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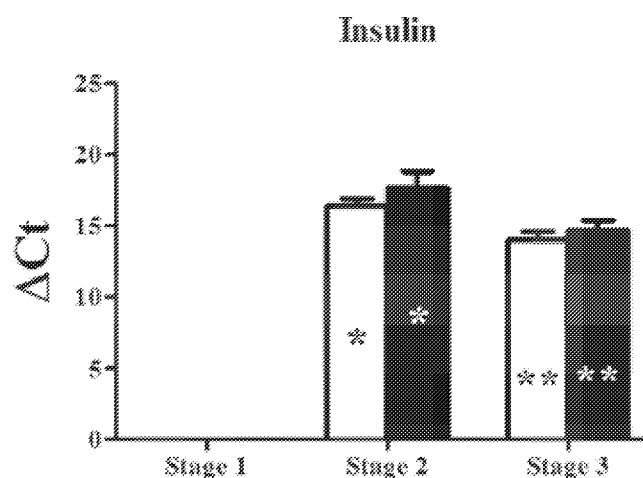


FIG. 10A

(57) Abstract: Compositions and methods are described herein for transdifferentiation of multipotent stromal cells into cells that can express insulin.



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FUNCTIONAL FELINE PANCREATIC CELLS FROM ADIPOSE TISSUE

Priority

This application claims benefit of the priority filing date of U.S. Provisional Patent Application Ser. No. 62/537,712, filed July 27, 2017, the contents of which are
5 specifically incorporated by reference herein in their entirety.

Background

Diabetes mellitus is one of the most prevalent feline endocrinopathies. About 15-20% of feline diabetes cases are caused by decreased β -cell numbers or insulin resistance (Rand et al., J Nutrition 134:1072S-1080S (2004); Sittinger et al. Curr.
10 Opin. Biotech. 15:411-418 (2004)). The condition affects all breeds and sexes and is associated with reproductive sterilization ^{5,6}, obesity, physical inactivity ⁷, and advancing age ⁸ and drug side effects (McCann et al., J. Feline Med & Surg 9: 289-299 (2007); Pancciera et al., J Am Vet Med Assoc 197: 1504-1508 (1990); Prah et al., J. Feline Med & Surg 9: 351-358 (2007); O'Brien, Molec Cell Endocrinol 197:
15 213-219 (2002)). To date, no single cause or effective cure has been identified.

Serious complications associated with unregulated glucose levels include increased bone fractures, cardiovascular disease, and neurological dysfunction, among others (Schwartz Calcified Tissue Internat 73:515-519 (2003); Shehadeh & Regan, Clin. Cardiol. 18: 301-305 (1995); Mizisin et al., J Neuropath & Exp. Neurol. 61-872-
20 884 (2002)). Contemporary treatment consists of diet and weight management with exogenous insulin administration to replace that normally produced by pancreatic β cells (Fu et al., Curr. Diabetes Rev. 9: 25 (2013)). Available insulin formulations do not share the feline amino acid sequence. Though insulin maintains biological activity across species, sequence differences may affect activity and stimulate the
25 immune system (Betsholtz et al., Diabetes 39: 118-122 (1990); Chance et al., Science 161: 165-167 (1968)). Additionally, insulin administration is typically customized for individual patients, a challenging and time-consuming process. The time and costs associated with establishing and revising insulin therapy and daily injections can be a burden on the owners.

Summary

Methods and compositions are described herein for transdifferentiation of feline starting cells such as adipose-derived multipotent stromal cells (ASCs) to generate pancreatic β cells. Such starting cells (e.g., ASCs) can be obtained from adipose tissues, for example, from subcutaneous adipose tissue or from reproductive organs (e.g., when they are removed during routine feline sterilization procedures). Results are described herein that quantify the transdifferentiation capability of feline starting cells such as ASCs. The feline starting cells cultured in pancreatic β cell induction medium had better insulin production and glucose response compared to that cultured in stromal medium.

One method described to generate pancreatic β cells can include:

- a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, activin A, sodium butyrate, and 2-mercapethanol to generate a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2;
- b. Stage 2: culturing the first population of cells for 4 to 8 days in a second culture medium comprising insulin-transferrin-selenium, and taurine to generate a second population of cells; and
- c. Stage 3: culturing the second population of cells for 2 to 6 days in a third culture medium comprising insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, pentagastrin, and betacellulin to generate a third population of cells.

Cells in the second population typically express Nkx 6.1, Pax6, Isl1, and/or Glut-2 at greater expression levels than cells in the first population. Cells in the third population also typically express Nkx 6.1, Pax6, Isl1, and/or Glut-2 at greater expression levels than cells in the second population.

The methods can also include administering the third population of cells (or a portion thereof) to a subject, for example, a feline subject.

In some cases, the first culture medium, the second culture medium, and the third culture medium can further include at least one, at least two, at least three, or at least four of the following: basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), laminin, N-2 supplement, or B-27 supplement.

In some cases the first culture medium, the second culture medium, and the third culture medium can also contain glucose, and/or serum albumin (e.g., bovine serum albumin).

Cells in the second population of cells and/or the third population of cells can
5 express insulin. However, cells in the first population of cells typically do not express detectable amounts of insulin mRNA as detected by quantitative polymerase chain polymerase reaction.

Compositions are also described herein.

For example, one type of cell composition can include a first population of
10 cells made by: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) from feline adipose tissue for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, activin A, sodium butyrate, and 2-mercapethanol to generate a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2.

15 Another type of cell composition can include a second population of cells made by:

- a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, activin A, sodium butyrate, and 2-
20 mercapethanol to generate a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2; and
- b. Stage 2: culturing the first population of cells for 4 to 8 days in a second culture medium comprising insulin-transferrin-selenium, and taurine to generate a second population of cells.

25 Another type of cell composition can include a third population of cells made by:

- a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, activin A, sodium butyrate, and 2-
30 mercapethanol to generate a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2;
- b. Stage 2: culturing the first population of cells for 4 to 8 days in a second culture medium comprising insulin-transferrin-selenium, and taurine to generate a second population of cells; and

- c. Stage 3: culturing the second population of cells for 2 to 6 days in a third culture medium comprising insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, pentagastrin, and betacellulin to generate a third population of cells.

5 Composition are also included herein that are useful for transdifferentiation of cells. Such compositions can be formulated as a culture medium, or as a dry composition or a concentrated liquid that is later rehydrated or diluted, respectively, to generate the culture medium.

For example, one type of composition can include at least four, or at least five,
10 or at least six, or at least seven, or at least eight, or at least nine, or at least ten of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, activin A, sodium butyrate, 2-mercapethanol, N-2 supplement, B-27 supplement, laminin, hepatocyte growth factor (HGF), or basic fibroblast growth factor (bFGF).

Another type of composition can include at least four, or at least five, or at
15 least six, or at least seven, or at least eight of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, taurine, laminin, basic fibroblast growth factor (bFGF), N-2 supplement, B-27 supplement, or human hepatocyte growth factor (HGF).

Another type of composition can include at least four, or at least five, or at
20 least six, or at least seven, or at least eight, or at least nine, or at least ten, or at least eleven, or at least twelve of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, non-essential amino acids, pentagastrin, N-2 supplement, B-27 supplement, human hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), laminin, or
25 betacellulin.

Description of the Figures

FIGs. 1A-1C are photomicrographs of feline adipose-derived multipotent stromal (stem) cells (ASCs) following culture in stromal medium. FIG. 1A is a photomicrograph showing ASCs after culturing in stromal medium. The scale is a 5 ×
30 scale, and the scale bar = 500 μm. FIG. 1B is a photomicrograph showing alizarin red stained ASCs after culturing in osteogenic medium. The scale is a 5 × scale, and the scale bar = 500μm. Alizarin red staining illustrates calcium deposition. FIG. 1C is a photomicrograph showing oil red O lipid stained ASCs after culture in adipogenic medium. The scale is a 40 × scale, and the scale bar = 50 μm.

FIG. 2A-2C are light photomicrographs of fresh feline P3 ASCs. FIG. 2A shows a light photomicrograph of fresh male feline P3 ASCs cultured in stromal medium. FIG. 2B shows a light photomicrograph of fresh male feline P3 ASCs cultured in pancreatic β -cell induction medium and transferred to the normal culture plate. FIG. 2C shows a light photomicrograph of fresh female feline P3 ASCs cultured in pancreatic β -cell induction medium and transferred to the normal culture plate. 5 $\times$ magnification. Scale bar=500 μ m.

FIGs. 3A-3C are light photomicrographs of dithizone-stained fresh feline P3 ASCs. FIG. 3A shows a light photomicrograph of fresh male feline P3 ASCs stained with dithizone cultured in stromal medium. FIG. 3B shows a light photomicrograph of fresh male feline P3 dithizone-stained ASCs cultured in pancreatic β -cell induction medium. FIG. 3C shows a light photomicrograph of fresh female feline P3 dithizone-stained ASCs cultured in pancreatic β -cell induction medium. Dithizone binds zinc ions present in the islet's beta cells, and therefore stains the islets. 20 $\times$ magnification, scale bar = 100 μ m (A, B); 40 $\times$ magnification, scale bar = 50 μ m.

FIG. 4 illustrates fluorescent photomicrographs of cells cultured in stromal (top three panels) or induction medium (bottom three panels) and labeled with antibodies against insulin (middle two panels, red in the original) and actin (left two panels, blue in original). As illustrated, cells cultured in stromal media do not express insulin, but cells incubated in induction medium do express insulin.

FIG. 5 graphically illustrates results of a glucose challenge assay of cells generated by the methods described herein. Insulin secretion from differentiated islet-like cell clusters exposed to distinct glucose concentrations in the medium. Columns with distinct superscripts are significantly different among differentiation stages within gender. Insulin secretion from differentiated islet-like cell clusters at stimulated concentrations of (25 and 55 mM) glucose are shown.

FIG. 6A-6B illustrate transmission electron photomicrographs of feline ASCs. FIG. 6A illustrates a transmission electron photomicrograph of feline ASCs cultured in stromal medium. FIG. 6B illustrates a transmission electron photomicrograph of feline ASCs cultured in induction medium. Legend: M – mitochondria, G – secretory granules. Scale bar = 2 μ m.

FIG. 7A-7B show transmission electron photomicrographs demonstrating insulin labeled with anti-insulin antibodies (arrows) in feline ASCs cultured in β pancreatic cell medium. FIG. 7A shows transmission electron photomicrographs of

feline ASCs cultured in stromal medium. FIG. 7B shows transmission electron photomicrographs of feline ASCs cultured in β cell induction medium. Scale bar = 0.5 μ m.

FIG. 8A-8B show scanning electron photomicrographs of feline ASCs. FIG. 8A shows scanning electron photomicrographs of feline ASCs cultured in stromal medium. FIG. 8B shows scanning electron photomicrographs of feline ASCs cultured in β cell induction medium. White arrows illustrate cell cluster morphology. Black arrows illustrate proteinaceous material on the surface of clusters cultured in induction medium. Scale bar = 5 μ m.

FIG. 9A-9D graphically illustrate expression of various feline genes in feline male (open bars) and female (dark bars) ASCs following three stages of pancreatic β cell culture induction. FIG. 9A graphically illustrates expression of feline NK6 homeobox 1 (NK6.1) in feline ASCs following three stages of pancreatic β cell culture induction. FIG. 9B graphically illustrates expression of feline paired box 6 (Pax6) in feline ASCs following three stages of pancreatic β cell culture induction. FIG. 9C graphically illustrates expression of feline ISL LIM homeobox 1 (Isl1) in feline ASCs following three stages of pancreatic β cell culture induction. FIG. 9D graphically illustrates expression of feline glucose transporter 2 (Glut2) in feline ASCs following three stages of pancreatic β cell culture induction. Expression levels (LS mean $\pm$ SEM) are shown. Columns with distinct superscripts are significantly different between sexes within stages. Different asterisk numbers (*) are significantly different among stages within gender.

FIGs. 10A-10C graphically illustrate expression of various genes in feline male (open bars) and female (dark bars) ASCs following three stages of pancreatic β cell culture induction. FIG. 10A graphically illustrates expression of feline insulin in feline ASCs following three stages of pancreatic β cell culture induction. FIG. 10B graphically illustrates expression of feline glucagon in feline ASCs following three stages of pancreatic β cell culture induction. FIG. 10C graphically illustrates expression of feline somatostatin in feline ASCs following three stages of pancreatic β cell culture induction. Expression levels (LS mean $\pm$ SEM) are shown. Columns with different asterisk numbers (*) are significantly different among stages within gender.

FIGs. 11A-11D graphically illustrate expression of various genes in feline male (open bars) and female (dark bars) ASCs following three stages of pancreatic β cell culture induction. FIG. 11A graphically illustrates expression of feline proto-

oncogene tyrosine-protein kinase ROS1 in feline ASCs following three stages of pancreatic β cell culture induction. FIG. 11B graphically illustrates expression of feline serine/threonine kinase 1 AKT1 in feline ASCs following three stages of pancreatic β cell culture induction. FIG. 11C graphically illustrates expression of feline Ras-related protein RAB2A in feline ASCs following three stages of pancreatic β cell culture induction. FIG. 11D graphically illustrates expression of feline hexokinase 1 HK1 in feline ASCs following three stages of pancreatic β cell culture induction. Expression levels (LS mean $\pm$ SEM) are shown.

FIG. 12 shows an image of a western blot confirming insulin production by feline pancreatic cell clusters generated from adipose derived stem cells by the methods described herein. The blot shows the protein ladder left, two samples from cell clusters and two samples from paired samples cultured in stromal (basal) medium (left to right).

Detailed Description

Diabetes mellitus is among the most common feline endocrinopathies, there is no known cure, and the prevalence is steadily increasing. Unregulated glucose levels contribute to serious, permanent health problems in cats of all breeds and ages. Exogenous insulin administration is necessary to maintain healthy glucose levels when pancreatic β cells cease to function. Therapy is often a complex, time consuming process that relies on non-feline insulin formulations. Treatment with xenogeneic insulin is fraught with complications and is time consuming. As many as 30% of cats succumb to consequences of the disease within 1 year of diagnosis.

As described herein, starting feline cells such as adipose-derived multipotent stromal (stem) cells (ASCs) can be transdifferentiated into pancreatic islet cells that secrete insulin in response to glucose. The cells can be used to create implantable tissue grafts to restore β cell function that can be applied in a permanent or removable configuration. The methods and compositions can be used to generate exogenous feline insulin for direct administration to cats.

The pancreatic islet cells produced using the methods and compositions described herein can restore natural insulin production after minimally invasive implantation of custom tissue grafts generated from individual stem cells. Such custom insulin producing grafts (e.g., made from ASCs) that are conducive to minimally invasive implantation may cure diabetes in cats.

As illustrated herein, feline ASCs isolated from subcutaneous adipose tissue or from reproductive organs have endodermal transdifferentiation capability. However, feline ASCs from male donors often have distinct morphology compared to female donors. Induced cells appear to form functional clusters based on zinc accumulation secretion of insulin in response to glucose stimulation, the presence of intracellular insulin, and the pancreatic β -cell specific gene expression. The methods and compositions described herein significantly advance the potential to produce custom feline insulin for exogenous administrations as well as a cell-based therapy for temporary or long term restoration of feline pancreatic β cell function.

The compositions and methods have been customized for feline cells and have been designed to replicate *in vivo* pancreatic formation and development in cats. In view of species differences, culture methods are most effective when designed for the target species (Buang et al., Arch. Med. Res. 43: 83-88 (2012); Dubey et al., Repro. Fertil. Devel. 26: 213 (2014); Dang et al., Biomed. Res. Ther. 2: 184-192 (2015); Chandra et al., Stem Cells 27: 1941-1953 (2009)).

In the developed a three-stage induction system, N-2 and B-27 can enhance proliferation and protect against reactive oxygen species in the absence of FBS. FBS can provide growth factors, nutrients and hormones for cell proliferation and adhesion. Additionally, there are several extrinsic factors used in the system that have beneficial effects on differentiation of MSCs into insulin producing cells.

Stage I

Stage 1 can involve culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) for about 1-4 days in a first culture medium comprising activin A, sodium butyrate, and 2-mercapethanol.

At Stage 1, activin A and sodium butyrate can direct starting cells such as ASCs to undergo endoderm differentiation. Insulin-transferrin-selenium (ITS), 2-mercaptoethanol, and the supplements can protect starting cells such as ASCs and can reduce the risk of apoptosis. Ultra-low attachment culture plates or culture vessels can be used to enhance formation of three-dimensional cell aggregates. For example, protein coated culture plates or culture vessels can be used. Examples of proteins that can coat the culture plates or culture vessels include collagen, fibronectin, laminin, polylysine, poly-ornithine, or a combination thereof.

