(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 April 2011 (28.04.2011)

PCT

(10) International Publication Number
WO 2011/048602 A1

(51) **International Patent Classification**:
A61K 38/28 (2006.01) *C07K 14/62* (2006.01)

(21) **International Application Number**:
PCT/IL2010/000877

(22) **International Filing Date**:
24 October 2010 (24.10.2010)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/254,363 23 October 2009 (23.10.2009) US

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2011/048602 A1

(54) **Title**: COMPOUNDS RESTORING PANCREATIC BETA CELLS

(57) **Abstract**: The use of proinsulin as inducer of de-novo generation of beta cells via the differentiation of pancreatic pluripotent cells or embryonic cells is provided. Additionally, a method of inducing the generation of beta cells and/or restoring endogenic production and secretion of insulin in a patient suffering from insulin dependent diabetes by administering to the patient a composition which includes proinsulin, is provided.

COMPOUNDS RESTORING PANCREATIC BETA CELLS

FIELD OF INVENTION

[001] A method of inducing the generation of insulin secreting cells in a subject by administering to the subject a pharmaceutical composition comprising proinsulin, is provided.

BACKGROUND OF THE INVENTION

[002] Induction of β-cell differentiation in cultured human β-cells was achieved by stimulating multiple signaling pathways, including those downstream of the homeodomain transcription factors NeuroD/BETA2 and PDX-1, cell-cell contact, and the glucagon-like peptide-1 (GLP-1) receptor. Synergistic activation of those pathways resulted in differentiation of the cultured human β-cells, which initially express no detectable pancreatic hormones, into fully functional β-cells that exhibit glucose-responsive insulin secretion. Furthermore, these cells can be transplanted in vivo and demonstrate glucose-responsive expression of insulin. The ability to grow unlimited quantities of functional human β-cells in vitro provides the means for a definitive cell transplantation therapy for treatment of diabetes.

[003] The expression of the transcription factor NeuroD/BETA2 in human β-cells that express PDX-1, are in cell to cell contact, and are contacted with a GLP-1 receptor agonist resulted in certain desirable characteristics. For example, surprisingly, the resulting cells produce high levels of insulin compared to cells that do not express NeuroD/BETA2. Moreover, the cells expressing NeuroD/BETA2 are highly stable in cell culture and can be grown in culture for multiple generations. Thus, in some embodiments of the invention, the present invention provides a method for inducing insulin gene expression in cultured endocrine pancreas cells, the method comprising the steps of (i) expressing a recombinant NeuroD/BETA2 polynucleotide and a recombinant PDX-1 gene in cells that have been cultured under conditions such that the cells are in contact with other cells in the culture; and (ii) contacting the cells with a GLP-1 receptor agonist, thereby inducing insulin gene expression in the cells. In some embodiments, the cells do not initially produce any detectable pancreatic hormones such as insulin and glucagon.

SUMMARY OF THE INVENTION

[004] In one embodiment, the present invention provides a method of inducing the generation of insulin secreting cells in a subject, comprising the step of administering to said subject from

0.05 to 1.00 unit of proinsulin per kg body weight in a pharmaceutical composition, thereby inducing the generation of beta cells.

[005] In another embodiment, the present invention further provides a method of treating insulin dependent diabetes mellitus in a subject, comprising the step of administering to said subject from 0.05 to 1.00 unit of proinsulin per kg body weight in a pharmaceutical composition, thereby treating insulin dependent diabetes mellitus in a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[006] FIG. 1 is a graph showing hypoglycemizing effects of human proinsulin in intact rats. The bars show mean ± SD concentration/time profiles of plasma glucose following the administration of human proinsulin or its vehicle to intact rats. A - vehicle; B - human proinsulin, 10 mg/kg; C - human proinsulin, 100 mg/kg.

[007] FIG. 2 is a graph showing Human proinsulin persistence in the bloodstream of intact rats. The bars show mean ± SD concentration/time profiles of plasma human proinsulin following the administration of human proinsulin or its vehicle to intact rats. A - vehicle; B - human proinsulin, 10 mg/kg; C - human proinsulin, 100 mg/kg.

[008] FIG. 3 is a flow chart of the study "Evaluation of Therapeutic Effects of Human Proinsulin in Rats with Chemically Induced Insulin-Dependent Diabetes".

[009] FIG. 4 is a graph showing Blood Glucose Concentrations in All Treatment Subgroups and in the Control Group on the 1st Day of the Treatment Period. The bars show mean ± SD concentrations of non-fasting blood glucose in rats with streptozotocin-induced diabetes before initiation of combination treatment with human insulin and human proinsulin. Non-fasting blood glucose concentrations were measured at 10:30 a.m., prior to injections of insulin and proinsulin. IA - insulin alone group (N=4), I/LpI - insulin+low-dose proinsulin group (N = 12), I/HpI - insulin+high-dose proinsulin group (N = 10), Control - control group (N = 7).

[010] FIG. 5 is a graph showing Blood Glucose Concentrations in All Treatment Subgroups and in the Control Group on the 8th Day of the Treatment Period. The bars show mean ± SD concentrations of non-fasting blood glucose in rats with streptozotocin-induced diabetes on the 8th day of combination treatment with human insulin and human proinsulin. Non-fasting blood glucose concentrations were measured at 7:30 a.m. IA - insulin alone group (N=4), I/LpI - insulin+low-dose proinsulin group (N = 12), I/HpI - insulin+high-dose proinsulin group (N = 10), Control - control group (N = 7).

[011] FIG. 6 is a graph showing Blood Glucose Concentrations in All Treatment Subgroups and in the Control Group on the 10th Day of the Treatment Period. The bars show mean ± SD concentrations of fasting blood glucose in rats with streptozotocin-induced diabetes on the 10th day of combination treatment with human insulin and human proinsulin. After 30-hr fasting, blood glucose concentrations were measured at 10:30 a.m. Treatment subgroups received neither insulin nor proinsulin injections on a previous day. IA - insulin alone group (N=4), I/LpI - insulin+low-dose proinsulin group (N = 12), I/HpI - insulin+high-dose proinsulin group (N = 10), Control - control group (N = 7).

[012] FIG. 7 is a graph showing Blood Glucose Concentrations in All Treatment Subgroups and in the Control Group on the 18th Day of the Treatment Period. The bars show mean ± SD concentrations of fasting blood glucose in rats with streptozotocin-induced diabetes on the 18th day of combination treatment with human insulin and human proinsulin. After 30-hr fasting, blood glucose concentrations were measured at 11:00 a.m. Treatment subgroups received neither insulin nor proinsulin injections on a previous day. IA - insulin alone group (N=4), I/LpI - insulin+low-dose proinsulin group (N = 12), I/HpI - insulin+high-dose proinsulin group (N = 10), Control - control group (N = 7).

[013] FIG. 8 is a graph showing Blood Glucose Concentrations in All Treatment Subgroups and in the Control Group on the 24th Day of the Treatment Period. The bars show mean ± SD concentrations of fasting blood glucose in rats with streptozotocin-induced diabetes on the 24th day of combination treatment with human insulin and human proinsulin. After 30-hr fasting, blood glucose concentrations were measured at 10:30 a.m. Treatment subgroups received neither insulin nor proinsulin injections over 2 previous days. IA - insulin alone group (N=4), I/LpI - insulin+low-dose proinsulin group (N = 12), I/HpI - insulin+high-dose proinsulin group (N = 10), Control - control group (N = 7).

[014] FIG. 9 is a graph showing Blood Glucose Concentrations in All Treatment Subgroups and in the Control Group on the 14th Day of the Washout Period. The bars show mean ± SD concentrations of non-fasting blood glucose in rats with streptozotocin-induced diabetes on the 14th day after discontinuation of combination treatment with human insulin and human proinsulin. Non-fasting blood glucose concentrations were measured at 10:30 a.m. IA - insulin alone group (N=4), I/LpI - insulin+low-dose proinsulin group (N = 12), I/HpI - insulin+high-dose proinsulin group (N = 10), Control - control group (N = 7).

