cold Liberase (Roche) at 1mg/ml. The tissue was subsequently placed in a 37 C water bath with vigorous shaking. The supernatant from the pancreatic tissue digest was collected every 10 minutes and fresh Liberase added to the remaining tissue until complete digestion of the tissue occurred. Each collected supernatant fraction was immediately diluted into an equal volume of ice-cold 10% BSA/1X HBSS (without calcium and magnesium) and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the cell pellet resuspended in cold 1X HBSS (without calcium and magnesium) and kept on ice. The digested tissue fractions were later pooled together and washed in HBSS (without calcium and magnesium) three times. The tissue

suspension was then layered at a 1:1:1 volume ratio on top of a prepared percoll gradient which consisted of two layers; an upper layer containing 35% percoll and a lower layer containing 45% percoll. The gradient was centrifuged at 1970 rpm for 10 minutes. The duct fraction, collected at the interface between the top aqueous layer and the 35% percoll layer, was washed three times with 1X HBSS (without calcium and magnesium) to remove any residual percoll.

2. Dissociation of the collected ducts.

The final isolated duct tissue was collected by centrifugation at 1500 rpm and resuspended into 1X HBSS (containing calcium and magnesium). The suspension was then centrifuged at 1500 rpm. The cell pellet was resuspended into an enzyme cocktail containing 1.33 mg/ml Trypsin, 0.7 mg/ml Hyaluronidase, 0.2 mg/ml Kynerenic Acid, and 200 U/ml DNase in 1X HBSS (containing calcium and magnesium) and incubated at 37 C for 6 minutes followed by 4 minutes of incubation at room temperature. The digestion was terminated by addition of 1/4 volume of 10% BSA in 1X HBSS (containing calcium and magnesium). The cells were then centrifuged for 5 minutes at 1500 rpm and the cell pellet resuspended in 10% BSA/1X HBSS (containing calcium and magnesium). The cell suspension was again centrifuged at 1500 rpm and the cell pellet re-suspended in 1X HBSS (containing calcium and magnesium) supplemented with DNase (200 U/ml) and placed at 37 C for 10 minutes. The dissociated single duct cells were centrifuged at 1500 rpm and the cell pellet re-suspended in culture medium and plated.

3. Passage and culture of cells.

The dissociated human duct cells were seeded in T-75 Nunc flasks or on non-tissue culture treated petri dishes in DMEM/F12 culture medium containing 15% Knockout Serum Replacement (Gibco/BRL), 10 ng/ml bFGF, 20 ng/ml EGF, 40 ng/ml HGF, 8 ng/ml TGF-α, 30 ng/ml IGF-I and IGF-II, 2% B27 supplement (Gibco/BRL), 8 mM HEPES, 2 mM Glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin. The cell flasks or non-tissue culture petri dishes were kept in a tissue culture incubator containing 3% oxygen, 5% CO2 at 37C. Supplemental growth factors were added to the medium every 48 hours after the initial plating of the cells. The resulting cell aggregates

were successively passaged by triturating the cell pellet and re-suspending the cells into fresh culture medium or by completely dissociating the cells with Collagenase I.

Human pancreatic ducts were isolated by Liberase (Roche) and purified through a percoll gradient. The ducts were then dissociated into single cells and were plated for sphere culture, in non-adhesive, T-75 Nunc flasks in the DMEM/F12 basal medium containing 15% Knockout Serum Replacement (KO-SR), and 6 growth factors, namely EGF, bFGF, HGF, TGFα, IGF-I and IGF-II. The spheres grow larger over time (Figure 72).

Duct spheres were grown in serum-free growth medium as described above, and the cells were passaged every 5 days on average. There was an enrichment of CK-7, a duct epithelial marker, over time under this culture condition. The figure shows ~50% cells stained positive for CK-7 at passage 1. In contrast, there were ~6% CK-7 positive cells prior to the culture. (Figure 73).