Activin A is available commercially from various suppliers, for example, from Invitrogen, PeproTech, StemRD, R&D Systems, and other vendors.

In some cases, agents that have activity like activin A can be used instead of activin A. Activin A is a member of the TGF β family first identified in late 1980s as an inducer of follicle-stimulating hormone. Activin A is highly conserved in evolution and throughout the animal kingdom. It regulates a variety of biologic processes including cell proliferation, hematopoiesis, wound healing, and fibrosis. Activin A signals through the activin type I (Alk2, 4, or 7) and type II (ActRII or ActRIIB) receptors and shares with TGF β the activation of the Smad cascade. *See*, Phillips et al., *Cytokine Growth Factor Rev.* 20(2): 153-64 (2009); Werner, *Cytokine Growth Factor Rev.* 17(3): 157-71 (2006). Examples of TGF- β family members that can be used instead of activin A include the decapentaplegic-Vg-related (DVR) related subfamily of proteins (including bone morphogenetic proteins and the growth differentiation factors), and the TGF- β subfamily.

Activin A and/or other TGF β family members can be used at a variety of concentrations, for example, at about 0.1 nanomolar to about 20 nanomolar, or from about 0.5 nanomolar to about 15 nanomolar, or from about 1 nanomolar to about 10 nanomolar, or from about 2 nanomolar to about 8 nanomolar, or about 4 nanomolar.

Agents with activities like sodium butyrate can also be used instead of sodium butyrate (or in combination with sodium butyrate). Sodium butyrate is an inhibitor histone deacetylases (HDACs), which are a class of enzymes that remove acetyl groups from an ϵ -N-acetyl lysine amino acid on a histone. Examples of HDAC inhibitors that can be used include, for example, butyrate, small molecular weight carboxylates (e.g., less than about 250 amu), hydroxamic acids, benzamides, epoxyketones, cyclic peptides, and hybrid molecules. (See, for example, Drummond et al., *Annu Rev Pharmacol Toxicol* 45: 495-528 (2005), (including specific examples therein) which is hereby incorporated by reference in its entirety). Other examples of negative regulators of type I/II HDACs include: sodium butyrate, phenyl butyrate, or butyrate, suberoylanilide hydroxamic Acid (SAHA; also called Vorinostat and MK0683), valproic acid (and other short chain fatty acids), suramin (e.g., suramin sodium), and combinations thereof.

The HDAC inhibitor can be employed in the compositions and methods described herein in a variety of amounts and/or concentrations. For example, the HDAC inhibitor can be employed at a concentration of about 1 micromolar to about 20 millimolar, or about 10 micromolar to about 15 millimolar, or about 50 micromolar to about 10 millimolar, or about 100 micromolar to about 10 millimolar,

or about 0.5 millimolar to about 2 millimolar, or about 1 millimolar in a culture medium solution.

The stage 1 culture medium can include beta-mercaptoethanol at a variety of concentrations. For example, beta-mercaptoethanol can be employed at a
5 concentration of from about 1 micromolar to about 200 micromolar, or from about 5 micromolar to about 100 micromolar, or from about 10 micromolar to about 80 micromolar, or at about 50 micromolar in a culture medium solution.

The stage 1 compositions and methods can include use of other agents including insulin-transferrin-selenium (ITS), supplements, proteins and/or growth
10 factors.

Insulin-transferrin-selenium can be present in various amounts. Insulin-Transferrin-Selenium is available from various commercial vendors. Insulin is typically present in culture media at a concentration of about 1 µg/ml to about 100 µg/ml, or from about 3 µg/ml to about 50 µg/ml, or from about 5 µg/ml to about 20
15 µg/ml, or at about 10 µg/ml. Transferrin is typically present in culture media at a concentration of about 5 µg/ml to about 150 µg/ml, or from about 20 µg/ml to about 100 µg/ml, or from about 30 µg/ml to about 750 µg/ml, or at about 55 µg/ml. Selenium is typically present in culture media at a concentration of about 1 µg/ml to about 100 µg/ml, or from about 3 µg/ml to about 50 µg/ml, or from about 5 µg/ml to
20 about 20 µg/ml, or at about 10 µg/ml. Selenium can be used at a variety of concentrations, for example, at about 5 ng/ml to about 200 ng/ml, or from about 20 ng/ml to about 150 ng/ml, or from about 40 ng/ml to about 100 ng/ml, or from about 50 ng/ml to about 80 ng/ml, or at about 67 ng/ml. In some cases, a commercial preparation of insulin-transferrin-selenium can be employed, for example, from Gibco
25 BRL (Gaithersburg, MD), which is often supplied in concentrated form. For example, 100-fold concentrated insulin-transferrin-selenium preparations can be diluted 1:100 into culture medium.

Examples of proteins that can be included in the stage 1 compositions and methods include serum albumin, collagen, fibronectin, laminin, polylysine, poly-
30 ornithine, or a combination thereof. Examples of growth factors that can be included in the stage 1 compositions and methods include hepatocyte growth factor (HGF) and/or fibroblast growth factor (bFGF).

In some cases, the stage 1 compositions and methods can include supplements such as N-2 supplement, B-27 supplement, or a combination thereof.

Proteins can be included in the stage 1 compositions at a variety of concentrations. For example, proteins (e.g., laminin, collagen, or a combination thereof) can be employed at a concentration of from about 0.1 µg/ml to about 100 µg/ml, or from about 1 µg/ml to about 10 µg/ml, or from about 2 µg/ml to about 7 µg/ml, or at about 5 µg/ml in a culture medium solution. Serum albumin (e.g., BSA) can be employed at a concentration of from about 0.05% to about 10%, or from about 0.1% to about 5%, or from about 0.5% to about 2%, or at about 1% in a culture medium solution.

Growth factors can be included in the stage 1 compositions are a variety of concentrations. For example, growth factors (e.g., HGF, bFGF, or a combination thereof) can be employed at a concentration of from about 0.1 ng/ml to about 200 ng/ml, or from about 1 ng/ml to about 150 ng/ml, or from about 10 ng/ml to about 70 ng/ml, or at about 20 ng/ml to about 50 ng/ml in a culture medium solution.

For example, the stage 1 media can include 4 nM avidin A, 1 mM sodium butyrate, 50 µM 2-mercapethanol, 1% N-2 supplement, 1% B-27 supplement, 5 µg/ml laminin (Corning), 50 ng/ml recombinant human hepatocyte growth factor (HGF), and 20ng/ml basic fibroblast growth factor (bFGF).

Stage 2

Stage 2 can induce the endoderm cell clusters formed at Stage 1 to provide cells of the pancreatic endodermal lineage. Cells at this stage can be cultured on protein coated culture plates or in protein coated culture vessels.

Stage 2 can involve culturing the first population of cells for 4 to 8 days in a second culture medium comprising insulin-transferrin-selenium, and taurine.

The insulin-transferrin-selenium can be present in various amounts. Insulin-Transferrin-Selenium is available from various commercial venders. Insulin is typically present in culture media at a concentration of about 1 µg/ml to about 100 µg/ml, or from about 3 µg/ml to about 50 µg/ml, or from about 5 µg/ml to about 20 µg/ml, or at about 10 µg/ml. Transferrin is typically present in culture media at a concentration of about 5 µg/ml to about 150 µg/ml, or from about 20 µg/ml to about 100 µg/ml, or from about 30 µg/ml to about 750 µg/ml, or at about 55 µg/ml. Selenium is typically present in culture media at a concentration of about 1 µg/ml to about 100 µg/ml, or from about 3 µg/ml to about 50 µg/ml, or from about 5 µg/ml to about 20 µg/ml, or at about 10 µg/ml. Selenium can be used at a variety of concentrations, for example, at about 5 ng/ml to about 200 ng/ml, or from about 20

ng/ml to about 150 ng/ml, or from about 40 ng/ml to about 100 ng/ml, or from about 50 ng/ml to about 80 ng/ml, or at about 67 ng/ml. In some cases, a commercial preparation of insulin-transferrin-selenium can be employed, for example, from Gibco BRL (Gaithersburg, MD). In some cases, a commercial preparation of insulin-
5 transferrin-selenium can be employed, for example, from Gibco BRL (Gaithersburg, MD), which is often supplied in concentrated form. For example, 100-fold concentrated insulin-transferrin-selenium preparations can be diluted 1:100 into culture medium.

10 Taurine, or 2-aminoethanesulfonic acid, is an organic compound that is widely distributed in animal tissues. It is a major constituent of bile and can be found in the large intestine.

 Taurine introduced into the induction medium can facilitate development of functional β cells. During the induction process, the induced cell clusters can have a morphology similar to the induced β -cell cluster morphology from other species (see,
15 e.g., Okura et al., J. Artificial Organs 12:123-130 (2009)) and may lose the capacity to attach to the plastic culture. Loss of the ability to attach to plastic is one criterion for identifying mesenchymal stromal cells (MSCs) (Dominici et al., Cytotherapy 8 (2006)). Cells cultured in stromal medium retained their plastic affinity after culture on normal culture plates while those in induction medium did not, further confirming
20 differentiation and maturation.

 Taurine can be employed in the compositions and methods described herein in a variety of amounts and/or concentrations. For example, taurine can be employed at a concentration of about 0.01 mM to about 10 mM, or from about 0.03 mM to about 5 mM, or from about 0.05 mM to about 1 mM, or from about 0.1 mM to about 0.7 mM,
25 or from about 0.2 mM to about 0.5 mM, or at about 0.3 mM.

 The compositions and methods can include use of other agents including one or more types of sugar, one or more types of protein, one or more types of growth factors, one or more types of supplements, and combinations thereof.

30 Sugars such as glucose or sucrose can be included in the stage 2 compositions and methods. Sugars can be employed in the compositions and methods described herein in a variety of amounts and/or concentrations. For example, sugars (e.g., glucose) can be employed at a concentration of about 0.1 mM to about 100 mM, or from about 1 mM to about 50 mM, or from about 5 mM to about 30 mM, or from

about 10 mM to about 25 mM, or from about 15 mM to about 20 mM, or at about 17.5 mM.

Proteins can be included in the stage 1 compositions at a variety of concentrations. For example, proteins (e.g., laminin, collagen, or a combination thereof) can be employed at a concentration of from about 0.1 µg/ml to about 100 µg/ml, or from about 1 µg/ml to about 10 µg/ml, or from about 2 µg/ml to about 7 µg/ml, or at about 5 µg/ml in a culture medium solution. Serum albumin (e.g., BSA) can be employed at a concentration of from about 0.05% to about 10%, or from about 0.1% to about 5%, or from about 0.5% to about 2%, or at about 1% in a culture medium solution.

Growth factors can be included in the stage 1 compositions are a variety of concentrations. For example, growth factors (e.g., HGF, bFGF, or a combination thereof) can be employed at a concentration of from about 0.1 ng/ml to about 200 ng/ml, or from about 1 ng/ml to about 150 ng/ml, or from about 10 ng/ml to about 70 ng/ml, or at about 20 ng/ml to about 50 ng/ml in a culture medium solution.

In some cases, the stage 1 compositions and methods can include supplements such as N-2 supplement, B-27 supplement, or a combination thereof.

For example, the stage 2 media can include serum free medium (SFM 2) (6 days): DMEM, 17.5mM glucose, 1% BSA, 1 × ITS, 0.3mM taurine (ACROS Organics, Morris Plains, NJ), 5 µg/ml laminin, 20 ng/ml bFGF, 1% N-2 supplement, 1% B-27 supplement, 50 ng/ml HGF;

Stage 3

Stage 3 can involve culturing the second population of cells for 2 to 6 days in a third culture medium comprising insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, pentagastrin, and betacellulin.

The insulin-transferrin-selenium can be used at somewhat higher concentrations than employed for stages 1 and 2. For example, insulin is typically present in culture media at a concentration of about 1.5 µg/ml to about 150 µg/ml, or from about 3 µg/ml to about 50 µg/ml, or from about 7 µg/ml to about 30 µg/ml, or at about 15 µg/ml. Transferrin is typically present in culture media at a concentration of about 7.5 µg/ml to about 200 µg/ml, or from about 25 µg/ml to about 150 µg/ml, or from about 50 µg/ml to about 100 µg/ml, or at about 75-80 µg/ml. Selenium can be used at a variety of concentrations, for example, at about 10 ng/ml to about 400 ng/ml, or from about 20 ng/ml to about 300 ng/ml, or from about 40 ng/ml to about 200

ng/ml, or from about 50 ng/ml to about 150 ng/ml, or at about 100 ng/ml. In some cases, a commercial preparation of insulin-transferrin-selenium can be employed, for example, from Gibco BRL (Gaithersburg, MD). In some cases, a commercial preparation of insulin-transferrin-selenium can be employed, for example, from Gibco BRL (Gaithersburg, MD), which is often supplied in concentrated form. For example, 100-fold concentrated insulin-transferrin-selenium preparations can be diluted 1.5:100 into culture medium.

Taurine can be used in amounts that are somewhat greater than the amounts used for Stage 2. For example, taurine can be used in amounts that are about two-fold, or five-fold, or ten-fold, or about fifteen-fold greater than the amounts used for Stage 2. Taurine can be employed in the stage 3 compositions and methods at a concentration of about 0.005 mM to about 20 mM, or from about 0.01 mM to about 15 mM, or from about 0.05 mM to about 10 mM, or from about 0.1 mM to about 5 mM, or from about 0.2 mM to about 1 mM, or at about 3 mM.

The glucagon-like peptide 1 (GLP-1) can be employed in the compositions and methods described herein in a variety of amounts and/or concentrations. For example, the GLP-1 can be employed at a concentration of about 1 nanomolar to about 300 nanomolar, or about 10 nanomolar to about 200 nanomolar, or about 30 nanomolar to about 150 nanomolar, or about 50 nanomolar to about 120 nanomolar, or at about 100 nanomolar in a culture medium solution.

Nicotinamide is a polyADP-ribose synthetase inhibitor. Other types of polyADP-ribose synthetase inhibitors can be used instead of or in combination with nicotinamide. Examples of polyADP-ribose synthetase inhibitors therefore include nicotinamide, 3-aminobenzamide, 1,5-isoquinolinediol and combinations thereof. The polyADP-ribose synthetase inhibitors (e.g., nicotinamide) can be employed at a concentration of about 0.05 millimolar to about 100 millimolar, or about 0.1 millimolar to about 20 millimolar, or about 0.3 millimolar to about 10 millimolar, or about 0.5 millimolar to about 5 millimolar, or about 1 millimolar in a culture medium solution.

Pentagastrin is a synthetic polypeptide that has effects like gastrin. When administered, it can stimulate the secretion of gastric acid, pepsin, and intrinsic factor. Pentagastrin can be employed in the compositions and methods described herein in a variety of amounts and/or concentrations. For example, pentagastrin can be employed at a concentration of from about 0.1 nanomolar to about 30 nanomolar, or about 1

nanomolar to about 20 nanomolar, or about 3 nanomolar to about 15 nanomolar, or about 5 nanomolar to about 12 nanomolar, or at about 10 nanomolar in a culture medium solution.

Betacellulin is a member of the EGF family of growth factors. It is synthesized primarily as a transmembrane precursor, which is then processed to mature molecule by proteolytic events. It can be a ligand for EGF receptor. Betacellulin can be employed in the compositions and methods described herein in a variety of amounts and/or concentrations. For example, betacellulin can be employed at a concentration of from about 0.1 ng/ml to about 200 ng/ml, or from about 1 ng/ml to about 100 ng/ml, or from about 3 ng/ml to about 50 ng/ml, or from about 5 ng/ml to about 25 ng/ml, or at about 100 ng/ml in a culture medium solution.

The stage 3 compositions and methods can include use of other agents such as sugars, proteins, supplements, and amino acids.

Sugars such as glucose or sucrose can be included in the stage 2 compositions and methods. Sugars can be employed in the compositions and methods described herein in a variety of amounts and/or concentrations. For example, sugars (e.g., glucose) can be employed at a concentration of about 0.1 mM to about 100 mM, or from about 1 mM to about 50 mM, or from about 5 mM to about 30 mM, or from about 10 mM to about 25 mM, or from about 15 mM to about 20 mM, or at about 17.5 mM.

Proteins can be included in the stage 1 compositions at a variety of concentrations. For example, proteins (e.g., laminin, collagen, or a combination thereof) can be employed at a concentration of from about 0.1 µg/ml to about 100 µg/ml, or from about 1 µg/ml to about 10 µg/ml, or from about 2 µg/ml to about 7 µg/ml, or at about 5 µg/ml in a culture medium solution. Serum albumin (e.g., BSA) can be employed at a concentration of from about 0.05% to about 10%, or from about 0.1% to about 5%, or from about 1% to about 3%, or at about 1.5% in a culture medium solution.