DETAILED DESCRIPTION OF THE INVENTION

[015] In one embodiment, the present invention provides a method of inducing the generation of beta cells, comprising the step of contacting pancreatic pluripotent cells with proinsulin, thereby inducing the generation of beta cells. In another embodiment, the present invention provides a method of inducing the differentiation of pancreatic pluripotent cells to beta cells, comprising the step of contacting pancreatic pluripotent cells with proinsulin. In another embodiment, the present invention provides a method of directing the differentiation of pancreatic pluripotent cells to beta cells, comprising the step of contacting pancreatic pluripotent cells with proinsulin. In another embodiment, the present invention provides a method for induction of beta cell differentiation in pancreatic pluripotent cells. In another embodiment, the present invention provides a method for induction of beta cell differentiation in human pancreatic pluripotent cells.

[016] In another embodiment, beta cells are pancreatic beta cells. In another embodiment, beta cells are pancreatic beta cells located in the islets of Langerhans. In another embodiment, beta cells are pancreatic insulin-producing cells. In another embodiment, beta cells are pancreatic primary beta cells. In one embodiment, the present invention provides a method of inducing the production and the release of insulin from cells, comprising the step of contacting pancreatic pluripotent cells with proinsulin. In another embodiment, the present invention provides a method of inducing the release of C-peptide, a byproduct of insulin production, into the bloodstream, comprising the step of contacting pancreatic pluripotent cells with proinsulin. In another embodiment, the present invention provides a method of inducing the production of Amylin (IAPP), comprising the step of contacting pancreatic pluripotent cells with proinsulin.

[017] In another embodiment, the method of the invention comprising contacting ES cells with proinsulin induces ES cells to differentiate into pancreatic beta cells. In another embodiment, the method of the invention comprising contacting pancreatic pluripotent cells with proinsulin induces pancreatic pluripotent cells to differentiate into pancreatic beta cells.

[018] In another embodiment, the term "pancreatic pluripotent cells" comprises cells that can differentiate to alpha cells or beta cells. In another embodiment, the term "pancreatic pluripotent cells" comprises pancreatic stem cells. In another embodiment, the term "pancreatic pluripotent cells" comprises pancreatic progenitor cells.

[019] In another embodiment, the method of the invention comprises contacting pancreatic pluripotent cells with proinsulin which induces pancreatic pluripotent cells to differentiate into pancreatic beta cells and transplanting the pancreatic beta cells in a subject in need thereof. In another embodiment, the method of the invention comprises contacting pancreatic pluripotent

cells with proinsulin which induces pancreatic pluripotent cells to differentiate into pancreatic beta cells and transplanting the pancreatic beta cells exhibiting glucose-responsive insulin secretion to cure diabetes in a subject in need. In another embodiment, the method of the invention comprises transplanting newly differentiated beta cells of the invention into a subject in need, thus inducing the production of insulin in a glucose responsive manner. In another embodiment, the invention provides methods of creating cells that produce a high level of insulin. In another embodiment, the invention provides methods of creating cells that produce a high level of insulin over many generations. In another embodiment, the method of the invention further comprises expanding the newly formed beta cells.

[020] In another embodiment, the method of the invention comprises the induction of complete beta cell function in-vitro. In another embodiment, the method of the invention comprises the induction of complete beta cell function in-vivo. In another embodiment, the method of the invention comprises the induction of complete beta cell function ex-vivo.

[021] In another embodiment, this invention relies upon routine techniques in the field of cell culture, and suitable methods can be determined by those of skill in the art using known methodology (see, e.g., Freshney et al., Culture of Animal Cells (3rd ed. 1994)). In general, the cell culture environment includes consideration of such factors as the substrate for cell growth, cell density and cell contact, the gas phase, the medium, and temperature.

[022] In another embodiment, pancreatic pluripotent cells or ES cells of the invention are grown under conditions that provide for maximal cell to cell contact. In another embodiment, the cell-to-cell contact occurs to a greater degree than found in monolayer cell cultures. In another embodiment, the cells are grown in suspension as three dimensional aggregates In another embodiment, the cells are grown in Costar dishes that have been coated with a hydrogel to prevent them from adhering to the bottom of the dish. In another embodiment, the cells are cultured under adherent conditions, plastic dishes, flasks, roller bottles, or microcarriers in suspension. In another embodiment, artificial substrates are used such as glass and metals. In another embodiment, the substrate is treated by etching, or by coating with substances such as collagen, chondronectin, fibronectin, and laminin. In another embodiment, the type of culture vessel depends on the culture conditions, e.g., multi-well plates, petri dishes, tissue culture tubes, flasks, roller bottles, and the like. In another embodiment, cultured cells are normally grown in an incubator that provides a suitable temperature, e.g., the body temperature of the animal from which the cells were obtained, accounting for regional variations in temperature. In another embodiment, an incubator is a humidified incubator. In

another embodiment, atmospheric oxygen tensions are used for cell cultures. In another embodiment, carbon dioxide plays a role in pH stabilization, along with buffer in the cell media and is typically present at a concentration of 1-10% in the incubator.

[023] In another embodiment, known cell media are used. In another embodiment, defined cell media comprises premixed powders or presterilized solutions. In another embodiment, cell media comprises DME, RPMI 1640, DMEM, Iscove's complete media, or McCoy's Medium. In another embodiment, cell media comprises low glucose DME or RPMI 1640. In another embodiment, cell media is supplemented with 5-20% serum, typically heat inactivated, e.g., human horse, calf, and fetal bovine serum. In another embodiment, cell media is supplemented with 10% fetal bovine serum. In another embodiment, cell media is buffered to maintain the cells at a pH preferably from 7.0-7.8. In another embodiment, cell media is supplemented with antibiotics, amino acids, sugars, growth factors, or any combination thereof.

[024] In another embodiment, provided herein a method of inducing the generation of insulin secreting cells in a subject, comprising the step of administering to a subject from 0.05 to 1.00 unit of proinsulin per kg body weight in a pharmaceutical composition, thereby inducing the generation of beta cells. In another embodiment, provided herein a method of inducing the generation of insulin secreting cells in a subject, comprising the step of administering to a subject from 10 to 100 μg per kg body weight in a pharmaceutical composition, thereby inducing the generation of beta cells. In another embodiment, a subject is a subject suffering from a deficiency in insulin secretion. In another embodiment, induction of the generation of insulin secreting cells occurs in the pancreas. In another embodiment, inducing the generation of insulin secreting cells is in the isles of Langerhans. In another embodiment, induction of the generation of insulin secreting cells comprises the induction of the differentiation of pancreatic pluripotent cells into beta cells. In another embodiment, insulin secreting cells as described herein further secrete C-peptide, produce Amylin, or any combination thereof.

[025] In another embodiment, provided herein a method of treating insulin dependent diabetes mellitus in a subject, comprising the step of administering to a subject a composition as described herein in a dosage of proinsulin as described herein, thereby treating insulin dependent diabetes mellitus in a subject. In another embodiment, treating insulin dependent diabetes mellitus is inducing the generation of insulin secreting cells in a pancreas.

[026] In another embodiment, provided herein a method of reducing a dosing frequency of insulin, comprising the step of administering to a subject afflicted with diabetes a composition comprising proinsulin, thereby reducing a dosing frequency of insulin. In another embodiment,

provided herein a method of reducing the dosage of insulin, comprising the step of administering to a subject afflicted with diabetes a composition comprising proinsulin, thereby reducing the dosage of insulin.

[027] In another embodiment, administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg per kg body weight in a pharmaceutical composition comprises administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to breakfast. In another embodiment, administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition comprises administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to dinner. In another embodiment, administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition comprises administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to lunch time. In another embodiment, administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition comprises administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to bedtime. In another embodiment, about 1 hour is 40 to 80 minutes. In another embodiment, about 1 hour is 45 to 75 minutes. In another embodiment, about 1 hour is 50 to 70 minutes. In another embodiment, about 1 hour is 55 to 65 minutes.