Duct spheres were seeded in 96-well plate coated with HTB-9 matrix in differentiation medium containing 5% FBS, 30 uM Forskolin and 200ng/ml PYY in Iscove's Modified Dulbecco's Medium with 25 mM glucose for four days. The cells were fixed with 1% PFA and co-stained for anti-insulin and anti-pdx-1 antibodies. The figure shows most cytoplasmic insulin expressing cells also express pdx-1; there are a few insulin expressing cells that do not express pdx-1. Both these two types of insulin positive cells have been well documented as a marker for β cells in the islet. (Figure 74).

More insulin expressing cells appeared after culture for 11 days in differentiation medium on HTB-9 matrix, the same differentiation condition described above. Figure 75 shows bigger clusters of insulin positive cells compared with those showed in Figure 74. There are ~ 10% insulin positive cells in each well.

Some cells co-expressed pdx-1 and somatostatin. There are also distinctive somatostatin positive cells, which are another important islet cell type (δ cells). Those somatostatin expressing cells appeared in the pdx-1 expressing clusters, but the two markers are not expressed in the same cells, suggesting that these two cell types might derive from the same progenitor cells. (Figure 76).

Vimentin is a marker for fibroblasts. Figure 77 shows that Glut2 protein

expressing cells are in clusters, and they are excluded from Vimentin protein expressing cells. There were about ~50% Glut2-clusters in a 96-well. Given the fact that there were only ~10% insulin expressing cells in the population, the large number of glut-2 expressing cells could be an indication for progenitor cells.

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Table 1: Immunostaining in Duct Cultures

Marker expression in isolated ducts and Insulin in duct cultures. The average cell number per duct fragment was 3450 ± 1860 (n=10), comprising a single layer of epithelial cells surrounded by mesenchymal/stromal cells. Positive immunostaining on whole isolated ducts (n=60-90) was as follows: Insulin 7/90 ducts, 32 positive cells; PDX-1 4/75 ducts, 15 positive cells; PYY 5/60 ducts, 31 cells; Amylase 10/69 ducts, 49 cells. n=2 isolations for PDX-1, PYY, amylase; n=4 isolations for insulin. NAC yield generally ranged from 80,000 to 130,000 cells/prep; maximum seen was 160,000 although theoretical maximum based on an observed cell density is estimated to be 300,000 cells/prep (10,000 NACs/cm^2).

Marker Expression in Pancreatic Ducts at the Start of Culture

	Insulin	PDX-1	PYY	Amylase
% Positive Ducts	7.8%	5.3%	8.3%%	14.5%
% Positive Cells	0.01%	0.01%	0.015%	0.02%

Immunostaining for Insulin in Duct Cultures

	Total Cells Prep	Total Ins Positive Cells/Prep	% Insulin-Positive
T=0, Primary Ducts	800K	80	0.01 %

T=7 days	Control	Monolayer	2,000K	400 (5x ↑)	0.02 %
	Activated (+ DCE)	Monolayer	2,400K	1000 (12.5x ↑)	0.04 %
		NACs	100K	40,000 (500x ↑)	40 %

Table 2: Immunostaining for PDX-1, PYY and Amylase in Duct Cultures

96-100% of Insulin-positive cells in culture were also PDX-1-positive. In addition, PDX-1-positive, insulin-negative cells appear in all states of culture; however, in DCE-activated monolayers, the number of these cells is increased (20-50% more cells) *and* there are more cytoplasmic PDX-1 cells. There is little or no apparent change in PYY in the monolayer; however, PYY appears to be present in emerging NACs.

			Total PDX-1 Positive Cells/Prep	% PDX-1 Positive
T=0, Primary Ducts			80	0.01%
T=7 days	Control	Monolayer	480 (6x ↑)	0.02 %
	Activated (+ DCE)	Monolayer	1200 (15x ↑)	0.05 %

			Total PYY Positive Cells/Prep	% PYY Positive
T=0, Primary Ducts			120	0.015%
T=7 days	Control	Monolayer	60 (2x ↓)	0.003 %
	Activated (+ DCE)	Monolayer	100 (approx. no change)	0.004 %

			Total Amylase Positive Cells/Prep	% Amylase Positive
T=0, Primary Ducts			160	0.02%
T=7 days	Control	Monolayer	* (> 10x ↓)	**
	Activated (+ DCE)	Monolayer	ND	ND

* Only two out of ten preparations contained any Amylase-positive cells at all.