Growth factors can be included in the stage 1 compositions are a variety of concentrations. For example, growth factors (e.g., HGF, bFGF, or a combination thereof) can be employed at a concentration of from about 0.1 ng/ml to about 200 ng/ml, or from about 1 ng/ml to about 150 ng/ml, or from about 10 ng/ml to about 70 ng/ml, or at about 20 ng/ml to about 50 ng/ml in a culture medium solution.

In some cases, the stage 1 compositions and methods can include supplements such as N-2 supplement, B-27 supplement, or a combination thereof.

In some cases, the third stage compositions and methods can include use of the following serum free media (SFM 3) for 4 days: DMEM, 17.5 mM glucose, 1.5%
 5 BSA, $1.5 \times$ ITS, 3 mM taurine, 100 nM glucagon-like peptide 1 (GLP-1), 1 mM nicotinamide, $1 \times$ non-essential amino acids (NEAA, e.g., from Gibco), 10 nM pentagastrin, 1% N-2 supplement, 1% B-27 supplement, 50ng/ml HGF, 20ng/ml bFGF, 5 μ g/ml laminin, 10 ng/ml betacellulin.

At a third stage, the cell clusters are converted into functional pancreatic
 10 hormone-expressing islet-like cell aggregates with the help of glucagon-like peptide 1 (GLP-1), nicotinamide, taurine, and β -cellulin. Nicotinamide can promote the maturation of precursor cells into insulin-producing cells and increase the rate of proinsulin biosynthesis. Addition of β -cellulin and nicotinamide to the Stage 3 induction medium can promote β cell maturation and can generate cells that express
 15 endocrine hormones including insulin and glucagon. Theophylline can also be used in the Stage 3 induction medium.

As illustrated herein, the induced cell clusters produced during stage 3 were specifically labeled with the zinc-chelating dye dithizone (DTZ), which stains β -cells due to the presence of zinc in insulin-containing secretory granules (D'Amour et al.,
 20 Nature Biotech. 24: 1392 (2006)). Immunofluorescence, DTZ staining, and immune electron microscopy results confirmed that the induced cells produced using the compositions and methods described herein expressed insulin. As shown herein, RT-PCR showed that the induced cells coexpressed insulin, glucagon, and somatostatin. These results are in accordance with normal pancreatic development, in which
 25 immature islets are known to coexpress pancreatic hormones (Chandra et al. Stem Cells 27: 1941-1953 (2009)).

Male / Female

Surprisingly, the mRNA levels of Pax6 and Glut2 were different in male and female ASCs at such an early induction stage, indicating that the cells from male cats
 30 may have higher sensitivity than that from female cats. For example, human male ASCs may have greater osteogenic potential than female ASCs. Pax6 is a transcription factor that can transactivate the insulin promoters (Sander et al., *Genes & development* 11:1662-1673 (1997)). The higher expression of Pax6 in male ASCs

at early induction stages may indicate that the male ASCs undergo pancreatic β cell differentiation more easily than female.

The results described herein show that male and female ASCs have endodermal transdifferentiation capability. The induced cell clusters can secrete insulin with the glucose stimulation. In the clinical context, these results demonstrate the potential for stem cell-based therapy to treat feline diabetes. The mechanisms of the transdifferentiation in feline ASCs are not clear. Hence, it may not be possible to predict what compositions and methods would successfully transdifferentiate feline starting cells into cells that can secrete insulin cells. However, the methods and compositions described herein do transdifferentiate feline ASCs into functional insulin-producing cells, demonstrating that such methods and compositions are provide a viable therapeutic option for feline diabetes.

Kits

Also provided are kits for generating pancreatic precursor cells and/or pancreatic cells. The kits can contain any of the compositions described herein and instructions for using the compositions for generating definitive endoderm cells and/or pancreatic precursor cells and/or pancreatic cells. Each of the compositions can be separately packaged. Each composition can contain any of the compounds or proteins described herein at a concentration that is convenient for addition to a culture of cells. For example, compositions can be concentrated to about 10X, 50X, 100X, or 1000X of the concentration at which it would be employed to pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic beta cells. The instructions can provide guidance for appropriate addition (dilution) of the compositions into a cell culture, and/or guidance on other culture conditions (e.g., appropriate cell culture media, an appropriate duration of exposure to the compositions, etc.). The instructions can also provide guidance on the selection of starting cells for generating pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic cells. In addition, the instructions can provide information for testing and/or recognition of the generated pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic cells.

The kits can also provide components and instructions for administering pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic beta cells to mammalian (e.g., feline) subjects. The instructions can provide guidance on the numbers and the type(s) (phenotype) of cells to be administered. The

instructions can also provide instructions for administration of pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic beta cells by surgical implantation or by infusion. For example, the kits can provide diluents, pharmaceutically acceptable carriers, scalpels, syringes, catheters, bandages, antiseptics, and the like to permit administration of cells.

Mixtures

The pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic cells can be present in any of the foregoing compositions. The pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic cells can also be present in a therapeutically acceptable carrier such as saline, phosphate buffered saline, or other aqueous carrier. Such a combination of the compositions described herein, or a therapeutically acceptable carrier, with pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic cells can be referred to as a mixture.

The mixtures can contain about 1 to about 10^{10} pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic (e.g., beta) cells.

The pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic cells generated as described herein can be isolated, separated, or purified from culture media, compositions, or other mixtures in which they are generated. The pancreatic precursor cells and/or cells that can secrete insulin cells and/or pancreatic cells generated as described herein can be enriched or cultured to increase the proportion or numbers of desired cells in the population. Any such isolate, separation, purification, enrichment, or culture is a mixture as described herein.

An isolating step can include providing the cells in the cell culture with a reagent which binds to a marker expressed in the desired cell type (e.g., pancreatic precursor cells, and/or cells that can secrete insulin cells and/or pancreatic cells) but which is not substantially expressed in other cells present in the cell culture. The reagent-bound cells can be separated from the non-reagent-bound cells by numerous methods. For example, an antibody against a marker that is selectively present on the desired cells can be provided to cells in a cell culture. Antibody-bound cells can then be separated from other cells in the culture by, for example, fluorescent activated cell sorting (FACS), binding the antibody to a solid support or isolating appropriately tagged antibody in a magnetic field. In some embodiments, the antibody is released from the cells after the separation process.

As an alternative means of separation, at least some of the desired cells can be separated from at least some of the other cells in the culture by specifically fluorescently labeling the desired cells in culture and then separating the labeled cells from the unlabeled cells by FACS.

5 An enriched cell population of pancreatic progenitor cells, and/or cells that can secrete insulin cells, and/or pancreatic beta cells produced, for example, by an isolating step can be substantially free of cells other than pancreatic progenitor cells, and/or cells that can secrete insulin cells and/or pancreatic beta cells. In other
10 embodiments, the enriched cell populations can have at least about 50% to at least about 100% pancreatic progenitor cells, and/or cells that can secrete insulin cells and/or pancreatic beta cells. In still other embodiments, the enriched cell populations comprise from at least about 75 %, at least about 80%, at least about 85%, at least
15 about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 99%, or at least about 100% pancreatic progenitor cells, and/or cells that can secrete insulin cells, and/or pancreatic beta cells.

In some instances, the pancreatic progenitor cells, cells that can secrete insulin cells, and/or pancreatic beta cells are expanded, for example, by culturing the cells under conditions that permit cell division. For example, some embodiments include a
20 culturing step that comprises plating a cell population on a surface such as a culture plate. In some embodiments, the cells are plated on a surface coated with a protein, poly-amino acid or carbohydrate (e.g., collagen, fibronectin, laminin, polylysine, poly-ornithine, or a combination thereof).

In other embodiments, the culturing step comprises incubating the cell population or portion thereof in an expansion medium comprising about 2% (v/v)
25 serum. In some embodiments, the serum concentration can range from about 0% (v/v) to about 20% (v/v). For example, in some methods described herein, the serum concentration of the medium can be about 0.05% (v/v), about 0.1% (v/v), about 0.2% (v/v), about 0.3% (v/v), about 0.4% (v/v), about 0.5% (v/v), about 0.6% (v/v), about
30 0.7% (v/v), about 0.8% (v/v), about 0.9% (v/v), about 1% (v/v), about 2% (v/v), about 3% (v/v), about 4% (v/v), about 5% (v/v), about 6% (v/v), about 7% (v/v), about 8% (v/v), about 9% (v/v), about 10% (v/v), about 15% (v/v) or about 20% (v/v). In some embodiments, serum replacement is included in the medium, and no serum is employed.

Using the methods described herein, cell populations or cell cultures can be enriched in pancreatic progenitor (precursor) cells, cells that secrete insulin, and/or pancreatic beta cell content by at least about 2-fold to about 1000-fold as compared to cell populations or cell cultures produced by the methods and compositions described herein. In some embodiments, pancreatic progenitor (precursor) cells, cells that secrete insulin, and/or pancreatic beta cells can be enriched by at least about 5-fold to about 500-fold as compared to cell populations or cell cultures produced by the methods and compositions described herein. In other embodiments, pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells can be enriched from at least about 10- to about 200-fold as compared to cell populations or cell cultures produced by the methods and compositions described herein. In still other embodiments, pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells can be enriched from at least about 20- to about 100-fold as compared to cell populations or cell cultures produced by the methods and compositions described herein. In yet other embodiments, pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells can be enriched from at least about 40- to about 80-fold as compared to cell populations or cell cultures produced by the methods and compositions described herein. In certain embodiments, pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells can be enriched from at least about 2- to about 20-fold as compared to cell populations or cell cultures produced by the methods and compositions described herein.

Some embodiments described herein relate to cell cultures or cell populations comprising from at least about 5% pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells to at least about 95% pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells. In some embodiments the cell cultures or cell populations comprise mammalian cells. In preferred embodiments, the cell cultures or cell populations comprise feline cells. For example, certain specific embodiments relate to cell cultures comprising feline cells, wherein from at least about 5% to at least about 95% of the cells are pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells. Other embodiments relate to cell cultures comprising feline cells, wherein at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about

80%, at least about 85%, at least about 90% or greater than 90% of the cells are pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells. In embodiments where the cell cultures or cell populations comprise mammalian feeder cells, the above percentages are calculated without respect to the feeder cells in the cell cultures or cell populations.

Therapy

Also described herein is a method for treating a subject (e.g., a feline subject) suffering from, or at risk of developing, diabetes. This method involves generating pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells as described herein, and administering or implanting the cells into a mammalian subject (e.g., a feline subject).

The pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells can be implanted as dispersed cells or formed into clusters. Alternatively, pancreatic progenitor, cells that secrete insulin, and/or pancreatic beta cells can be infused into the subject, for example, via a hepatic portal vein. Alternatively, cells may be provided in biocompatible degradable polymeric supports, porous non-degradable devices or encapsulated to protect from host immune response. Cells may be implanted into an appropriate site in a subject. The implantation sites include, for example, the liver, natural pancreas, renal subcapsular space, omentum, peritoneum, subserosal space, intestine, stomach, or a subcutaneous pocket.

The amount of cells used in implantation depends on a number of various factors including the subject's condition and response to the therapy, and can be determined by one skilled in the art. For example, the number of cells administered can range from about 1000 to about 10^9 , or from about 1000 to about 10^8 , or from about 1000 to about 10^7 , or from about 1000 to about 10^6 , or from about 10000 to about 10^7 .

In one aspect, a method is provided for treating a subject suffering from, or at risk of developing diabetes. This method involves culturing a starting cell population, differentiating or redirecting the cultured cells *in vitro* into a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2; differentiating the first population of cells into a second population containing pancreatic progenitor cells and administering the second population of cells to a subject. In some instances pancreatic progenitor cells are enriched within the second

population or purified from the second population of cells to generate a third population of cells that is substantially free of non-pancreatic cells.

The cells to be administered can be incorporated into a three-dimensional support. The cells can be maintained *in vitro* on this support prior to implantation into the subject. Alternatively, the support containing the cells can be directly implanted in the subject without additional *in vitro* culturing. The support can optionally be incorporated with at least one pharmaceutical agent that facilitates the survival and function of the transplanted cells.

Support materials suitable for use include tissue templates, conduits, barriers, and reservoirs useful for tissue repair. In particular, synthetic and natural materials in the form of foams, sponges, gels, hydrogels, textiles, and nonwoven structures, which have been used *in vitro* and *in vivo* to reconstruct or regenerate biological tissue, as well as to deliver chemotactic agents for inducing tissue growth, are suitable for use in practicing the methods described herein. See, for example, the materials disclosed in U.S. Pat. No. 5,770,417, U.S. Pat. No. 6,022,743, U.S. Pat. No. 5,567,612, U.S. Pat. No. 5,759,830, U.S. Pat. No. 6,626,950, U.S. Pat. No. 6,534,084, U.S. Pat. No. 6,306,424, U.S. Pat. No. 6,365,149, U.S. Pat. No. 6,599,323, U.S. Pat. No. 6,656,488, U.S. Published Application 2004/0062753 A1, U.S. Pat. No. 4,557,264 and U.S. Pat. No. 6,333,029, each of which is specifically incorporated by reference herein in its entirety.

The mammalian subject can be a domestic animal, or a laboratory animal. In some cases, the subject is a feline subject.

Definitions

As used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to “a compound,” “a cell,” “a nucleic acid” or “a polypeptide” includes a plurality of such compounds, cells, nucleic acids or polypeptides (for example, a solution of cells, nucleic acids or polypeptides, a suspension of cells, or a series of compound, cell, nucleic acid or polypeptide preparations), and so forth.

The following Examples describe some experimental work performed during development of the invention. Appendix A may provide additional information about the invention.

Example 1: Materials and Methods

This Example describes some of the materials and methods employed in the development of the invention.

Feline ASC Isolation

5 Feline ASCs were isolated during routine sterilization of 8 male and 8 female adult cats. Adipose tissue was isolated with sharp dissection, minced, and digested in type I collagenase (33 ml/g adipose tissue, Worthington Biochemical, Lakewood, NJ) within 4 h of harvest. Tissues from each cat ($n = 5$) were divided into three equal portions, and each portion was digested by one of three methods: 1) 0.1 % type I
10 collagenase in DMEM, 0.5 h, 60 rpm (Classic); 2) 0.3 % type I collagenase in Kreb's Ringer buffer (KRB), 0.5 h, 1,000 rpm stirring (New); and 3) 0.3 % type I collagenase in KRB, 1 h, 1,000 rpm stirring (Hour). Tissue was added to collagenase solution in a 30 ml glass jar and stirred with a stir bar at 37 °C for the New and Hour digestion methods. For the Classic digestion method, digestion mixtures within glass jars were
15 agitated on a three-dimensional plate shaker at 37 °C. Digests were filtered and then centrifuged (260 g, 5 min). Resulting SVF pellets were resuspended in 5 ml red cell lysis buffer (0.16 mol/L NH_4Cl , 0.01 mol/L KHCO_3 , 0.01 % EDTA) followed by cell seeding in 10 mm cell culture dishes in stromal medium (DMEM-Ham's F12, 10 % fetal bovine serum (FBS, Hyclone), 1 % antibiotic/antimycotic solution). Medium
20 was refreshed after overnight incubation under standard conditions (37 °C, 5 % CO_2) and then every 2–3 days. The total number of colonies with 20 or more cells was determined after 7 days of culture. MSC density was then calculated as colony number/adipose weight (g). The New method was selected to isolate ASCs for the remainder of the study based on the highest number of colonies/g tissue. When SVF
25 cells reached 80 % confluence, they were detached with 0.25 % trypsin and 0.1 % EDTA. Cells were seeded at a density of 5×10^3 cells/cm² for P0 and all subsequent passages for evaluation of fresh cells. To prepare revitalized cells, aliquots (5×10^5 cells) of P0 cells were frozen in cryopreservation medium (80 % FBS, 10%DMEM, 10 % dimethyl sulfoxide) in liquid nitrogen for 30 days. Cells were then revitalized
30 and seeded at a density of 5×10^3 cells/cm² in stromal medium. Revitalized cells were subsequently cultured and evaluated identically to fresh cells.

Study design

For the purposes of this study, the stromal vascular fraction was the primary cell isolate, and passage (P) 0 was the first cell passage. Fresh cells were expanded to

P3 and then paired samples were cultured in stromal or three-step islet cell induction medium. The zinc concentration, insulin production, and ultrastructure was compared between stromal cells and the induced cells from stage 3. Multipotent capacity and lineage specific gene expression (insulin, NK6 homeobox 1 (NK6.1), proto-oncogene tyrosine-protein kinase ROS1 (ROS1), somatostatin (STS), ISL LIM homeobox 1 (Isl1), glucagon (GCG), paired box 6 (Pax6), AKT serine/threonine kinase 1 (AKT1), Ras-related protein Rab-2A (RAB2A), hexokinase 1 (HK1), and glucose transporter 2 (Glut2)) were compared between culture conditions in male and female donors. All materials and reagents were from Sigma-Aldrich, St. Louis, MO unless otherwise noted.