[028] In another embodiment, administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition comprises administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to breakfast, administering to said subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to lunch, administering to said subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to dinner, administering to a subject from 0.05 to 1.00 unit or 5 to 200 μg of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to bedtime, or any combinations thereof.

[029] In another embodiment, proinsulin is administered in a concentration of 20-150 units/ml. In another embodiment, proinsulin is administered in a concentration of 20-40

units/ml. In another embodiment, proinsulin is administered in a concentration of 40-60 units/ml. In another embodiment, proinsulin is administered in a concentration of 60-100 units/ml. In another embodiment, proinsulin is administered in a concentration of 100-150 units/ml. In another embodiment, proinsulin is administered in a concentration of 40 or 100 units/ml.

[030] In another embodiment, one unit of proinsulin corresponds quantitatively to one international accepted unit of insulin, i.e. one unit of proinsulin is equimolar to one unit of insulin. In another embodiment, 1 unit of insulin is 10-35 times more active than 1 unit of proinsulin. In another embodiment, 1 unit of insulin is 20-30 times more active than 1 unit of proinsulin. In another embodiment, 1 unit of insulin is 25 times more active than 1 unit of proinsulin.

[031] In another embodiment, proinsulin is administered in a composition. In another embodiment, proinsulin is administered in a pharmaceutical composition. In another embodiment, proinsulin is administered in a solution. In another embodiment, proinsulin is administered in an infusion. In another embodiment, proinsulin is administered in an injectable solution. In another embodiment, proinsulin solution comprises 10-80 units of proinsulin in one milliliter of a solution. In another embodiment, proinsulin solution comprises 10-60 units of proinsulin in one milliliter of a solution. In another embodiment, proinsulin solution comprises 20-60 units of proinsulin in one milliliter of a solution. In another embodiment, proinsulin solution comprises 30-50 units of proinsulin in one milliliter of a solution. In another embodiment, proinsulin solution comprises 35-45 units of proinsulin in one milliliter of a solution. In another embodiment, proinsulin solution comprises 38-42 units of proinsulin in one milliliter of a solution. In another embodiment, proinsulin solution further comprises pharmaceutical accepted excipients.

[032] In another embodiment, proinsulin solution is administered in a dose of 0.05 to 1.0 unit proinsulin per kg body weight. In another embodiment, proinsulin solution is administered in a dose of 0.05 to 0.10 unit or 5 to 50 μg proinsulin per kg body weight. In another embodiment, proinsulin solution is administered in a dose of 0.10 to 0.50 unit or 50 to 100 μg proinsulin per kg body weight. In another embodiment, proinsulin solution is administered in a dose of 0.20 to 0.40 unit or 20 to 80 μg proinsulin per kg body weight.

[033] In another embodiment, a dose of proinsulin as described herein is administered once a day. In another embodiment, a dose of proinsulin as described herein is administered twice a

day. In another embodiment, a dose of proinsulin as described herein is administered three times a day. In another embodiment, a dose of proinsulin as described herein is administered four times a day. In another embodiment, a dose of proinsulin as described herein is administered five times a day. In another embodiment, a dose of proinsulin as described herein is administered 0.5-1.5 hours prior to a meal. In another embodiment, a dose of proinsulin as described herein is administered 0.8-1.2 hours prior to a meal. In another embodiment, a dose of proinsulin as described herein is administered about an hour prior to a meal.

[034] In another embodiment, a subject is a human subject. In another embodiment, a subject is a pet. In another embodiment, a subject is a laboratory animal. In another embodiment, a subject is a rodent. In another embodiment, a subject is a farm animal. In another embodiment, a subject is a diabetic subject. In another embodiment, a subject is a subject afflicted with any type of diabetes, including primary and secondary diabetes, type 1 IDDM, type 2 IDDM-transient, and type 2 MODY, as described in Harrison's Internal Medicine, 14th ed. 1998.

[035] In another embodiment, "contacting" comprises the addition of proinsulin to a media of pancreatic pluripotent cells or ES cells. In another embodiment, "contacting" comprises contacting intact pancreatic pluripotent cells with proinsulin, in-vivo, by means of drug delivery. In another embodiment, "contacting" comprises contacting intact pancreatic pluripotent cells with a composition comprising proinsulin, in-vivo, by means of drug delivery. In another embodiment, "contacting" comprises contacting intact pancreatic pluripotent cells with a composition comprising proinsulin, in-vivo, by means of intravenous injection. In another embodiment, "contacting" comprises contacting intact pancreatic pluripotent cells with a composition comprising proinsulin, in-vivo, by means of oral peptide drug delivery. In another embodiment, "contacting" comprises contacting intact pancreatic pluripotent cells with a composition comprising proinsulin, in-vivo, by means of subcutaneous injection. In another embodiment, "contacting" comprises contacting intact pancreatic pluripotent cells with a composition comprising proinsulin, in-vivo, by means of intramuscular injection. In another embodiment, "contacting" comprises contacting intact pancreatic pluripotent cells with a composition comprising proinsulin, in-vivo, by means of intraperitoneal injection. In another embodiment, "contacting" comprises contacting intact pancreatic pluripotent cells within the pancreas in a subject. In another embodiment, "contacting" comprises contacting intact pancreatic pluripotent cells within the pancreas in a subject afflicted with diabetes.

[036] In another embodiment, "inducing the generation of beta cells" comprises inducing and/or shifting the differentiation of pancreatic pluripotent cells towards beta cells. In another

embodiment, "inducing the generation of beta cells" comprises inducing and/or shifting the differentiation of ES cells towards pancreatic beta cells. In another embodiment, "inducing the generation of beta cells" comprises inducing and/or shifting the differentiation of pancreatic pluripotent cells towards insulin producing cells. In another embodiment, "inducing the generation of beta cells" comprises inducing and/or shifting the differentiation of pancreatic pluripotent cells towards insulin secreting cells.

[037] In another embodiment, proinsulin is a pro-hormone precursor of insulin. In another embodiment, proinsulin is made in the beta cell of the islets of Langerhans. In another embodiment, proinsulin is the precursor of insulin and the C-peptide. In another embodiment, the C-peptide is abstracted from the end of the proinsulin sequence.

[038] In another embodiment, proinsulin is a polypeptide of 9390 MW (86 amino acids). In another embodiment, proinsulin is a recombinant Proinsulin. In another embodiment, proinsulin is a recombinant human Proinsulin. In another embodiment, recombinant Proinsulin forms are known to one of skill in the art. In another embodiment, proinsulin is proinsulin precursor. In another embodiment, proinsulin is encoded by the NCBI Reference Sequence: NP_000198.1. In another embodiment, proinsulin is encoded by the following amino acid sequence: MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALY LVCGERGFFYTPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTS ICSLYQLENYCN (SEQ ID NO: 1). In another embodiment, proinsulin is encoded by a sequence comprising the following amino acid sequence: MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRR EAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN (SEQ ID NO: 2). In another embodiment, proinsulin is encoded by a sequence comprising the following amino acid sequence: MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRR EAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN (SEQ ID NO: 3). In another embodiment, proinsulin is encoded by a sequence comprising the following amino acid sequence: MALWMRFLPLLALLFLWESHPTQAFVKQHLCGSHLVEALYLVCGERGFFYTPMSRRE VEDPQVAQLELGGGPGAGDLQTLALEVAQQKRGIVDQCCTSICSLYQLENYCN (SEQ ID NO: 4). In another embodiment, proinsulin is encoded by a sequence comprising the following amino acid sequence: MALWMRFLPLLALLFLWESHPTQAFVKQHLCGSHLVEALYLVCGERGFFYTPMSRRE VEDPQVAQLELGGGPGAGDLQTLALEVAQQKRGIVDQCCTSICSLYQLENYCN (SEQ

ID NO: 5). In another embodiment, proinsulin is encoded by a sequence comprising the following amino acid sequence: MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRR EAEDLQ (SEQ ID NO: 6). In another embodiment, proinsulin is encoded by a sequence comprising the following amino acid sequence: MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRR EAEDLQV (SEQ ID NO: 7). In another embodiment, proinsulin is encoded by a sequence comprising the following amino acid sequence: MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRR EAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN (SEQ ID NO: 8). In another embodiment, proinsulin is a single chain protein. In another embodiment, proinsulin as used herein comprises 110 amino acids preproprecursor that contains a 24 amino acids signal sequence and an 86 amino acids proinsulin. In another embodiment, proinsulin as used herein comprises 86 amino acids of proinsulin.