** % Amylase-positive is on the order of 0.001% *for the positive preps.* ND, not detected.

Table 3: Marker staining in Spheres on Cytospin slides (undifferentiated cells - cultured for 24 days)

Markers	Positive cells/Total cells	Average
Pdx1 +	20/627	
	16/520	
	10/366	
	3/493	2.2 +/- 0.4%
Insulin +	10/627	
	8/520	
	6/366	
	3/493	0.11 +/- 0.03%
Pdx+ per Insulin +		60%
Glut2 +	5/562	
	6/495	
	2/503	0.82 +/- 0.03%
Nestin +	314/562	
	222/495	
	250/503	52 +/- 3.2%
Glut2+ per Nestin +		0

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All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Claims

1. A method for obtaining differentiated pancreatic cell types comprising contacting a pancreatic progenitor cell with an adherent matrix and a differentiation medium comprising a cAMP elevating agent, a PYY polypeptide, and glucose, wherein said progenitor cell gives rise to at least one differentiated pancreatic cell type.

2. A method of claim 1, wherein said pancreatic progenitor cell is obtained as a non-adherent cell.

3. A method of claim 1 or 2, wherein said cAMP elevating agent comprises forskolin.

4. A pancreatic differentiation medium comprising a cAMP elevating agent, at least 150 ng/ml PYY and at least 3% fetal bovine serum.

5. The medium of claim 4, wherein said cAMP elevating agent is forskolin.

6. The medium of claim 4 or 5, comprising at least 25 mM forskolin, at least 150 ng/ml PYY and at least 3% fetal bovine serum.

7. A method of obtaining glucagon-expressing cells, comprising:
propagating a progenitor cell in the presence of 15% KO-SR; and
differentiating said cell by contacting it with an adherent matrix and a differentiation medium;
wherein said progenitor cell gives rise to glucagon expressing cells.

8. A method of claim 7, wherein said progenitor cell is propagated without adhering to a substrate.

9. A method of obtaining somatostatin-expressing cells, comprising:

propagating a progenitor cell in the presence of LIF; and
differentiating said cell by contacting it with an adherent matrix and a differentiation medium; wherein said progenitor cell gives rise to somatostatin expressing cells.

10. A method for expanding a non-adherent population of progenitor cells comprising:
obtaining a non-adherent population of progenitor cells;
dissociating the non-adherent population of progenitor cells; and
culturing the dissociated cells in a medium comprising TGFα, HGF, IGF-1, and IGF-II, thereby expanding the progenitor cell population.

11. The method of claim 10, wherein the medium further comprises EGF and FGF.

12. The method of claim 10 or 11, wherein the medium further comprises KO-SR.

13. A hematopoietic cell culture medium, comprising EGF, FGF, and SCF.

14. The culture medium of claim 13, further comprising LIF.

15. The culture medium of claim 13 or 14, further comprising Flt3.

16. A method for preparing a non-adherent population of cells enriched in hematopoietic progenitor cells comprising:

obtaining a cell suspension from an animal tissue, wherein said cell suspension comprises at least one progenitor cell;

treating the cell suspension with a growth factor preparation comprising SCF; and

allowing proliferation of said at least one progenitor cell such that a substantially pure non-adherent progenitor cell population is obtained,

thereby obtaining a population of non-adherent cells enriched in hematopoietic progenitor cells.

17. The method of claim 16, wherein the growth factor preparation further comprises EGF and FGF.

18. The method of claim 16 or 17, wherein the growth factor preparation further comprises LIF.

19. The method of any one of claims 16 to 18, wherein the animal tissue is embryonic, fetal, or adult tissue.

20. The method of any one of claims 16 to 19, wherein the hematopoietic progenitor cells are Sca+, c-kit+, and Lin-.

21. The method of any one of claims 16 to 20, wherein the animal tissue is liver.