Cell Isolation

Tissue was minced and digested with 0.3% type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ) in Kreb's Ringer buffer (KRB) for 30 minutes, 1,000 rpm stirring with a stir bar at 37 °C. After filtering (100 µm nylon cell strainers, BD Falcon, Bedford, MA) and centrifugation ($260 \times g$, 5 min), the resulting SVF pellets were resuspended in 5 ml red blood cell lysis buffer (0.16 mol/L NH_4Cl , 0.01 mol/L KHCO_3 , 0.01% ethylenediaminetetraacetic acid (EDTA)) for 5 min. The SVF was collected after centrifugation ($260 \times g$, 5 min) and seeded in 10-mm cell culture dishes in stromal medium (Dulbecco's modified Eagle's medium F-12 (DMEM/F-12, Hyclone, Logan, UT), 1% antibiotic/antimycotic solution (MP Biomedical, Irvine, CA), 10% fetal bovine serum (FBS, Hyclone)). Stromal medium was refreshed after 24 hours and then every 3 days. After 70-80% confluence, the SVF cells were detached with 0.05% trypsin (Hyclone) and cells were seeded in T75 flasks with the density of 5×10^3 cells/cm² for P0 and all subsequent passages. Procedures performed at temperatures other than room temperature are indicated.

Multipotentiality

Cell isolates were tested to confirm multipotentiality, P1 fibroblastic colony formation and adipogenic and osteogenic differentiation. For fibroblastic colony formation, P1 cells were cultured in stromal medium for 7 days, fixed with 4% formalin, and stained with 0.1% toluidine blue. To assess adipogenesis, cells were cultured in stromal medium to 70-80% confluence, washed with phosphate buffered saline (PBS, Hyclone) and then cultured in adipogenic medium (Table 1) for 10 days. They were then fixed with 4% neutral paraformaldehyde (PFA) and stained with oil red O. For osteogenesis, the cells were cultured in stromal medium as above, then in

osteogenic preinduction medium for 10 days followed by osteogenic induction medium (Table 1) for another 10 days. Colonies were fixed with 70% ice cold ethanol and stained with 2% alizarin red.

Differentiation of feline ASCs into IPCs.

5 Cell isolates were culture expanded to P3. At 70-80% confluence, they were seeded in 6-well ultra-low attachment plates (Corning, Corning, NY) at 1×10^6 cells/well and cultured for 7 days in stromal medium. A three-stage protocol was used to induce β -cell islet-like clusters (Table 1). The cells in ultra-low attachment plates were incubated in serum free medium (SFM) 1 for 2 days as stage 1 and then in
10 SFM 2 for another 6 days at stage 2. Cells were then manually transferred to a standard 6-well plate (Thermo Fisher Scientific, Waltham, MA, USA) and cultured in stage 3 induction medium for another 4 days in standard 6-well plates. All media were refreshed every 2 days and the medium was refreshed every 24 hours in stage 3. Paired cells were cultured in stromal medium in standard 6-well plates throughout the
15 induction process.

Table 1: Composition of induction medium for differentiation

Medium	Composition
Adipogenic medium	Minimum essential medium alpha (α -MEM), 10% rabbit serum, 10% FBS, 10nM dexamethasone, 5 μ g/ml insulin, 50 μ M 5,8,11,14-eicosatetraynoic acid (ETYA, Cayman, Ann Arbor, MI), 100 μ M indomethacin
Osteogenic preinduction medium	DMEM, 10% FBS, 100 nM dexamethasone, 0.25 mM L-ascorbic acid
Osteogenic induction medium	Osteogenic preinduction medium supplemented with 10 mM β -glycerophosphate
β -cell induction medium	Stage 1: SFM 1 (2 days): DMEM, 17.5mM glucose, 1% BSA (bovine serum albumin), $1 \times$ insulin-transferrin-selenium (ITS, Gibco BRL, Gaithersburg, MD), 4nM activin A (R&D Systems Inc., Minneapolis), 1 mM sodium butyrate, 50 μM 2-mercapethanol, 1% N-2 supplement (R&D Systems Inc.), 1% B-27 supplement (Gibco), 5 μg/ml laminin (Corning), 50 ng/ml recombinant human hepatocyte growth factor (HGF, EMD Millipore, Temecula, CA), 20ng/ml basic fibroblast growth factor (bFGF, Gibco); Stage 2: SFM 2 (6 days): DMEM, 17.5mM glucose, 1% BSA, $1 \times$ ITS, 0.3mM taurine (ACROS Organics, Morris Plains, NJ), 5 μg/ml laminin, 20 ng/ml bFGF, 1% N-2 supplement, 1% B-27 supplement, 50 ng/ml HGF;

	Stage 3 SFM 3 (4 days): DMEM, 17.5 mM glucose, 1.5% BSA, $1.5 \times$ ITS, 3 mM taurine, 100 nM glucagon-like peptide 1 (GLP-1, TOCRIS bioscience, Ellisville, MO), 1 mM nicotinamide (ACROS Organics), $1 \times$ non-essential amino acids (NEAA, Gibco), 10 nM pentagastrin (TOCRIS bioscience), 1% N-2 supplement, 1% B-27 supplement, 50ng/ml HGF, 20ng/ml bFGF, 5 μ g/ml laminin, 10 ng/ml betacellulin (R&D Systems)
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Dithizone Staining – Zinc Concentration

Following the induction process, cells were incubated with dithizone (DTZ, Fisher Scientific, Fairlawn, NJ) solution (10 μ l DTZ working solution (1 mg/ml in dimethyl sulfoxide (Fisher Scientific)) in 1 ml of the culture medium) for 30 min at 37 °C. Cells were washed in PBS after incubation and imaged with light microscope.

Immunohistochemistry – Intra-cellular Insulin

The Dylight 633 antibody labeling kit (Thermo Fisher Scientific, Somerset, NJ) was used to label the antibody goat anti insulin according to the manufacturer's instruction. Briefly, 100 μ l antibody solution (1 mg/ml) was mixed with 8 μ l supplied borate buffer and the mixture was incubated with Dylight Reagent for 60 min and protected from light. The labeling reaction was mixed with resin and then centrifuged (1000 $\times$ g, 1 min) to collect the labeled antibody.

Cells were washed with PBS and fixed overnight in 4% neutral PFA. They were permeabilized with 0.5% Triton X-100 in PBS and then incubated with the labeled antibody goat anti insulin (1:200 in PBS, Santa Cruz Technologies, CA) for 30 min. After incubation, the cells were washed with PBS and then cytoskeletal actins was stained with β actin-FITC for 30 min (1: 500 in PBS, Neomarkers, Fremont, CA). Photomicrographs were obtained for all labeled cells with confocal laser scanning microscopy (CLSM, Leica TCS SP2, Leica, Wetzlar, Germany).

Glucose Challenge Assay – Glucose Sensitivity

The induced islet-like cell clusters were collected and washed twice with PBS. The islet-like cell clusters were incubated with KRB buffer for 1 hour at 37°C, followed by incubation with KRB buffer supplemented with different glucose concentrations (25 and 55 mM) for 30 min at 37°C. Following incubation, the medium was collected and stored at -80°C until further use. The stored medium was analyzed for insulin content using a feline specific enzyme-linked immunosorbent

assay (ELISA) kit (Mybiosource, San Diego, CA). The cells cultured in stromal medium were used as a control.

Transmission Electron Microscopy – Ultrastructure

Following a PBS rinse, cells were fixed in 2% PFA and 2.5% glutaraldehyde in 0.1 M PBS for 10 min. The samples were centrifuged ($350 \times g$, 5 min) and then fixed with fresh fixative with gentle agitation for 2 hours. They were mixed with equal amounts of 3% agarose and the mixture was placed to a glass slide. When the mixture solidified, it was sliced into cubes. The cubes were washed with 0.1 M PBS and 0.08M glycine 5 times (15 min/time) followed by incubation with 2% osmium tetroxide in 0.1 M PBS in the darkness for 1 hour to fix the cells. The samples were washed with H₂O and dehydrated in a series of ethanol-distilled water solutions. The dehydrated samples were infiltrated with 1:1 ethanol and LR white resin for 2 hours, and then infiltrated with 100% LR white resin for another 2 hours. Embedded samples were placed into the bottom of a beam capsule and incubated in an 18 °C oven for 24 hours. Ultra-thin sections (90 nm) were cut and stained with 2% uranyl acetate and lead citrate. Some sections were directly evaluated with transmission electron microscopy (JEOL JEM-1400, Japan). The other sections were blocked in 5% BSA in PBS for 30 min and then incubated with goat anti insulin in 0.1% BSA in PBS (1:20) for another 90 min. After incubation, the sections were washed in 0.1% BSA in PBS 6 times (5 min/time) and then incubated in secondary antibody (rabbit anti goat IgG-Gold, Sigma-Aldrich) in 0.1% BSA in PBS for another 90 min. After incubation, the sections were washed with 0.1% BSA buffer and PBS, respectively. The sections were then fixed in 2% glutaraldehyde in PBS for 5 min and contrasted with 2% uranyl acetate and lead citrate after being thoroughly washed in distilled water. Gold labeled sections were observed with TEM.

Scanning Electron Microscopy – Surface Ultrastructure

Cells were collected by filtration and fixed in 2% PFA and 2% glutaraldehyde in 0.1 M PBS for 15 min. The solution was extracted into a 10 ml syringe with a Swinney filter holder fitted with a 2 μ m pore polycarbonate with 13 mm diameter. Entrapped cells on the filtered were fixed as before another 15 min and then rinsed with 0.1 M PBS and distilled water. The filter was removed from the syringe and dried with hexamethyldisilazane (HMDS, Electron Microscopy Sciences, Fort Washington, PA) for 30 min, 1:1 100% ethanol and HDMS, and 2 changes for 30 min each with 100% HDMS. Finally the HDMS was removed and the samples were

placed in a hood overnight to air-dry. The dried samples were mounted onto aluminum SEM stubs, coated with platinum in an EMS 550X sputter coater and imaged with JSM-6610 High vacuum mode SEM (JEOL Ltd., Japan).

RT-PCR – Gene Expression

5 Total RNA was isolated from cells harvested from each induction stage (EZNA® MicroElute Total RNA kit, Omega, Bio-Tek, Norcross, GA). The quality and concentration was determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE), and cDNA synthesized (Maxima First-Strand cDNA synthesis kit, Thermo Scientific, Waltham, MA). Feline pancreatic
10 target gene levels (insulin, Isl1, HK1, Glut-2, NK6.1, ROS1, STS, GCG, Pax6, AKT1, and RAB2A) (Table 2) were quantified with real-time RT-PCR using the Thermo Fisher Absolute™ Blue QPCR Rox Mix technology and an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) using feline-specific primers. The ΔC_t values were determined relative to the reference gene β -
15 actin.

Table 2 Primer Sequences

Lineage	Primer	Sequences	Accession No.
Housekeeping	β -actin	F: AGCCTTCCTTCCTGGGTATG SEQ ID NO:1 R: ACAGCACCGTGTTAGCGTAG SEQ ID NO:2	XM_006941899.3
Transcription Factor	Nkx 6.1	F: AACGAAATACTTGGCGG SEQ ID NO:3 R: CCAGAGGCTTGTTGTAGTCG SEQ ID NO:4	XM_019829291.1
Transcription Factor	Pax6	F: GGCAATCGGTGGTAGTAA SEQ ID NO:5 R: CTTGGTATGTTATCGTTGG SEQ ID NO:6	XM_019812231.1
Transcription Factor	Isl1	F: CAAGGACAAGAAGCGGAG SEQ ID NO:7 R: CTGGGTTTGCCTGTAAGC SEQ ID NO:8	XM_003981424.3
Transcription Factor	Glut 2	F: TTGGCTTGGATGAGTTACG SEQ ID NO:9 R: GACTTTCCTTTGGTTTCCG SEQ ID NO:10	XM_003991916.3
Protein	Insulin	F: CTTCGTCAACCAGCACC SEQ ID NO:11 R: ACAGCATTGCTCCACGA SEQ ID NO:12	XM_019811180.1
	Glucagon	F: TGAACACCAAGAGGAACAA	XM_006935320.2

Lineage	Primer	Sequences	Accession No.
		SEQ ID NO:13 R: ACCAGCCAAGCAATGAAT SEQ ID NO:14	
	Somato- statin	F: CCAGACAGAGAACGATGCC SEQ ID NO:15 R: CAGGGTTTGAGTTAGTGGA SEQ ID NO:16	XM_003991805.4
Oncogene	ROS1	F: AACAAACAGCCTCTACTACAG SEQ ID NO:17 R: TATCCTCCGACCGAATCC SEQ ID NO:18	XM_019831130.1 P
	Akt1	F: CCAACACCTTCATCATCCG SEQ ID NO:19 R: CCATCATTTCTCCTCCTG SEQ ID NO:20	NM_001322435.1
	RAB2A	F: ACAGACAAGAGGTTTCAGC SEQ ID NO:21 R: TATGACCGTGTGATGGAAC SEQ ID NO:22	XM_019822712.1
	HK1	F: TGAGAAGATGGTGAGTGGC SEQ ID NO:23 R: GGCAGAGCGAAATGAGAC SEQ ID NO:24	XM_006937834.3

Statistical Analysis

All results are presented as least squares (LS) mean $\pm$ SEM. Statistical analyses were performed with the JMP statistical package (v 13.0.0, SAS Institute Cary, NC). Mixed ANOVA models were used to evaluate insulin levels between glucose concentrations within genders and between genders within glucose concentrations. The same models were used to evaluate target gene expression among induction stages within genders and between genders within induction stages. Tukey's post hoc tests were applied for multiple group comparisons ($p < 0.05$).

Example 2: ASC Multipotentiality

All ASC isolates following culture in stromal medium displayed osteogenic and adipogenic differentiation based on histochemical staining. Cells had a fibroblastic shape when cultured in stromal medium (FIG. 1A). Colony calcium stained with alizarin red following culture in osteogenic medium (FIG. 1B), and lipid droplets stained with oil red O after adipogenic medium culture (FIG. 1C).

Example 3: Cell Morphology

All ACSs cultured in ultralow attachment plates formed cell clusters. Following transfer to a standard six well culture plates, cells cultured in stromal

medium attached to the plate in colony formation within 24 hours (FIG. 2A). The vast majority of those cultured in induction medium did not attach at any point and remained as detached cell clusters (FIGs. 2B and 2C).

Example 4: Dithizone Staining – Zinc Concentration

5 The cells cultured in induction medium formed clusters that stained dithizone (DTZ), confirming zinc accumulation (FIG. 3B-3C), and those cultured in stromal medium did not stain with DTZ (FIG. 3A). As before, cells cultured in stromal medium attached to standard culture ware while those cultured in induction medium did not (FIG. 3A).

Example 5: Immunohistochemistry

10 The cells cultured in stromal medium lacked insulin expression, while the cells cultured in induction medium had strong insulin expression (FIG. 4).

Example 6: Glucose Challenge Assay

15 Male and female ASCs cultured in induction medium released insulin in response to glucose (FIG. 5). Insulin secretion was slightly higher (55.7 versus 62.7) at the higher glucose concentration. Insulin secretion was significantly higher at high glucose concentrations (25 and 55mM) compared to a low glucose concentration (5.5mM).

Example 7: Transmission Electron Microscopy – Ultrastructure

20 Cell ultrastructure was distinct between cells cultured in stromal versus induction medium. Notable differences were the presence abundant perinuclear mitochondria in the cells cultured in stromal medium (FIG. 6A) and secretory granules in the induced cells (FIG. 6B).

Example 8: Electroimmunohistochemistry – Insulin Localization

25 Insulin labeling was localized to the cytoplasm of cells cultured in induction medium, while there was no labeling in those cultured in stromal medium (FIG. 7).

Example 9: Scanning Electron Microscopy – Surface Ultrastructure

30 Cells cultured in stromal medium tended to form loose, spherical clusters while those in β cell induction medium formed larger, highly organized clusters with an irregular shape (FIG. 8). Proteinaceous material was apparent on the surface of differentiated cell clusters (FIG. 8B).

Example 10: Gene Expression

This Example illustrates expression patterns of cells at various stages of transdifferentiation.

Transcription factor expression

The mRNA levels of transcription factors (Nkx 6.1, Pax6, Isl1, and Glut-2) at Stage 1 tended to be lower than other induction stages (Fig. 9). The mRNA levels of Pax6 and Glut2 in female samples had lower expression than male at induction stages 1 and 2 and induction stage 1, respectively (FIG. 9B and 9D).