[039] In another embodiment, the DNA sequence of proinsulin comprises the following nucleic acid sequence: ATGGCCCTGTGGATGCGCCTCCTGCCCCTGCTGGCGCTGCTGGCCCTCTGGGGACC TGACCCAGCCGCAGCCTTTGTGAACCAACACCTGTGCGGCTCACACCTGGTGGAA GCTCTCTACCTAGTGTGCGGGGAACGAGGCTTCTTCTACACACCCAAGACCCGCCG GGAGGCAGAGGACCTGCAGG (SEQ ID NO: 9). In another embodiment, the DNA sequence of proinsulin comprises the following nucleic acid sequence: ATGGCCCTGTGGATGCGCCTCCTGCCCCTGCTGGCGCTGCTGGCCCTCTGGGGACC TGACCCAGCCGCAGCCTTTGTGAACCAACACCTGTGCGGCTCACACCTGGTGGAA GCTCTCTACCTAGTGTGCGGGGAACGAGGCTTCTTCTACACACCCAAGACCCGCCG GGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAGCTGGGCGGGGGCCCTGGTGC AGGCAGCCTGCAGCCCTTGGCCCTGGAGGGGTCCCTGCAGAAGCGTGGCATTGTG GAACAATGCTGTACCAGCATCTGCTCCCTCTACCAGCTGGAGAACTACTGCAACTA G (SEQ ID NO: 10). In another embodiment, the DNA sequence of proinsulin comprises the following nucleic acid sequence: TGGGGCAGGTGGAGCTGGGCGGGGGCCCTGGTGCAGGCAGCCTGCAGCCCTTGGC CCTGGAGGGGTCCCTGCAGAAGCGTGGCATTGTGGAACAATGCTGTACCAGCATC TGCTCCCTCTACCAGCTGGAGAACTACTGCAACTAG (SEQ ID NO: 11). In another embodiment, the DNA sequence of proinsulin comprises the human Proinsulin (Phe 25 - Asn 110; Accession # NP_000198).

[040] In another embodiment, proinsulin is encoded by a sequence known to one of skill in the art. In another embodiment, proinsulin is encoded by a sequence deposited in a gene bank.

[041] In another embodiment, the proinsulin amino acid sequence of the present invention is at least 70% homologous to a native proinsulin amino acid sequence or a peptide thereof. In another embodiment, the proinsulin amino acid sequence of the present invention is at least 80% homologous to a native proinsulin amino acid sequence or a peptide thereof. In another embodiment, a proinsulin amino acid sequence of the present invention is at least 90% homologous to a native proinsulin amino acid sequence or a peptide thereof. In another embodiment, a proinsulin amino acid sequence of the present invention is at least 95% homologous to a native proinsulin amino acid sequence or a peptide thereof. In another embodiment, a proinsulin amino acid sequence of the present invention is 100% homologous to a native proinsulin amino acid sequence or a peptide thereof.

[042] In another embodiment, the proinsulin DNA sequence of the present invention is at least 70% homologous to the native human proinsulin DNA sequence or a peptide thereof. In another embodiment, the proinsulin DNA sequence of the present invention is at least 80% homologous to the native human proinsulin DNA sequence or a peptide thereof. In another embodiment, the proinsulin DNA sequence of the present invention is at least 90% homologous to the native proinsulin DNA sequence or a peptide thereof. In another embodiment, the proinsulin DNA sequence of the present invention is at least 95% homologous to the native proinsulin DNA sequence or a peptide thereof. In another embodiment, the proinsulin DNA sequence of the present invention is 100% homologous to the native proinsulin DNA sequence or a peptide thereof.

[043] In some embodiments, "proinsulin", as used herein encompasses native genetically engineered proinsulin including degradation products, synthetically synthesized polypeptides or recombinant polypeptides and peptidomimetics (typically, synthetically synthesized polypeptides), as well as peptoids and semipeptoids which are polypeptide analogs of proinsulin, which have, in some embodiments, modifications rendering the polypeptide comprising a proinsulin more stable while in a body or more capable of contacting pluripotent cells.

[044] In some embodiments, natural aromatic amino acids of the proinsulin such as Trp, Tyr and Phe, are substituted for synthetic non-natural acid such as Phenylglycine, TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-

methyl-Tyr. In some embodiments, the proinsulin of the present invention includes one or more modified amino acid or one or more non-amino acid monomers (e.g. fatty acid, complex carbohydrates etc).

[045] In one embodiment, "amino acid" or "amino acid sequence" is understood to include the 20 naturally occurring amino acid; those amino acid often modified post-translationally *in vivo*, including, for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acid including, but not limited to, 2-aminoadipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. In another embodiment, "amino acid" includes both D- and L-amino acid.

[046] In some embodiments, the proinsulin of the present invention is utilized in therapeutics which requires the proinsulin to be in a soluble form. In some embodiments, the proinsulin of the present invention includes one or more non-natural or natural polar amino acid, including but not limited to serine and threonine which are capable of increasing proinsulin solubility due to their hydroxyl-containing side chain.

[047] In some embodiments, proinsulin of the present invention is utilized in a linear form, although it will be appreciated by one skilled in the art that in cases where cyclicization does not severely interfere with proinsulin characteristics, cyclic forms of the proinsulin can also be utilized.

[048] In some embodiments, the proinsulin of present invention is biochemically synthesized such as by using standard solid phase techniques. In some embodiments, these biochemical methods include exclusive solid phase synthesis, partial solid phase synthesis, fragment condensation, or classical solution synthesis.

[049] In some embodiments, recombinant protein techniques are used to generate the proinsulin of the present invention. In some embodiments, recombinant protein techniques are used for the generation of relatively long polypeptides (e.g., longer than 18-25 amino acids). In some embodiments, recombinant protein techniques are used for the generation of large amounts of the proinsulin of the present invention. In some embodiments, recombinant techniques are described by Bitter et al., (1987) Methods in Enzymol. 153:516-544, Studier et al. (1990) Methods in Enzymol. 185:60-89, Brisson et al. (1984) Nature 310:511-514, Takamatsu et al. (1987) EMBO J. 6:307-311, Coruzzi et al. (1984) EMBO J. 3:1671-1680 and Brogli et al., (1984) Science 224:838-843, Gurley et al. (1986) Mol. Cell. Biol. 6:559-565 and

Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463.

[050] In another embodiment, the invention provides an expression vector comprising a polynucleotide molecule which encodes a proinsulin. In another embodiment, the invention provides a cell comprising the expression vector as described herein. In another embodiment, the invention provides a composition comprising the expression vector as described herein. In another embodiment, the invention provides a composition comprising the cell as described herein. In another embodiment, the cell is a eukaryotic cell. In another embodiment, the cell is a prokaryotic cell.

[051] In another embodiment, proinsulin of the present invention is synthesized using a polynucleotide molecule encoding a proinsulin. In some embodiments, the polynucleotide molecule encoding proinsulin of the present invention is ligated into an expression vector, comprising a transcriptional control of a cis-regulatory sequence (e.g., promoter sequence). In some embodiments, the cis-regulatory sequence is suitable for directing constitutive expression of the proinsulin of the present invention. In some embodiments, the cis-regulatory sequence is suitable for directing tissue specific expression of the proinsulin of the present invention. In some embodiments, the cis-regulatory sequence is suitable for directing inducible expression of the proinsulin of the present invention.

[052] In some embodiment, tissue-specific promoters suitable for use with the present invention include sequences which are functional in specific cell population, example include, but are not limited to promoters such as albumin that is liver specific [Pinkert et al., (1987) Genes Dev. 1:268-277], lymphoid specific promoters [Calame et al., (1988) Adv. Immunol. 43:235-275]; in particular promoters of T-cell receptors [Winoto et al., (1989) EMBO J. 8:729-733] and immunoglobulins; [Banerji et al. (1983) Cell 33729-740], neuron-specific promoters such as the neurofilament promoter [Byrne et al. (1989) Proc. Natl. Acad. Sci. USA 86:5473-5477], pancreas-specific promoters [Edlunch et al. (1985) Science 230:912-916] or mammary gland-specific promoters such as the milk whey promoter (U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Inducible promoters suitable for use with the present invention include for example the tetracycline-inducible promoter (Srour, M.A., et al., 2003. Thromb. Haemost. 90: 398-405).

[053] In one embodiment, the phrase "a polynucleotide molecule" refers to a single or double stranded nucleic acid sequence which can be isolated and provided in the form of an RNA

sequence, a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

[054] In one embodiment, following expression and secretion, the signal peptides are cleaved from the precursor proinsulin resulting in the mature proinsulin.

[055] In some embodiments, polynucleotides of the present invention are prepared using PCR techniques, or any other method or procedure known to one skilled in the art. In some embodiments, the procedure involves the ligation of two different DNA sequences (See, for example, "Current Protocols in Molecular Biology", eds. Ausubel et al., John Wiley & Sons, 1992).

[056] In one embodiment, polynucleotides of the present invention which encode the proinsulin are inserted into expression vectors (i.e., a nucleic acid construct) to enable expression of the recombinant proinsulin. In another embodiment, the expression vector of the present invention includes additional sequences which render this vector suitable for replication and integration in prokaryotes. In another embodiment, the expression vector of the present invention includes additional sequences which render this vector suitable for replication and integration in eukaryotes. In another embodiment, the expression vector of the present invention includes a shuttle vector which renders this vector suitable for replication and integration in both prokaryotes and eukaryotes. In some embodiments, cloning vectors comprise transcription and translation initiation sequences (e.g., promoters, enhancers) and transcription and translation terminators (e.g., polyadenylation signals).

[057] In one embodiment, a variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the proinsulin of the present invention. In some embodiments, these include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the proinsulin coding sequence; yeast transformed with recombinant yeast expression vectors containing the proinsulin coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors, such as Ti plasmid, containing the proinsulin coding sequence.

[058] In some embodiments, non-bacterial expression systems are used (e.g. mammalian expression systems such as CHO cells) to express the proinsulin of the present invention. In one embodiment, the expression vector used to express polynucleotides of the present

invention in mammalian cells is pCI-DHFR vector comprising a CMV promoter and a neomycin resistance gene.

[059] In some embodiments, in bacterial systems of the present invention, a number of expression vectors can be advantageously selected depending upon the use intended for the proinsulin expressed. In one embodiment, large quantities of proinsulin are desired. In another embodiment, vectors that direct the expression of high levels of the protein product, possibly as a fusion with a hydrophobic signal sequence, which directs the expressed product into the periplasm of the bacteria or the culture medium where the protein product is readily purified are desired. In one embodiment, certain fusion protein engineered with a specific cleavage site to aid in recovery of the proinsulin. In one embodiment, vectors adaptable to such manipulation include, but are not limited to, the pET series of *E. coli* expression vectors [Studier *et al.*, Methods in Enzymol. 185:60-89 (1990)].

[060] In one embodiment, yeast expression systems are used. In one embodiment, a number of vectors containing constitutive or inducible promoters can be used in yeast as disclosed in U.S. Pat. Application. No: 5,932,447. In another embodiment, vectors which promote integration of foreign DNA sequences into the yeast chromosome are used.

[061] In one embodiment, the expression vector of the present invention can further include additional polynucleotide sequences that allow, for example, the translation of several proteins from a single mRNA such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric polypeptide- proinsulin.

[062] In some embodiments, mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

[063] In some embodiments, expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses are used by the present invention. SV40 vectors include pSVT7 and pMT2. In some embodiments, vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A^+, pMTO10/A^+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40

early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[064] In some embodiments, recombinant viral vectors are useful for *in vivo* expression of the proinsulin of the present invention since they offer advantages such as lateral infection and targeting specificity. In one embodiment, lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. In one embodiment, the result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. In one embodiment, viral vectors are produced that are unable to spread laterally. In one embodiment, this characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

[065] In one embodiment, various methods can be used to introduce the expression vector of the present invention into cells. Such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

[066] In some embodiments, introduction of nucleic acid by viral infection offers several advantages over other methods such as lipofection and electroporation, since higher transfection efficiency can be obtained due to the infectious nature of viruses.

[067] In one embodiment, it will be appreciated that the proinsulin of the present invention can also be expressed from a nucleic acid construct administered to the individual employing any suitable mode of administration, described hereinabove (i.e., in-vivo gene therapy). In one embodiment, the nucleic acid construct is introduced into a suitable cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the individual (i.e., ex-vivo gene therapy).

[068] In one embodiment, plant expression vectors are used. In one embodiment, the expression of a proinsulin coding sequence is driven by a number of promoters. In some embodiments, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV [Brisson *et al.*, Nature 310:511-514 (1984)], or the coat protein promoter to TMV [Takamatsu *et al.*, EMBO J. 6:307-311 (1987)] are used. In another embodiment, plant promoters are used such as, for example, the small subunit of RUBISCO [Coruzzi *et al.*, EMBO J. 3:1671-1680 (1984); and Brogli *et al.*, Science 224:838-843 (1984)] or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B [Gurley *et al.*, Mol. Cell. Biol. 6:559-565 (1986)]. In one embodiment, constructs are introduced into plant cells using Ti plasmid, Ri plasmid, plant viral vectors, direct DNA transformation, microinjection, electroporation and other techniques well known to the skilled artisan. See, for example, Weissbach & Weissbach [Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463 (1988)]. Other expression systems such as insects and mammalian host cell systems, which are well known in the art, can also be used by the present invention.

[069] It will be appreciated that other than containing the necessary elements for the transcription and translation of the inserted coding sequence (encoding the proinsulin), the expression construct of the present invention can also include sequences engineered to optimize stability, production, purification, yield or activity of the expressed proinsulin.

[070] Various methods, in some embodiments, can be used to introduce the expression vector of the present invention into the host cell system. In some embodiments, such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

[071] In some embodiments, transformed cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant proinsulin. In some embodiments, effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. In one embodiment, an

effective medium refers to any medium in which a cell is cultured to produce the recombinant proinsulin of the present invention. In some embodiments, a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. In some embodiments, cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes and petri plates. In some embodiments, culturing is carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. In some embodiments, culturing conditions are within the expertise of one of ordinary skill in the art.

[072] In some embodiments, depending on the vector and host system used for production, resultant proinsulin of the present invention either remain within the recombinant cell, secreted into the fermentation medium, secreted into a space between two cellular membranes, such as the periplasmic space in *E. coli*; or retained on the outer surface of a cell or viral membrane.

[073] In one embodiment, following a predetermined time in culture, recovery of the proinsulin is effected.

[074] In one embodiment, recovering the proinsulin refers to collecting the whole fermentation medium containing the proinsulin and need not imply additional steps of separation or purification.