Pancreatic β -cell target gene expression

Insulin expression detectable only after stage 2 and 3 induction (FIG. 10A). Glucagon expression was higher after stage 3 induction versus the other stages (FIG. 10B). Somatostatin levels were lower after stage 1 induction compared to the others (FIG. 10C).

Oncogene expression

There was no difference in ROS1, AKT1, RAB2A, or HK1 expression among induction stages (FIG. 11).

Insulin expression

As shown in FIG. 12, pancreatic cells generated via the methods described herein (stages 1-3) express insulin. In particular, FIG. 12 shows a Western blot demonstrating insulin protein produced by feline pancreatic beta cells generated from adipose derived stem cells. Columns from left to right: Protein ladder, pancreatic beta cell cluster, pancreatic beta cell cluster, adipose derived stem cells cultured in stromal medium, adipose derived stem cells cultured in stromal medium.

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5 All patents and publications referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced patent or publication is hereby specifically incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to
10 physically incorporate into this specification any and all materials and information from any such cited patents or publications.

The following statements are intended to describe and summarize various embodiments of the invention according to the foregoing description in the specification.

15 **Statements:**

1) A method comprising:

- a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, a TGF β family member (e.g., activin A),
20 an HDAC inhibitor (e.g., sodium butyrate), and 2-mercapethanol to generate a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2;
- b. Stage 2: culturing the first population of cells for 4 to 8 days in a second culture medium comprising insulin-transferrin-selenium, and taurine to
25 generate a second population of cells; and
- c. Stage 3: culturing the second population of cells for 2 to 6 days in a third culture medium comprising insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, pentagastrin, and betacellulin to generate a third population of cells.

- 30 2) The method of statement 1, wherein the first culture medium, the second culture medium, and the third culture medium further comprise at least one, at least two, at least three, or at least four of the following: basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), laminin, N-2 supplement, or B-27 supplement.

- 3) The method of statement 1 or 2, wherein the first culture medium, the second culture medium, and the third culture medium further comprise glucose, serum albumin (e.g., bovine serum albumin).
- 4) The method of statement 1, 2, or 3, wherein the third culture medium further comprises theophylline.
- 5) The method of statement 1-3, or 4, where the second population of cells and/or the third population of cells express insulin.
- 6) The method of statement 1-4 or 5, where cells in the first population of cells do not express detectable amounts of insulin mRNA as detected by quantitative polymerase chain polymerase reaction.
- 7) The method of statement 1-5 or 6, further comprising administering the third population of cells, or a portion thereof, to a mammalian subject.
- 8) The method of statement 1-6 or 7, further comprising administering the third population of cells, or a portion thereof, to a feline subject.
- 9) The method of statement 1-7 or 8, wherein the adult adipose-derived multipotent stromal (stem) cells (ASCs) are cultured on an ultra-low attachment culture plate.
- 10) The method of statement 1-8 or 9, wherein cells in the first population are cultured on an ultra-low attachment culture plate.
- 11) The method of statement 1-9 or 10, wherein cells in the first population are cultured on culture plates or in culture vessels coated with protein.
- 12) The method of statement 1-10 or 11, wherein cells in the second population expresses higher or lower levels of at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2 than cells in the first population of cells.
- 13) The method of statement 1-11 or 12, further comprising culturing cells from the third population and isolating insulin.
- 14) The method of statement 13, wherein the insulin is isolated from the cultured cells.
- 15) The method of statement 13 or 14, wherein the insulin is isolated from the culture medium.
- 16) A composition comprising a first population of cells made by culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) from feline adipose tissue for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, a TGF β family member (e.g., activin A), an HDAC

inhibitor (e.g., sodium butyrate), and 2-mercapethanol to generate a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2.

- 5 17) The composition of statement 16, wherein the adult adipose-derived multipotent stromal (stem) cells (ASCs) are cultured on an ultra-low attachment culture plate.
- 10 18) A composition comprising a second population of cells made by:
- a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) from feline adipose tissue for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, a TGF β family member (e.g., activin A), an HDAC inhibitor (e.g., sodium butyrate), and 2-mercapethanol to generate a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2; and
 - 15 b. Stage 2: culturing the first population of cells for 4 to 8 days in a second culture medium comprising insulin-transferrin-selenium, and taurine to generate a second population of cells.
- 19) The composition of statement 18, wherein the adult adipose-derived multipotent stromal (stem) cells (ASCs) and/or cells in the first population are cultured on an ultra-low attachment culture plate.
- 20 20) The composition of statement 16-17 or 18, wherein cells in the second population express higher or lower levels of at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2 than cells in the first population of cells.
- 21) A composition comprising a third population of cells made by:
- 25 a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) from feline adipose tissue for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, a TGF β family member (e.g., activin A), an HDAC inhibitor (e.g., sodium butyrate), and 2-mercapethanol to generate a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2;
 - 30 b. Stage 2: culturing the first population of cells for 4 to 8 days in a second culture medium comprising insulin-transferrin-selenium, and taurine to generate a second population of cells; and
 - c. Stage 3: culturing the second population of cells for 2 to 6 days in a third culture medium comprising insulin-transferrin-selenium, taurine,

glucagon-like peptide 1 (GLP-1), nicotinamide, pentagastrin, and betacellulin to generate a third population of cells.

- 22) The composition of statement 21, wherein the adult adipose-derived multipotent stromal (stem) cells (ASCs), and/or cells in the first population are cultured on an ultra-low attachment culture plate.
- 23) A composition comprising at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least ten of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, a TGF β family member (e.g., activin A), an HDAC inhibitor (e.g., sodium butyrate), 2-mercapethanol, N-2 supplement, B-27 supplement, laminin, mammalian hepatocyte growth factor (HGF), or basic fibroblast growth factor (bFGF).
- 24) A composition comprising at least four, or at least five, or at least six, or at least seven, or at least eight of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, taurine, laminin, basic fibroblast growth factor (bFGF), N-2 supplement, B-27 supplement, or mammalian hepatocyte growth factor (HGF).
- 25) A composition comprising at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least ten, or at least eleven, or at least twelve of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, non-essential amino acids, pentagastrin, N-2 supplement, B-27 supplement, human hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), laminin, or betacellulin.
- 26) The composition of statement 23, 24, or 25, which is formulated as a culture medium.

The specific compositions, constructs, and methods described herein are representative of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or

limitations, which is not specifically disclosed herein as essential. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and the methods and processes are not necessarily restricted to the orders of steps indicated herein or in the claims.

5 The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present
10 invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims and statements of the invention. Under no circumstances may the patent be interpreted to
15 be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

20

What is Claimed:

1. A method comprising:
 - a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) for about 1-4 days in a first culture medium comprising at least four of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, activin A, sodium butyrate, 2-mercapethanol, N-2 supplement, B-27 supplement, laminin, human hepatocyte growth factor (HGF), or basic fibroblast growth factor (bFGF) to generate a first population of cells that express Nkx 6.1, Pax6, Isl1, and Glut-2;
 - b. Stage 2: culturing the first population of cells in a second culture medium comprising at least four of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, taurine, laminin, basic fibroblast growth factor (bFGF), N-2 supplement, B-27 supplement, or human hepatocyte growth factor (HGF) to generate a second population of cells; and
 - c. Stage 3: culturing the second population of cells in a third culture medium comprising at least four of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, non-essential amino acids, pentagastrin, N-2 supplement, B-27 supplement, mammalian hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), laminin, or betacellulin to generate a third population comprising cells that can express insulin.
2. The method of claim 1, further comprising selecting one or more cells that express insulin.
3. The method of claim 1, further comprising selecting one or more cells that express insulin and separately expanding one or more of the selected cells that express insulin to generate a population of insulin-producing cells.
4. The method of claim 1, further comprising administering cells from the second population to a mammal.
5. The method of claim 1, further comprising administering cells from the third population to a mammal.
6. The method of claim 1 further comprising culturing one or more cells from the third population and isolating insulin therefrom.

7. A composition comprising a first population of cells made by culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) from feline adipose tissue for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, activin A, sodium butyrate, and 2-mercapethanol to generate the first population of cells
8. The composition of claim 7, wherein cells in the first population of cells express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2.
9. A composition comprising a second population of cells made by:
 - a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) from feline adipose tissue for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, activin A, sodium butyrate, and 2-mercapethanol to generate a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2; and
 - b. Stage 2: culturing the first population of cells for 4 to 8 days in a second culture medium comprising insulin-transferrin-selenium, and taurine to generate the second population of cells.
10. A composition comprising a third population of cells made by:
 - a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) from feline adipose tissue for about 1-4 days in a first culture medium comprising insulin-transferrin-selenium, activin A, sodium butyrate, and 2-mercapethanol to generate a first population of cells that express at least one of the following genes: Nkx 6.1, Pax6, Isl1, or Glut-2;
 - b. Stage 2: culturing the first population of cells for 4 to 8 days in a second culture medium comprising insulin-transferrin-selenium, and taurine to generate a second population of cells; and
 - c. Stage 3: culturing the second population of cells for 2 to 6 days in a third culture medium comprising insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, pentagastrin, and betacellulin to generate the third population of cells.
11. A composition comprising at least eight of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, activin A, sodium butyrate, 2-

mercapethanol, N-2 supplement, B-27 supplement, laminin, human hepatocyte growth factor (HGF), or basic fibroblast growth factor (bFGF).

12. The composition of claim 11, which is formulated as a culture medium.
13. A composition comprising at least seven of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, taurine, laminin, basic fibroblast growth factor (bFGF), N-2 supplement, B-27 supplement, or human hepatocyte growth factor (HGF).
14. The composition of claim 13, which is formulated as a culture medium.
15. A composition comprising at least ten of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, non-essential amino acids, pentagastrin, N-2 supplement, B-27 supplement, human hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), laminin, or betacellulin.
16. The composition of claim 15, which is formulated as a culture medium.