[075] In one embodiment, proinsulin of the present invention is purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

[076] In one embodiment, to facilitate recovery, the expressed coding sequence can be engineered to encode the proinsulin of the present invention and fused cleavable moiety. In one embodiment, a fusion protein can be designed so that the proinsulin can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the cleavable moiety. In one embodiment, a cleavage site is engineered between the proinsulin and the cleavable moiety and the proinsulin can be released from the chromatographic column by treatment with an appropriate enzyme or agent that specifically cleaves the fusion protein at this site [e.g., see Booth *et al.*, Immunol. Lett. 19:65-70 (1988); and Gardella *et al.*, J. Biol. Chem. 265:15854-15859 (1990)].

[077] In one embodiment, the proinsulin of the present invention is retrieved in "substantially pure" form.

[078] In one embodiment, the phrase "substantially pure" refers to a purity that allows for the effective use of the protein in the applications described herein.

[079] In one embodiment, the proinsulin of the present invention is synthesized using *in vitro* expression systems. In one embodiment, *in vitro* synthesis methods are well known in the art and the components of the system are commercially available.

[080] In some embodiments, the proinsulin is synthesized and purified; its therapeutic efficacy is assayed either in vivo or in vitro. In one embodiment, the biological activities of proinsulin of the present invention can be ascertained using various assays as known to one of skill in the art.

[081] In another embodiment, the proinsulin of the present invention can be provided to the individual *per se*. In one embodiment, the proinsulin of the present invention can be provided to the individual as part of a pharmaceutical composition where it is mixed with a pharmaceutically acceptable carrier.

[082] In another embodiment, "the generation of beta cells" comprises the differentiation of pluripotent cells into beta cells. In another embodiment, "the generation of beta cells" comprises the differentiation of pluripotent cells into cells which produce and/or secrete insulin.

[083] In another embodiment, the invention provides a method of inducing the generation of beta cells in the pancreas in a subject in need thereof, comprising the step of administering to a subject a composition comprising proinsulin, thereby inducing the generation of beta cells in the pancreas in a subject in need thereof. In another embodiment, the invention provides a method of inducing the generation of cells which produce and/or secrete insulin in the pancreas in a subject in need thereof, comprising the step of administering to a subject a composition comprising proinsulin, thereby inducing the generation of cells which produce and/or secrete insulin in the pancreas in a subject in need thereof. In another embodiment, the subject is afflicted with insulin dependent diabetes mellitus.

[084] In another embodiment, inducing the generation of beta cells comprises restoring beta cells in the pancreas. In another embodiment, inducing the generation of beta cells comprises restoring beta cells in the pancreas in a subject suffering from diabetes. In another

embodiment, inducing the generation of beta cells comprises de-novo inducing the generation of beta cells in the pancreas in a subject. In another embodiment, inducing the generation of beta cells comprises de-novo inducing the differentiation of pancreatic pluripotent cells to beta cells. In another embodiment, inducing the generation of beta cells comprises de-novo inducing the differentiation of ES cells to beta cells. In another embodiment, inducing the generation of beta cells comprises inducing endogenous production and secretion of insulin in a subject.

[085] In another embodiment, the invention provides a method of treating insulin dependent diabetes mellitus in a subject, comprising the step of administering to a subject a composition comprising proinsulin, thereby treating insulin dependent diabetes mellitus in a subject. In another embodiment, the invention provides a method for inducing endogenous production and secretion of insulin in said subject, comprising the step of administering to a subject a composition comprising proinsulin. In another embodiment, treating insulin dependent diabetes mellitus in a subject comprises inducing endogenous production and secretion of insulin in a subject. In another embodiment, treating insulin dependent diabetes mellitus in a subject comprises de-novo induction of endogenous production and secretion of insulin in a subject.

[086] In another embodiment, treating insulin dependent diabetes mellitus in a subject comprises curing a subject afflicted with insulin dependent diabetes mellitus. In another embodiment, treating insulin dependent diabetes mellitus in a subject comprises reversing insulin production and/or secretion insufficiency. In another embodiment, treating insulin dependent diabetes mellitus in a subject comprises repopulating the pancreas with new beta cells derived from pluripotent cells. In another embodiment, treating insulin dependent diabetes mellitus in a subject comprises repopulating the pancreas with new beta cells derived from pancreatic pluripotent cells. In another embodiment, treating insulin dependent diabetes mellitus in a subject comprises repopulating the pancreas with new beta cells derived from ES cells. In another embodiment, treating insulin dependent diabetes mellitus in a subject comprises in-vivo repopulating the pancreas with new beta cells derived from intact, live, pancreatic pluripotent cells. In another embodiment, treating insulin dependent diabetes mellitus in a subject comprises repopulating the pancreas with new beta cells derived from ex-vivo induction pluripotent cells. In another embodiment, treating insulin dependent diabetes mellitus in a subject comprises repopulating the pancreas with new beta cells derived from in-vivo induction pluripotent cells.

[087] In another embodiment, the invention provides a method of preventing, treating, or abrogating symptoms of diabetes in a subject. In another embodiment, the invention provides a method of preventing, treating, or abrogating symptoms of diabetes related to vascular deterioration in a subject. In another embodiment, the invention provides a method of de-novo induction of endogenous production and secretion of C-peptide in a subject. In another embodiment, the invention provides a method of preventing, treating, or abrogating neuropathy. In another embodiment, the invention provides a method of preventing, treating, or abrogating diabetes induced neuropathy.

[088] In another embodiment, the invention provides a method of de-novo induction of endogenous production and secretion of amylin in a subject. In another embodiment, the invention provides a method of inducing glycemic control by amylin. In another embodiment, the invention provides a method of preventing, treating, or abrogating diabetes induced neuropathy. In another embodiment, the invention provides a method of preventing increase in food intake as amylin decreases food intake in the short term. In another embodiment, the invention provides a method of preventing increase in glucose intake in the stomach and the small intestine as amylin decreases glucose intake in the stomach and the small intestine in the short term.

[089] In another embodiment, provided herein a composition comprising the proinsulin as described herein. In another embodiment, provided herein a pharmaceutical composition comprising the proinsulin as described herein. In another embodiment, a therapeutically effective amount of a proinsulin is determined according to factors as the exact type of condition being treated, the condition of the patient being treated, as well as the other ingredients in the composition. In another embodiment, a therapeutically effective amount of a proinsulin is 0.05-1.00 unit of proinsulin per kg body weight in a pharmaceutical composition. In another embodiment, a therapeutically effective amount of a proinsulin is 0.05-1.00 unit of proinsulin per kg body weight in a pharmaceutical composition administered at least once a day. In another embodiment, a pharmaceutical composition comprising a proinsulin is formulated at strength effective for administration by various means to a human patient.

[090] In another embodiment, the proinsulin of the invention is in a composition. In another embodiment, the proinsulin of the invention is in a pharmaceutical composition. In another embodiment, a "pharmaceutical composition" refers to a preparation of the proinsulin described herein with other chemical components such as physiologically suitable carriers and excipients. In another embodiment, a "pharmaceutical composition" refers to a preparation of the

proinsulin and other active diabetes therapeutic ingredients with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

[091] In another embodiment, any of the compositions of this invention will comprise at least proinsulin in any form. In one embodiment, the present invention provides combined preparations. In one embodiment, "a combined preparation" defines especially a "kit of parts" in the sense that the combination partners as defined above can be dosed independently or by use of different fixed combinations with distinguished amounts of the combination partners i.e., simultaneously, concurrently, separately or sequentially. In some embodiments, the parts of the kit of parts can then, e.g., be administered simultaneously or chronologically staggered, that is at different time points and with equal or different time intervals for any part of the kit of parts. The ratio of the total amounts of the combination partners, in some embodiments, can be administered in the combined preparation. In one embodiment, the combined preparation can be varied, e.g., in order to cope with the needs of a patient subpopulation to be treated or the needs of the single patient whose different needs can be due to a particular disease, severity of a disease, age, sex, or body weight as can be readily made by a person skilled in the art.

[092] In another embodiment, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which can be interchangeably used, refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered proinsulin. An adjuvant is included under these phrases. In one embodiment, one of the ingredients included in the pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a biocompatible polymer with a wide range of solubility in both organic and aqueous media (Mutter et al. (1979).

[093] In another embodiment, "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient/ proinsulin. In one embodiment, excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

[094] Techniques for formulation and administration of drugs are found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

[095] In another embodiment, suitable routes of administration, for example, include oral, rectal, transmucosal, transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

[096] In another embodiment, the preparation is administered in a local rather than systemic manner, for example, via injection of the preparation directly into a specific region of a patient's body.

[097] Various embodiments of dosage ranges are contemplated by this invention. The dosage of proinsulin of the present invention, in one embodiment, is in the range of 0.005-100.0 mg/day. In another embodiment, the dosage is in the range of 0.005-5.0 mg/day. In another embodiment, the dosage is in the range of 0.01-50 mg/day. In another embodiment, the dosage is in the range of 0.1-20.0 mg/day. In another embodiment, the dosage is in the range of 0.1-10.0 mg/day. In another embodiment, the dosage is in the range of 0.01-5 mg/day. In another embodiment, the dosage is in the range of 0.001-0.01 mg/day. In another embodiment, the dosage is in the range of 0.001-0.10 mg/day. In another embodiment, the dosage is in the range of 0.1-5 mg/day. In another embodiment, the dosage is in the range of 0.5-50 mg/day. In another embodiment, the dosage is in the range of 0.2-15. 0 mg/day. In another embodiment, the dosage is in the range of 0.8-65 mg/day. In another embodiment, the dosage is in the range of 1-50 mg/day. In another embodiment, the dosage is in the range of 5-10 mg/day. In another embodiment, the dosage is in the range of 8-15 mg/day. In another embodiment, the dosage is in a range of 10-20.0 mg/day. In another embodiment, the dosage is in the range of 20-40 mg/day. In another embodiment, the dosage is in a range of 60-120 mg/day. In another embodiment, the dosage is in the range of 12-40 mg/day. In another embodiment, the dosage is in the range of 40-60 mg/day. In another embodiment, the dosage is in a range of 50-100mg/day. In another embodiment, the dosage is in a range of 1-60 mg/day. In another embodiment, the dosage is in the range of 15-25 mg/day. In another embodiment, the dosage is in the range of 5-10 mg/day. In another embodiment, the dosage is in the range of 55-65 mg/day.

[098] In another embodiment, proinsulin is formulated in an intranasal dosage form. In another embodiment, proinsulin is formulated in an injectable dosage form. In another embodiment, proinsulin is administered to a subject in a dose ranging from 0.0001 mg to 0.6 mg. In another embodiment, proinsulin is administered to a subject in a dose ranging from 0.001 mg to 0.005 mg. In another embodiment, proinsulin is administered to a subject in a dose ranging from 0.005 mg to 0.01 mg. In another embodiment, proinsulin is administered to a

subject in a dose ranging from 0.01 mg to 0.3 mg. In another embodiment, proinsulin is administered to a subject in a dose ranging from 0.2 mg to 0.6 mg.

[099] In another embodiment, proinsulin is administered to a subject in a dose ranging from 1-100 micrograms. In another embodiment, proinsulin is administered to a subject in a dose ranging from 10-80 micrograms. In another embodiment, proinsulin is administered to a subject in a dose ranging from 20-60 micrograms. In another embodiment, proinsulin is administered to a subject in a dose ranging from 10-50 micrograms. In another embodiment, proinsulin is administered to a subject in a dose ranging from 40-80 micrograms. In another embodiment, a proinsulin is administered to a subject in a dose ranging from 10-30 micrograms. In another embodiment, proinsulin is administered to a subject in a dose ranging from 30-60 micrograms.

[0100] In another embodiment, proinsulin is administered to a subject in a dose ranging from 0.2 mg to 2 mg. In another embodiment, proinsulin is administered to a subject in a dose ranging from 2 mg to 6 mg. In another embodiment, proinsulin is administered to a subject in a dose ranging from 4 mg to 10 mg.

[0101] In another embodiment, the daily dosage of proinsulin for a 70kg human subject is about 7.5mg. In another embodiment, the daily dosage of proinsulin for a 70kg human subject is about 6-8 mg. In another embodiment, the daily dosage of proinsulin for a 70kg human subject is about 0.1-9 mg.

[0102] In another embodiment, proinsulin is injected into the muscle (intramuscular injection). In another embodiment, proinsulin is injected below the skin (subcutaneous injection). In another embodiment, proinsulin is injected intravenously.

[0103] Oral administration, in one embodiment, comprises a unit dosage form comprising tablets, capsules, lozenges, chewable tablets, suspensions, emulsions and the like. Such unit dosage forms comprise a safe and effective amount of proinsulin of the invention as described herein. The pharmaceutically-acceptable carriers suitable for the preparation of unit dosage forms for peroral administration are well-known in the art. In some embodiments, tablets typically comprise conventional pharmaceutically-compatible adjuvants as inert diluents, such as calcium carbonate, sodium carbonate, mannitol, lactose and cellulose; binders such as starch, gelatin and sucrose; disintegrants such as starch, alginic acid and croscarmelose; lubricants such as magnesium stearate, stearic acid and talc. In one embodiment, glidants such as silicon dioxide can be used to improve flow characteristics of the powder-mixture. In one embodiment, coloring agents, such as

the FD&C dyes, can be added for appearance. Sweeteners and flavoring agents, such as aspartame, saccharin, menthol, peppermint, and fruit flavors, are useful adjuvants for chewable tablets. Capsules typically comprise one or more solid diluents disclosed above. In some embodiments, the selection of carrier components depends on secondary considerations like taste, cost, and shelf stability, which are not critical for the purposes of this invention, and can be readily made by a person skilled in the art.

[0104] In one embodiment, the oral dosage form comprises predefined release profile. In one embodiment, the oral dosage form of the present invention comprises an extended release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form of the present invention comprises a slow release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form of the present invention comprises an immediate release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form is formulated according to the desired release profile of the pharmaceutical active ingredient as known to one skilled in the art.

[0105] Peroral compositions, in some embodiments, comprise liquid solutions, emulsions, suspensions, and the like. In some embodiments, pharmaceutically-acceptable carriers suitable for preparation of such compositions are well known in the art. In some embodiments, liquid oral compositions comprise from about 0.001% to about 0.933% of proinsulin, or in another embodiment, from about 0.01% to about 10 %.

[0106] In some embodiments, compositions for use in the methods of this invention comprise solutions or emulsions, which in some embodiments are aqueous solutions or emulsions comprising a safe and effective amount of proinsulin and optionally, other compounds, intended for topical intranasal administration. In some embodiments, the compositions comprise from about 0.001% to about 10.0% w/v of proinsulin, more preferably from about 00.1% to about 2.0%, which is used for systemic delivery of the compounds by the intranasal route.

[0107] In another embodiment, the pharmaceutical compositions are administered by intravenous, intra-arterial, or intramuscular injection of a liquid preparation. In some embodiments, liquid formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In one embodiment, the pharmaceutical compositions are administered intravenously, and are thus formulated in a form suitable for intravenous administration. In another embodiment, the pharmaceutical compositions are administered intra-arterially, and are thus formulated in a form suitable for intra-arterial administration. In another embodiment, the pharmaceutical compositions

are administered intramuscularly, and are thus formulated in a form suitable for intramuscular administration.

[0108] In another embodiment, pharmaceutical compositions of the present invention are manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

[0109] In another embodiment, pharmaceutical compositions for use in accordance with the present invention is formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of proinsulin into preparations which can be used pharmaceutically. In one embodiment, formulation is dependent upon the route of administration chosen.