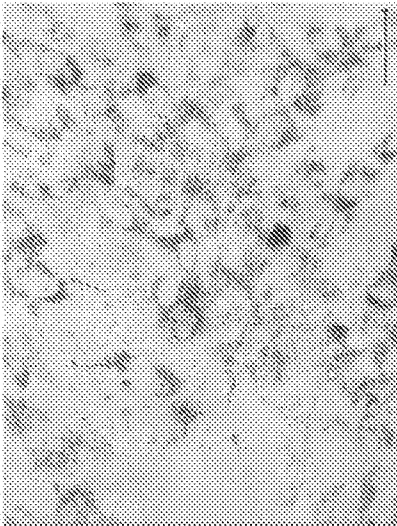


FIG. 1C

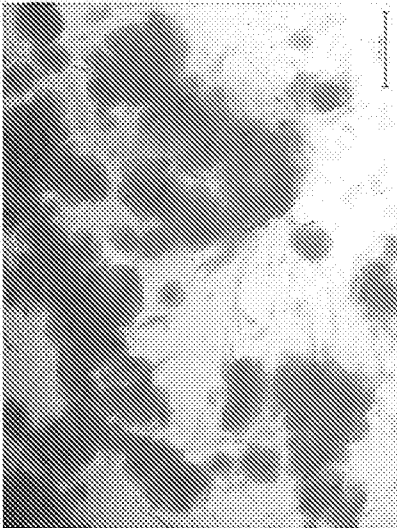


FIG. 1B

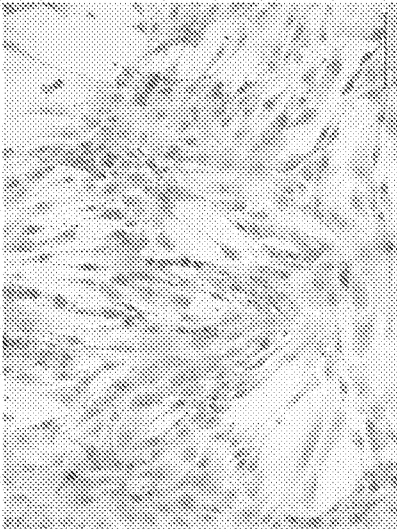


FIG. 1A

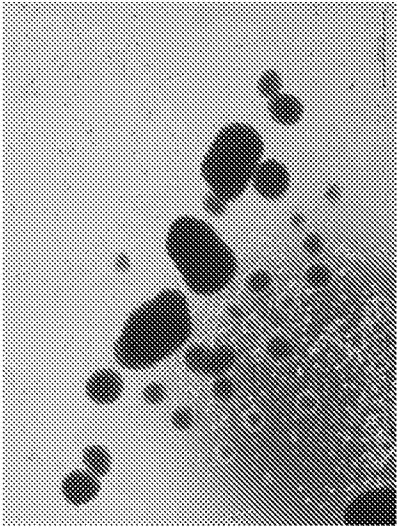


FIG. 2C

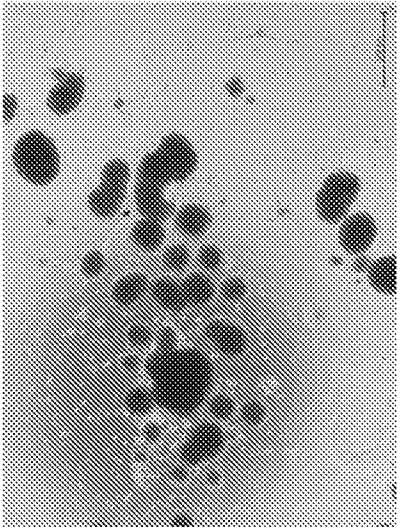


FIG. 2B

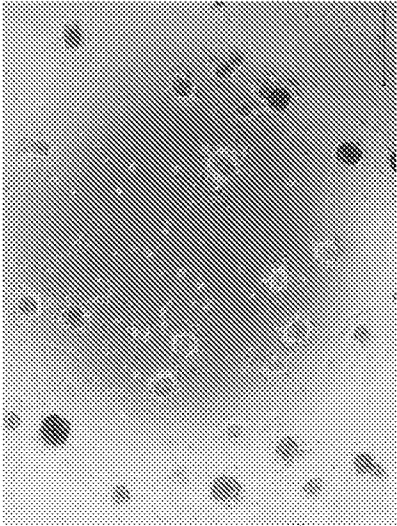


FIG. 2A

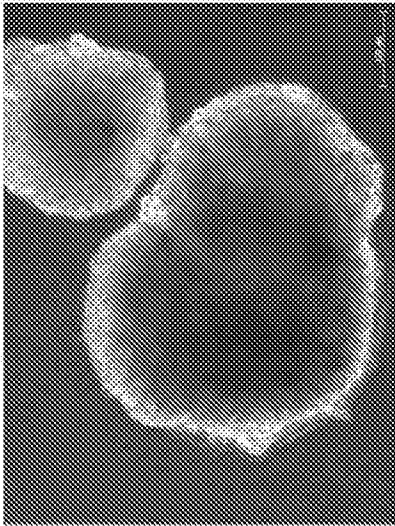


FIG. 3C

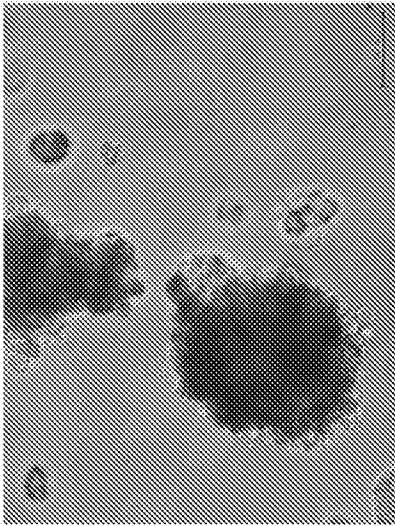


FIG. 3B

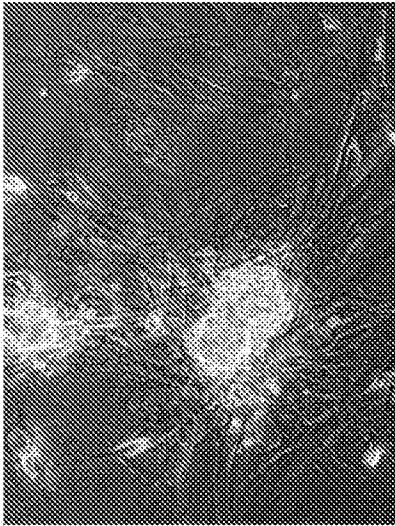


FIG. 3A

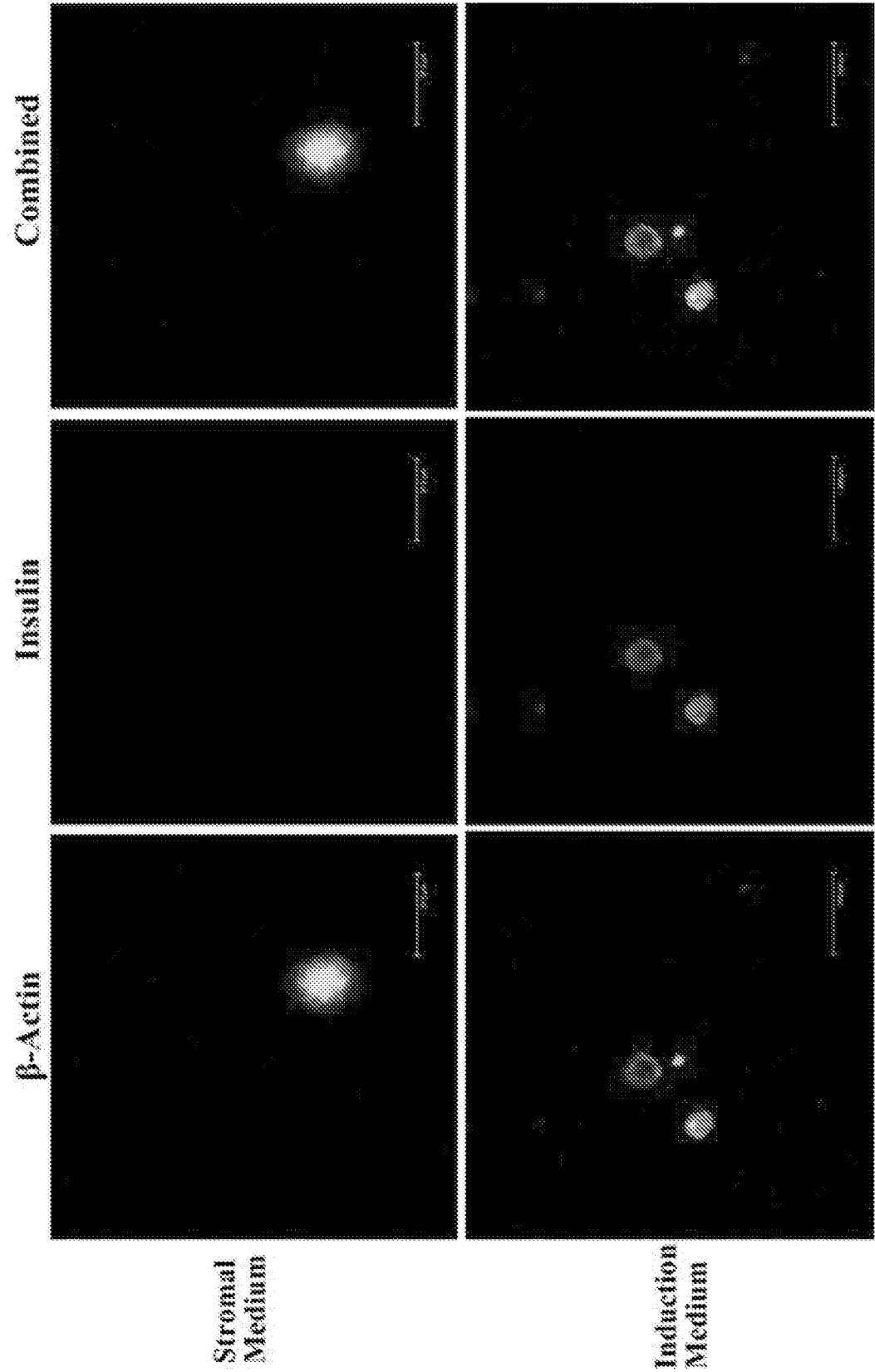


FIG. 4

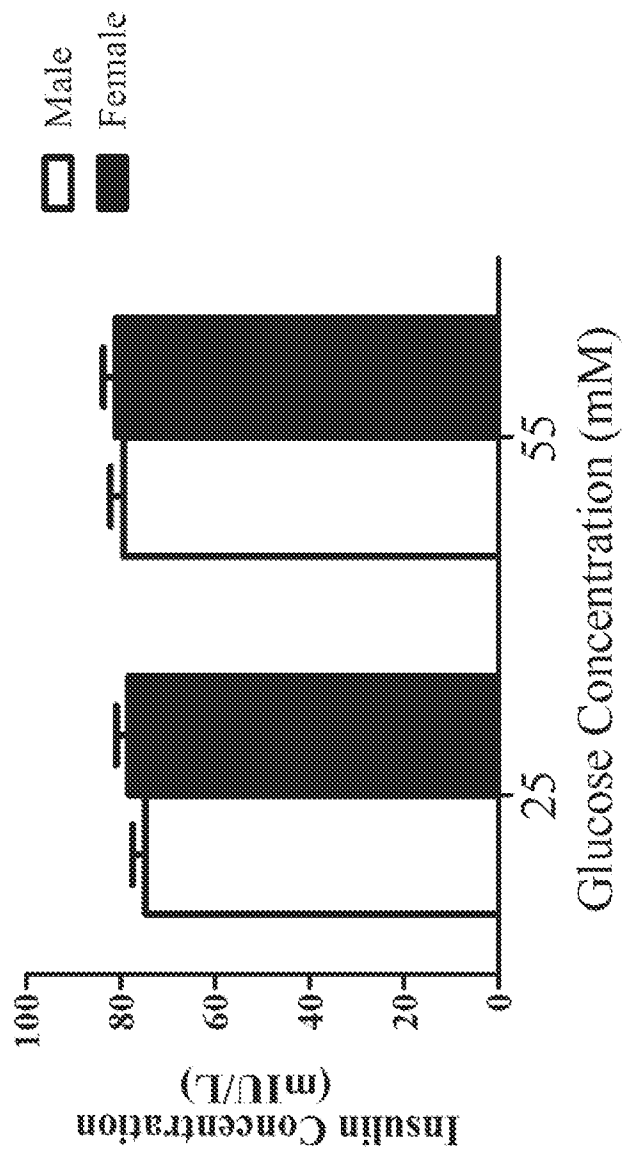


FIG. 5

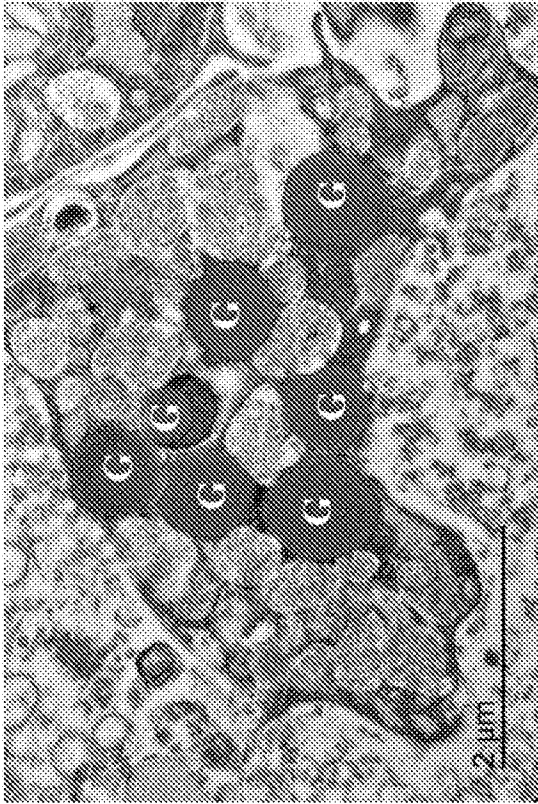


FIG. 6B

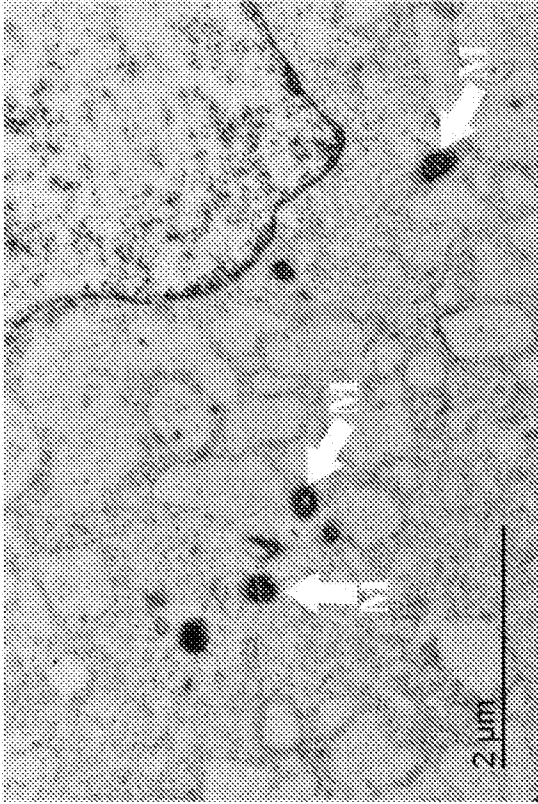


FIG. 6A

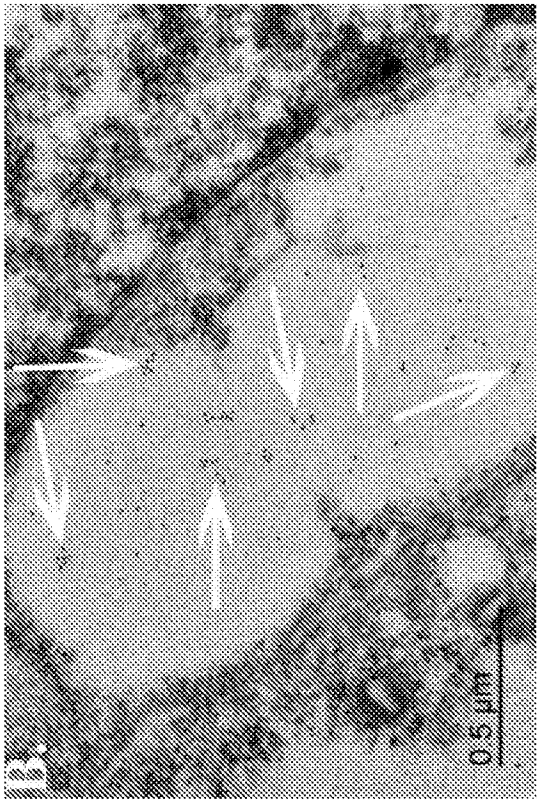


FIG. 7B

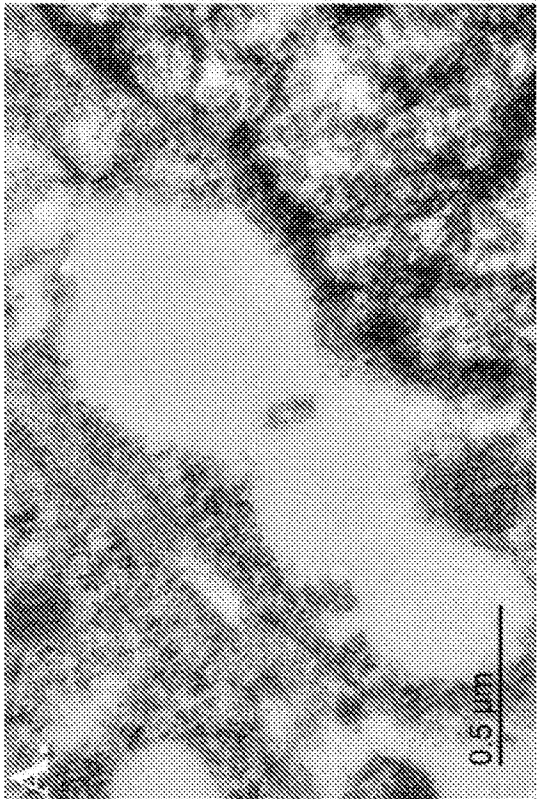


FIG. 7A

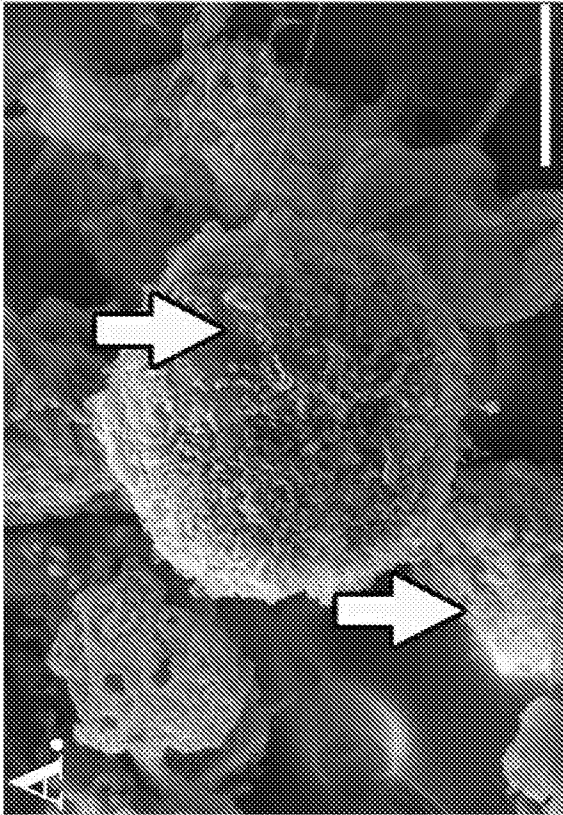


FIG. 8A

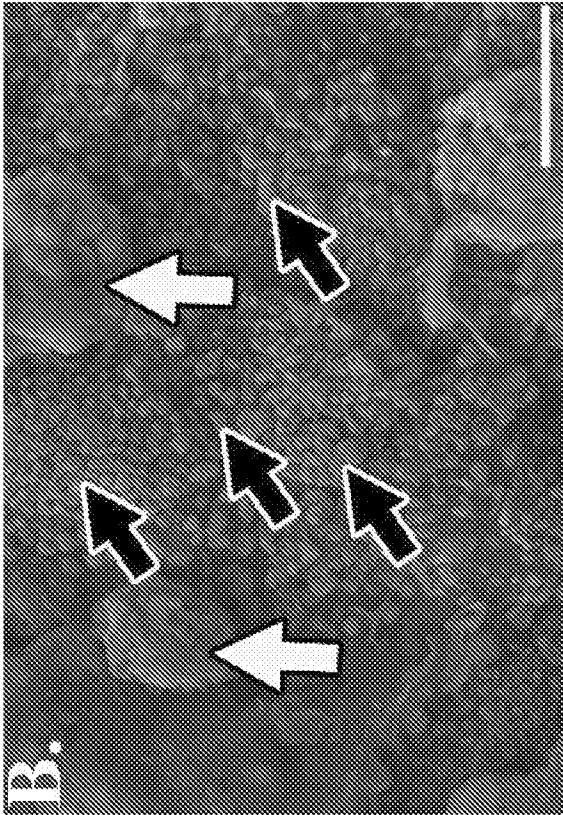


FIG. 8B

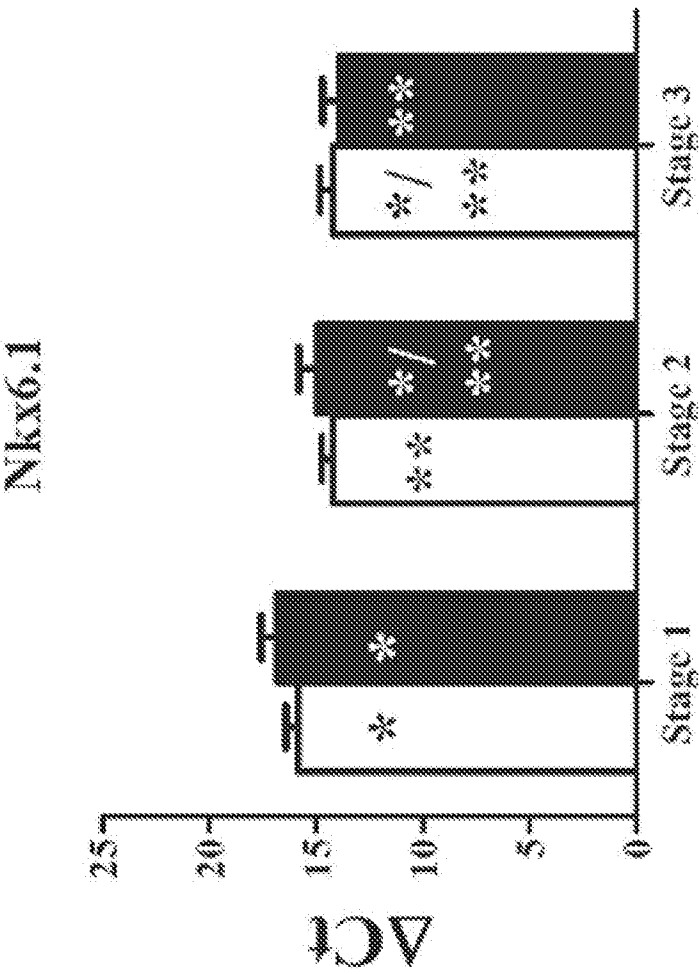


FIG. 9A

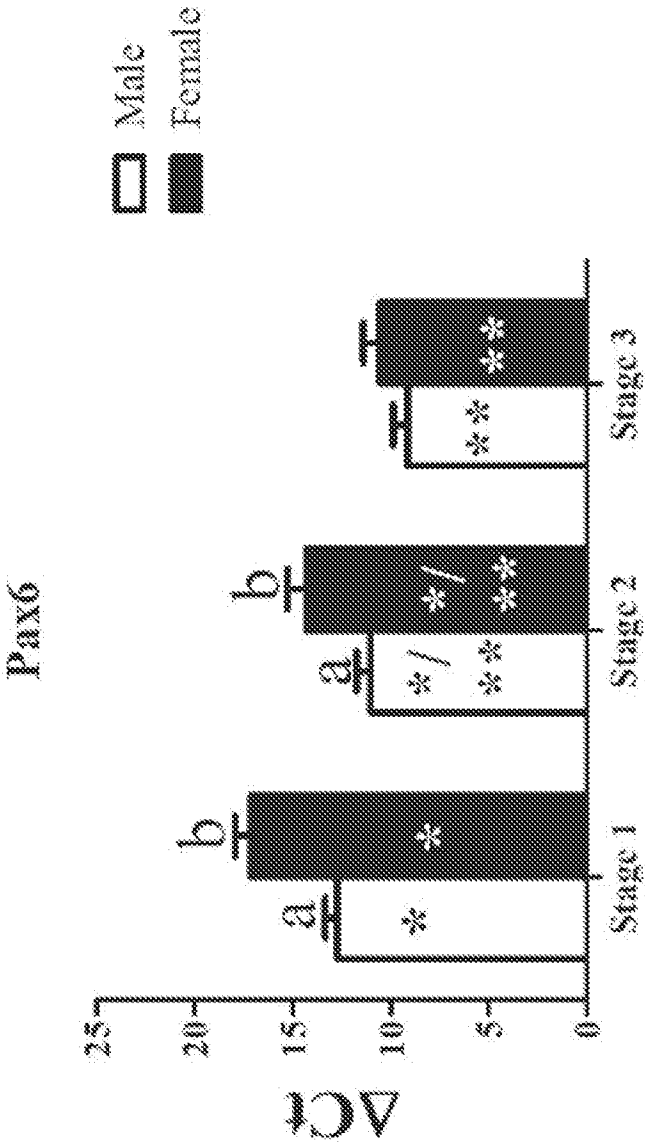


FIG. 9B

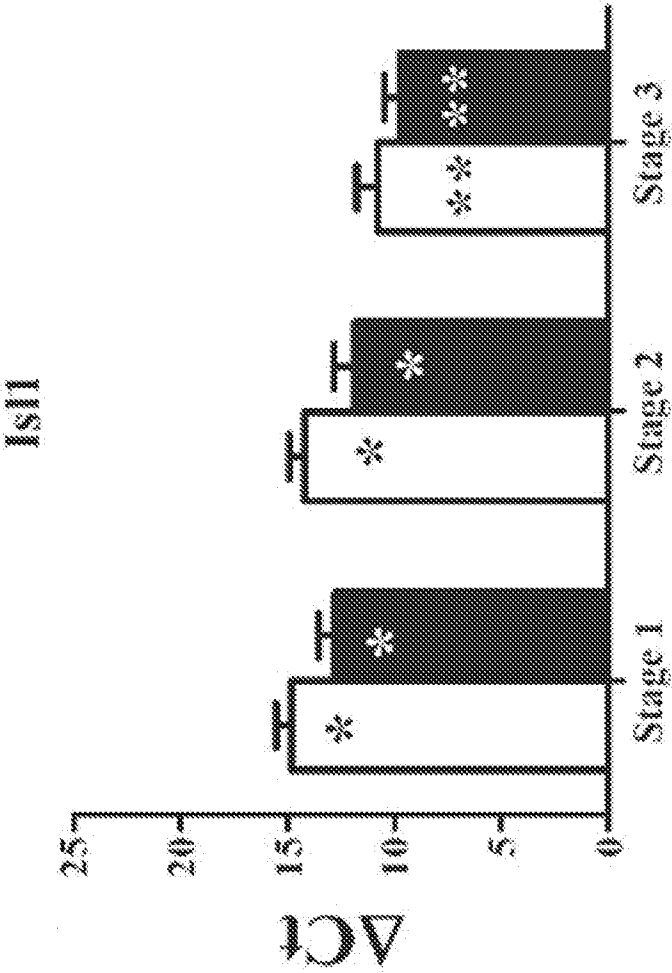


FIG. 9C

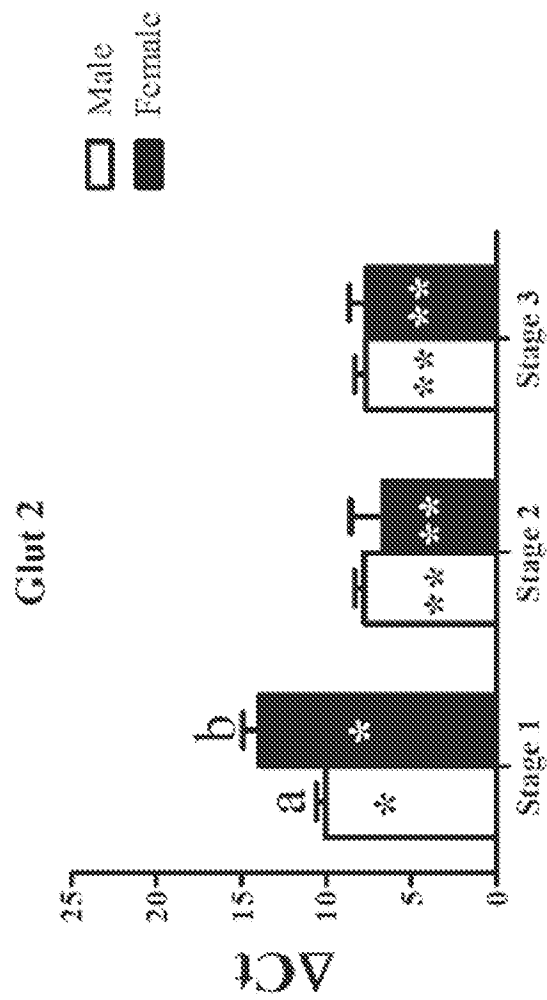


FIG. 9D

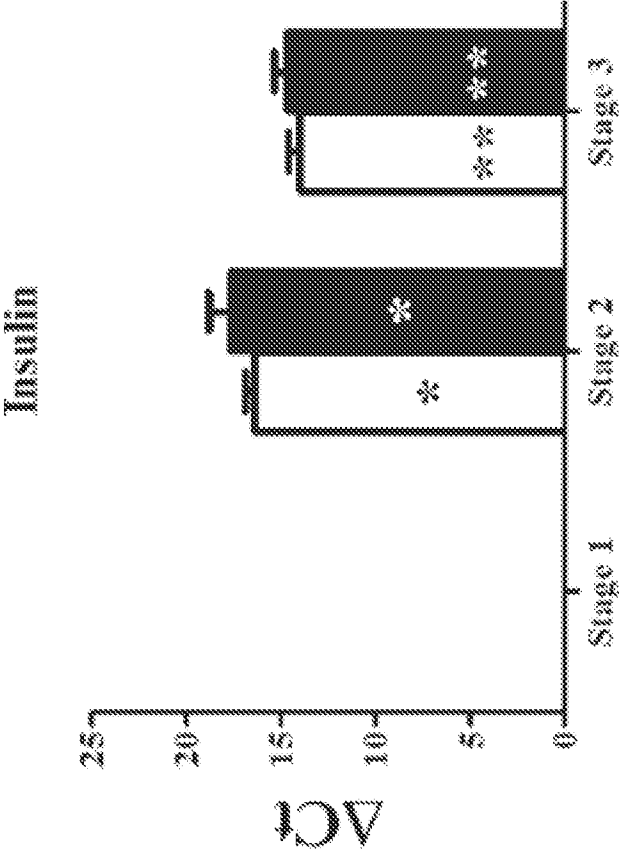


FIG. 10A

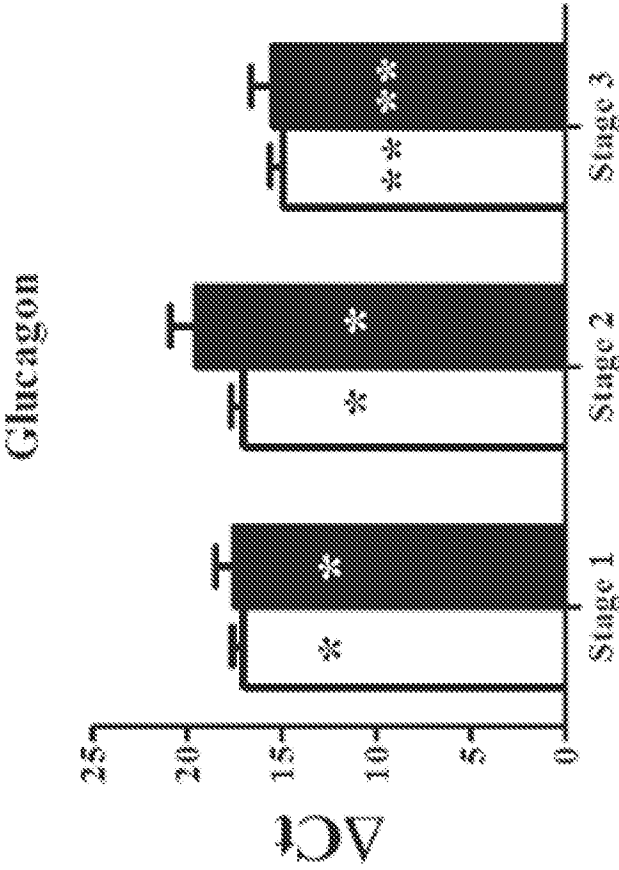


FIG. 10B

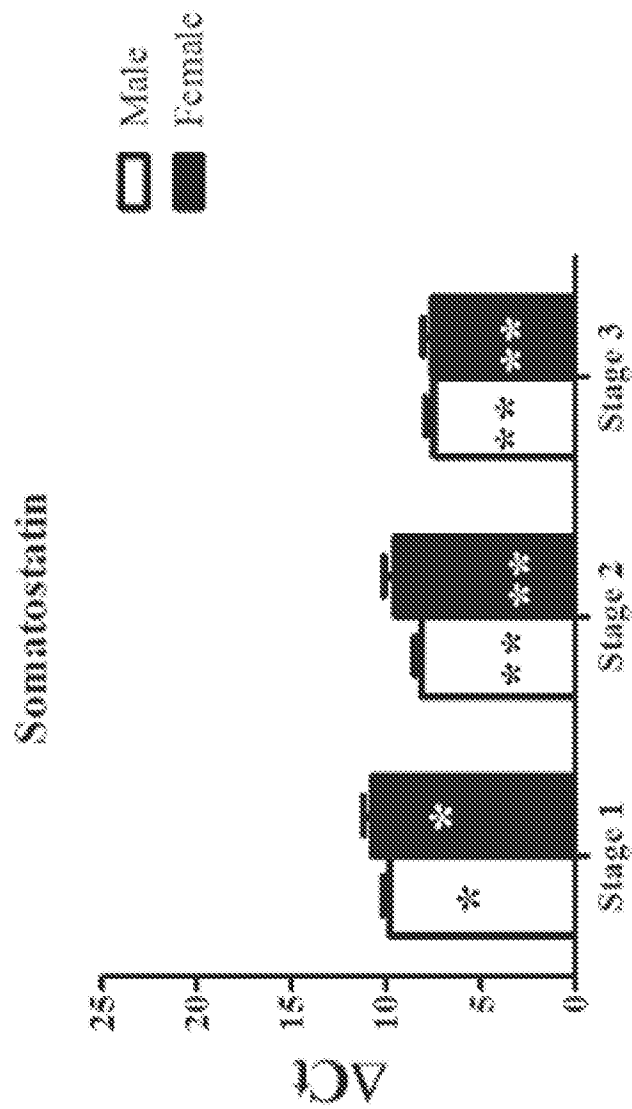


FIG. 10C

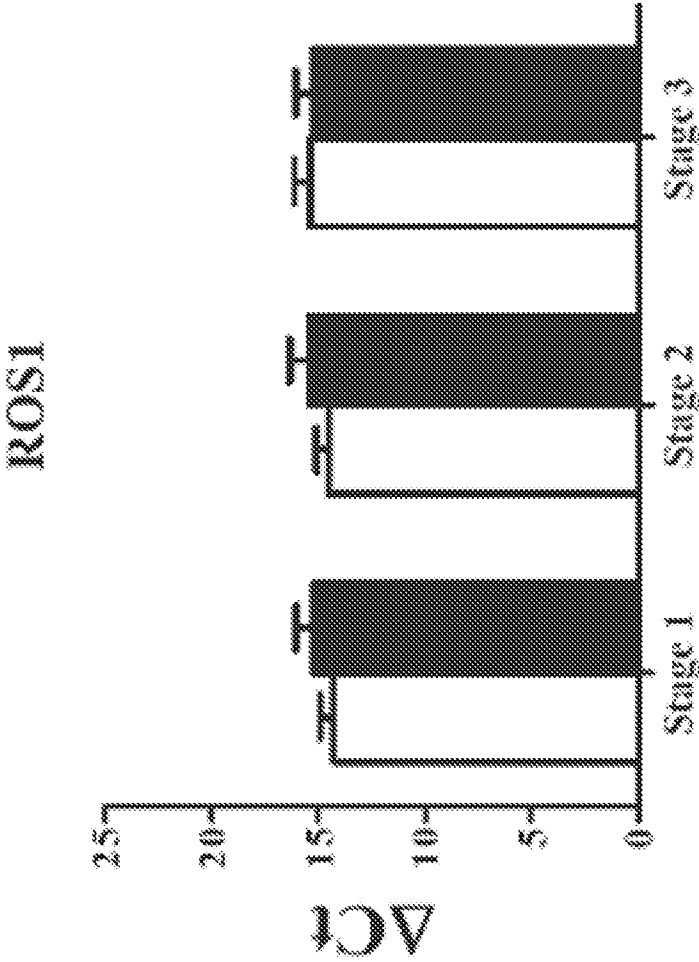


FIG. 11A

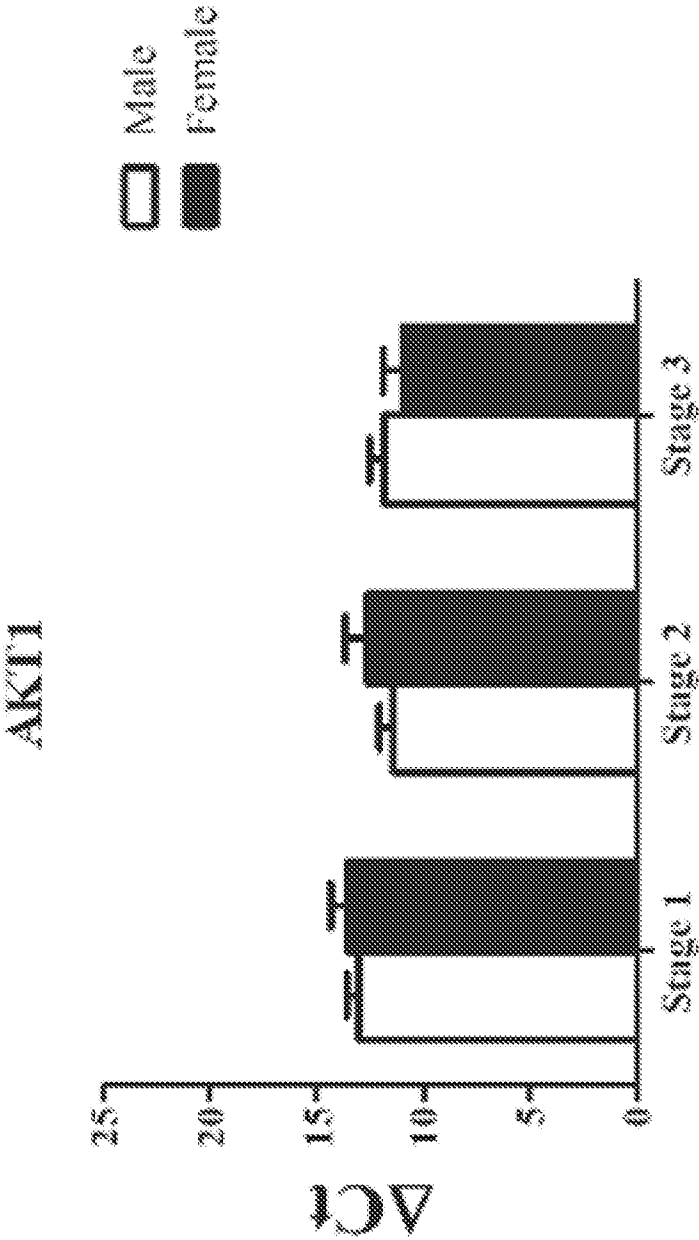


FIG. 11B

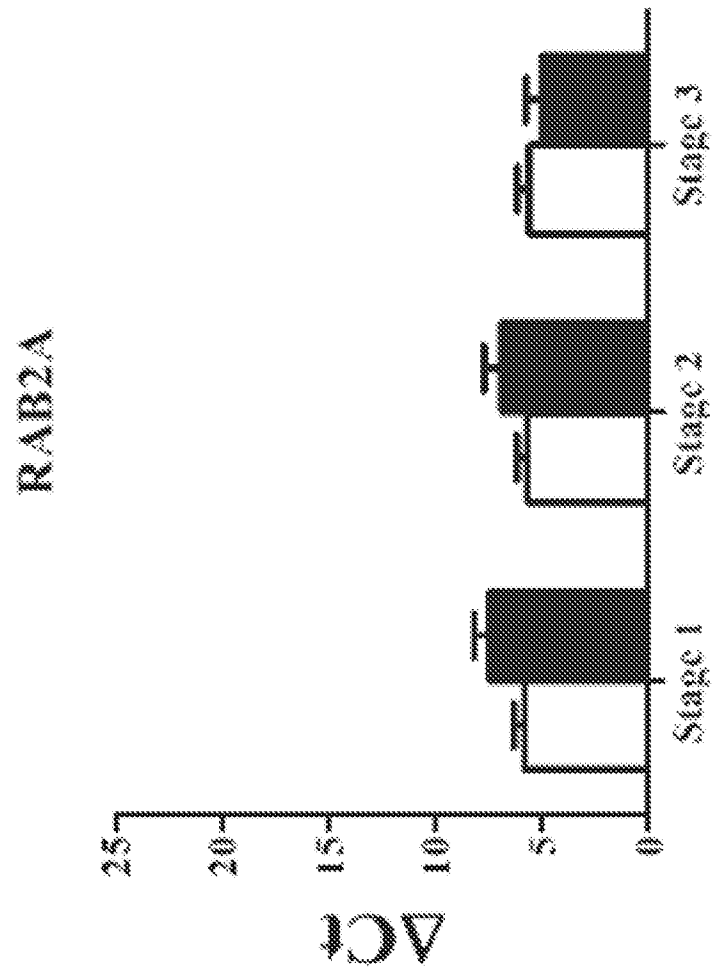


FIG. 11C

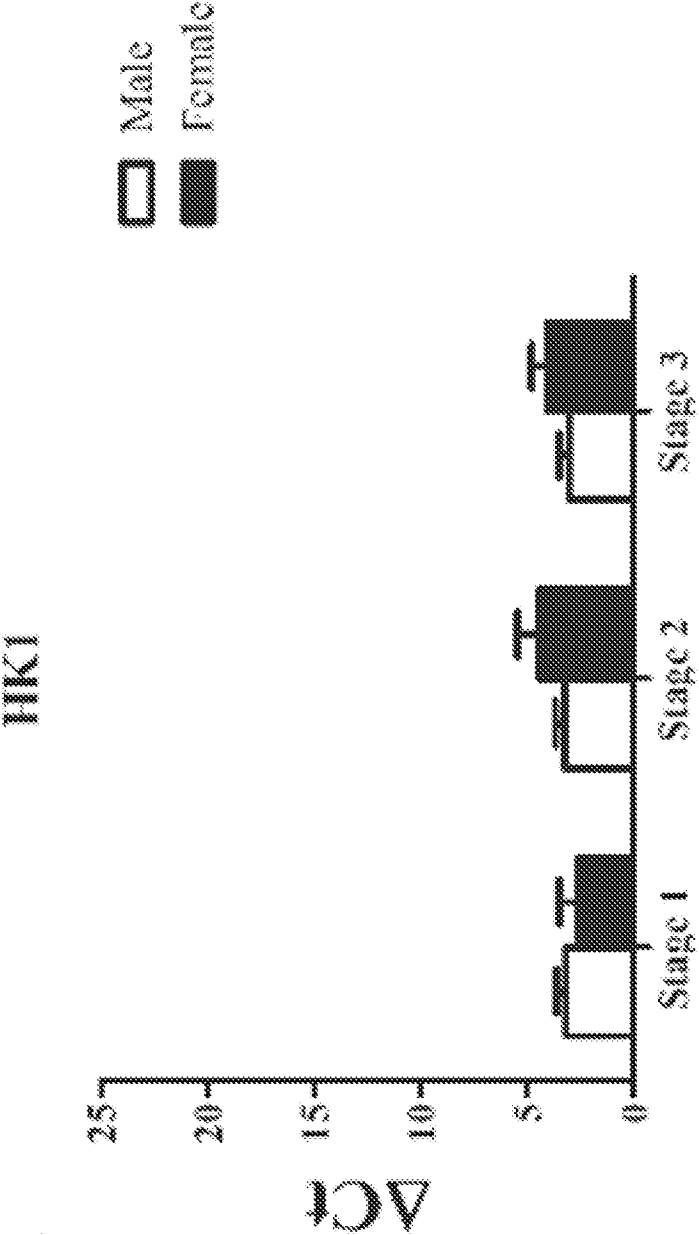


FIG. 11D

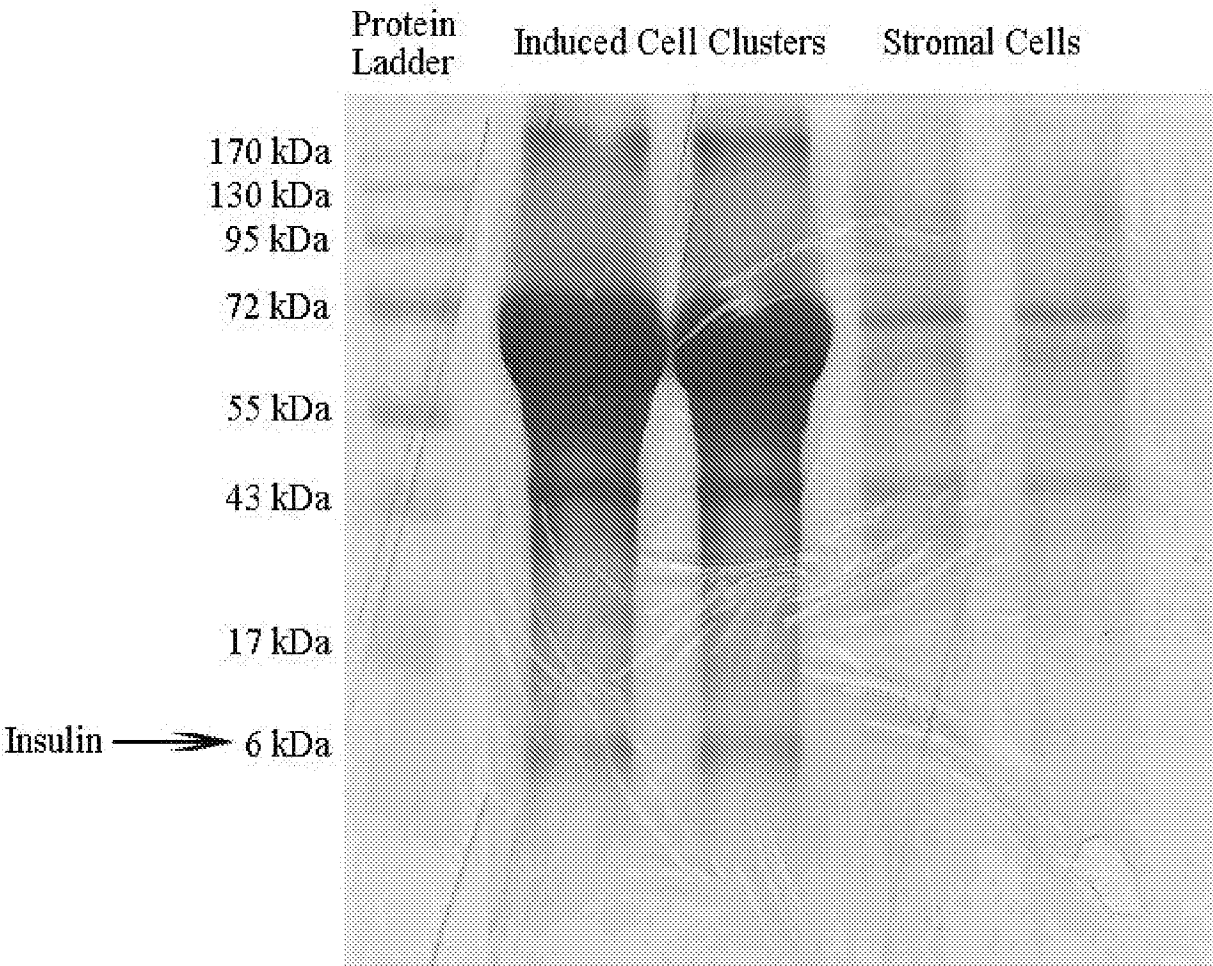


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/55136

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/28, C12N 5/02, C12N 5/074, C12N 5/0775 (2017.01)

CPC - A61K 35/28, C12N 5/0667, C12N 5/0018, C12N 5/0607, C12N 5/0653

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHANDRA et al., Islet-Like Cell Aggregates Generated from Human Adipose Tissue Derived Stem Cells Ameliorate Experimental Diabetes in Mice. PLoS ONE June, 2011. Vol. 6, No.6, pp e20615. doi:10.1371/journal.pone.0020615. Abstract; pg 4, col 1, para 1; pg 4, col 1, para 3; pg 4, col 2, para 2; pg 6, col 2, para 1; pg 8, col 2, last para; pg 8, Figure 8 legend; pg 9, col 2, para 3-5; pg 9, col 2, last para-p10, col 1, para 1; p10, col 1, para 1; pg 10, col 2, para 3	1-6

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

14 December 2017

Date of mailing of the international search report

09 FEB 2018

Name and mailing address of the ISA/US

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Facsimile No. 571-273-8300

Authorized officer:

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PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/55136

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/55136

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-6, drawn to a method of culturing cells to obtain a population comprising cells that can express insulin.

Group II: Claims 7-10, drawn to compositions comprising a population of cells.

Group III: Claims 11-16, drawn to compositions comprising agents for a culture medium.

-----Please see continuation in first extra sheet-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Continuation of Box No III. Observations where unity of invention is lacking

The inventions listed as Groups I, II, and III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I requires method steps for culturing cells, not required by Groups II, III.

Group II requires a composition of matter comprising a population of cells, not required by Groups I, III.

Group III requires a composition of matter comprising agents for a cell culture medium, not required by Groups I, II.

Common Technical Features

The feature shared by Groups I, II, III, is a culture medium comprising at least four of the following: bovine serum albumin, insulintransferrin-selenium, activin A, sodium butyrate, 2-mercapethanol, N-2 supplement, B-27 supplement, laminin, human hepatocyte growth factor (HGF), or basic fibroblast growth factor (bFGF).

The feature shared by Groups I and II is the steps of:

- a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) for about 1-4 days in a first culture medium comprising at least four of the following: glucose, bovine serum albumin, insulintransferrin-selenium, activin A, sodium butyrate, 2-mercapethanol to generate a first population of cells that express Nkx 6.1, Pax6, Isl1, and Glut-2;
- b. Stage 2: culturing the first population of cells in a second culture medium comprising insulin-transferrin-selenium, and taurine to generate a second population of cells; and
- c. Stage 3: culturing the second population of cells in a third culture medium comprising at least four of the following: insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, pentagastrin, betacellulin to generate a third population comprising cells that can express insulin.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by the article entitled 'Islet-Like Cell Aggregates Generated from Human Adipose Tissue Derived Stem Cells Ameliorate Experimental Diabetes in Mice' by Chandra et al., (hereinafter 'Chandra') (PLoS ONE 6(6): e20615. Doi:10.1371; Published June 7, 2011).

Chandra discloses a culture medium comprising at least four of the following: bovine serum albumin, insulintransferrin-selenium, activin A, sodium butyrate, 2-mercapethanol, N-2 supplement, B-27 supplement, laminin, human hepatocyte growth factor (HGF), or basic fibroblast growth factor (bFGF) (p 9, col 2, last para-p 10, col 1, para 1 - "Cells were counted for initial seeding density and 1 x 10⁶ cells/cm² were resuspended in SFM-A....SFM-A contained DMEM/F12 (1:1) (GIBCO) with 17.5 mM glucose, 1% BSA Cohn fraction V, fatty acid free, 1X Insulin-transferrin-selenium (ITS, 5 mg/L insulin+5 mg/L transferrin+5 mg/L selenium), 4 nM activin A, 1 mM sodium butyrate, 50 mM 2-mercaptoethanol and 2 ng/ml Fibroblast Growth Factor").

Chandra discloses:

- a. Stage 1: culturing adult adipose-derived multipotent stromal (stem) cells (ASCs) (p 9, col 2, para 3-5 - "ASCs Cell Isolation and Expansion....The pellet containing stromal vascular fraction was resuspended in expansion medium....Briefly, 10⁶ isolated human ASCs (h-ASCs) were suspended in 1 ml culture medium.") for about 1-4 days in a first culture medium comprising at least four of the following: glucose, bovine serum albumin, insulin, transferrin-selenium, activin A, sodium butyrate, 2-mercaptoethanol, N-2 supplement, B-27 supplement, laminin, human hepatocyte growth factor (HGF), or basic fibroblast growth factor (bFGF) to generate a first population of cells that express Nkx 6.1, Pax6, Isl1, and Glut-2 (p9, col 2, last para-p10, col1, para 1 - "Cells were counted for initial seeding density and 1 x 10⁶ cells/cm² were resuspended in SFM-A....SFM-A contained DMEM/F12 (1:1) (GIBCO) with 17.5 mM glucose, 1% BSA Cohn fraction V, fatty acid free, 1X Insulin-transferrin-selenium (ITS, 5 mg/L insulin+5 mg/L transferrin+5 mg/L selenium), 4 nM activin A, 1 mM sodium butyrate, 50 mM 2-mercaptoethanol and 2 ng/ml Fibroblast Growth Factor. The cells were cultured in this media for 2 days"; p 8, col 2, last para - "The taurine supplemented with nicotinamide and GLP-1 in the differentiation cocktail resulted in the up regulation of a number of genes (PDX1, Ngn3, NeuroD, Pax4, Nkx2.2, Nkx6.1, Pax-6, Isl-1 and pancreas specific glucose transporter Glut-2) involved in pancreatic beta cells development cascade. Additionally, detectable level of transcripts of two pancreatic progenitor specific transcription factors Nkx2.2/Nkx6.1 and Isl-1 in undifferentiated h-ASCs make it more suitable candidate stem cells to differentiate into pancreatic lineage"; p4, col 1, para 1 - "A significant up regulation in the expression of pancreatic endoderm markers as well as key transcription factors involved in pancreas development like PDX-1, Ngn3, NeuroD, Pax-4, Nkx2.2, Nkx6.1, Pax-6, Isl-1 and glucose transporter Glut-2 were observed in d14- ICAs over undifferentiated h-ASCs (Figure 5)");
- b. Stage 2: culturing the first population of cells in a second culture medium comprising insulin-transferrin-selenium, taurine to generate a second population of cells (p 10, col 1, para 1 - "On 3rd day media was changed to SFM-B that contained DMEM/F12 (1:1) with 17.5 mM glucose, 1% BSA, ITS and 0.3 mM Taurine. The cell aggregates were cultured in this medium for another 2 days"); and
- c. Stage 3: culturing the second population of cells in a third culture medium comprising at least four of the following: glucose, bovine serum albumin, insulin-transferrin-selenium, taurine, glucagon-like peptide 1 (GLP-1), nicotinamide, non-essential amino acids, pentagastrin, N-2 supplement, B-27 supplement, mammalian hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), laminin, or betacellulin to generate a third population comprising cells that can express insulin (p 10, col 1, para 1 - "and shifted to SFM-C on 5th day. SFM-C contained DMEM/F12 (1:1) with 17.5 mM glucose, 1.5% BSA, ITS, 3 mM Taurine, 100 nM Glucagon-like peptide 1 (GLP-1) (amide fragment 7-36, Sigma Aldrich), 1 mM nicotinamide and 16 non-essential amino acids (NEAA). The cell aggregates were fed with fresh SFM-C medium every 2 days for another 12-14 days."; p4, col 1, para 3 - "The mature day-14 ICAs showed enhanced transcript levels of insulin, glucagon, somatostatin, pan-polypeptide and ghrelin as compared to undifferentiated h-ASCs").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Groups I, II, and III therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.