[0110] In one embodiment, injectables, of the invention are formulated in aqueous solutions. In one embodiment, injectables, of the invention are formulated in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. In some embodiments, for transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[0111] In another embodiment, the preparations described herein are formulated for parenteral administration, e.g., by bolus injection or continuous infusion. In some embodiments, formulations for injection are presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. In some embodiments, compositions are suspensions, solutions or emulsions in oily or aqueous vehicles, and contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

[0112] The compositions also comprise, in some embodiments, preservatives, such as benzalkonium chloride and thimerosal and the like; chelating agents, such as edetate sodium and others; buffers such as phosphate, citrate and acetate; tonicity agents such as sodium chloride, potassium chloride, glycerin, mannitol and others; antioxidants such as ascorbic acid, acetylcystine, sodium metabisulfite and others; aromatic agents; viscosity adjustors, such as polymers, including cellulose and derivatives thereof; and polyvinyl alcohol and acid and bases to adjust the pH of these aqueous compositions as needed. The compositions also comprise, in some embodiments, local anesthetics or other actives. The compositions can be used as sprays, mists, drops, and the like.

[0113] In some embodiments, pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of proinsulin, in some embodiments, are prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include, in some embodiments, fatty oils such as sesame oil, or synthetic fatty acid esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions contain, in some embodiments, substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. In another embodiment, a suspension also contains suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

[0114] In another embodiment, proinsulin is delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez- Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid).

[0115] In another embodiment, the pharmaceutical composition delivered in a controlled release system is formulated for intravenous infusion, implantable osmotic pump, transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump is used (see Langer, supra; Sefton, CRC Crit. Ref. *Biomed. Eng.* 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used. In yet another embodiment, a controlled release system can be placed in proximity to the therapeutic target, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, supra, vol. 2, pp. 115-138 (1984)). Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

[0116] In some embodiments, proinsulin is in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use. Compositions are formulated, in some embodiments, for atomization and inhalation administration. In another embodiment, compositions are contained in a container with attached atomizing means.

[0117] In some embodiments, pharmaceutical compositions suitable for use in context of the present invention include compositions wherein proinsulin is contained in an amount effective to achieve the intended purpose. In some embodiments, a therapeutically effective amount means an amount of proinsulin effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

[0118] In one embodiment, determination of a therapeutically effective amount is well within the capability of those skilled in the art.

[0119] Some examples of substances which can serve as pharmaceutically-acceptable carriers or components thereof are sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose, and methyl cellulose; powdered tragacanth; malt; gelatin; talc; solid lubricants, such as stearic acid and magnesium stearate; calcium sulfate; vegetable oils, such as peanut oil, cottonseed oil, sesame oil, olive oil, corn oil and oil of theobroma; polyols such as propylene glycol, glycerine, sorbitol, mannitol, and polyethylene glycol; alginic acid; emulsifiers, such as the Tween™ brand emulsifiers; wetting agents, such sodium lauryl sulfate; coloring agents; flavoring agents; tableting agents, stabilizers; antioxidants; preservatives; pyrogen-free water; isotonic saline; and phosphate buffer solutions. The choice of a pharmaceutically-acceptable carrier to be used in conjunction with the compound is basically determined by the way the compound is to be administered. If the subject compound is to be injected, in one embodiment, the pharmaceutically-acceptable carrier is sterile, physiological saline, with a blood-compatible suspending agent, the pH of which has been adjusted to about 7.4.

[0120] In addition, the compositions further comprise binders (e.g. acacia, cornstarch, gelatin, carbomer, ethyl cellulose, guar gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, povidone), disintegrating agents (e.g. cornstarch, potato starch, alginic acid, silicon dioxide, croscarmelose sodium, crospovidone, guar gum, sodium starch glycolate), buffers (e.g., Tris-HCl., acetate, phosphate) of various pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), protease inhibitors, surfactants (e.g. sodium lauryl sulfate), permeation enhancers, solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite, butylated hydroxyanisole), stabilizers (e.g. hydroxypropyl cellulose, hyroxypropylmethyl cellulose), viscosity increasing agents(e.g. carbomer, colloidal silicon dioxide, ethyl cellulose, guar gum), sweeteners (e.g. aspartame, citric acid), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), lubricants (e.g. stearic acid, magnesium stearate, polyethylene glycol, sodium lauryl sulfate), flow-aids (e.g. colloidal silicon dioxide), plasticizers (e.g. diethyl phthalate, triethyl citrate), emulsifiers (e.g. carbomer, hydroxypropyl cellulose, sodium lauryl sulfate), polymer coatings (e.g., poloxamers or poloxamines), coating and film forming agents (e.g. ethyl cellulose, acrylates, polymethacrylates) and/or adjuvants.

[0121] Typical components of carriers for syrups, elixirs, emulsions and suspensions include ethanol, glycerol, propylene glycol, polyethylene glycol, liquid sucrose, sorbitol and water. For a suspension, typical suspending agents include methyl cellulose, sodium carboxymethyl cellulose, cellulose (e.g. Avicel™, RC-591), tragacanth and sodium alginate; typical wetting agents include lecithin and polyethylene oxide sorbitan (e.g. polysorbate 80). Typical preservatives include methyl paraben and sodium benzoate. In another embodiment, peroral liquid compositions also contain one or more components such as sweeteners, flavoring agents and colorants disclosed above.

[0122] The compositions also include incorporation of the active material into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts.) Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance.

[0123] Also comprehended by the invention are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors.

[0124] In some embodiments, compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline. In another embodiment, the modified compounds exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds. In one embodiment, modifications also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. In another embodiment, the desired *in vivo* biological activity is achieved by the administration of such polymer-compound abducts less frequently or in lower doses than with the unmodified compound.

[0125] In some embodiments, preparation of effective amount or dose can be estimated initially from in vitro assays. In one embodiment, a dose can be formulated in animal models and such information can be used to more accurately determine useful doses in humans.

[0126] In one embodiment, toxicity and therapeutic efficacy of proinsulin described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. In one embodiment, the data obtained from these *in vitro* and cell culture assays and

animal studies can be used in formulating a range of dosage for use in human. In one embodiment, the dosages vary depending upon the dosage form employed and the route of administration utilized. In one embodiment, the exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. [See e.g., Fingl, et al., (1975) "The Pharmacological Basis of Therapeutics", Ch. 1 p.1].

[0127] In one embodiment, depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

[0128] In another embodiment, the amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

[0129] In one embodiment, compositions including the preparation of the present invention are formulated in a compatible pharmaceutical carrier, placed in an appropriate container, and labeled for treatment of an indicated condition as described herein.

[0130] In another embodiment, a proinsulin as described herein is administered via systemic administration. In another embodiment, a proinsulin as described herein is lyophilized (i.e., freeze-dried) preparation in combination with complex organic excipients and stabilizers such as nonionic surface active agents (i.e., surfactants), various sugars, organic polyols and/or human serum albumin. In another embodiment, a pharmaceutical composition comprises a lyophilized proinsulin as described in sterile water for injection. In another embodiment, a pharmaceutical composition comprises a lyophilized proinsulin as described in sterile PBS for injection. In another embodiment, a pharmaceutical composition comprises a lyophilized proinsulin as described in sterile 0.9% NaCl for injection.

[0131] In another embodiment, the pharmaceutical composition comprises a proinsulin as described herein and complex carriers such as human serum albumin, polyols, sugars, and anionic surface active stabilizing agents. See, for example, WO 89/10756 (Hara et al.- containing polyol and p-hydroxybenzoate). In another embodiment, the pharmaceutical composition comprises a proinsulin as described herein and lactobionic acid and an acetate/glycine buffer. In another embodiment, the pharmaceutical composition comprises a proinsulin as described herein and amino acids, such as arginine or glutamate that increase the solubility of interferon compositions in water. In another embodiment, the pharmaceutical

composition comprises a lyophilized proinsulin as described herein and glycine or human serum albumin (HSA), a buffer (e.g., acetate) and an isotonic agent (e.g., NaCl). In another embodiment, the pharmaceutical composition comprises a lyophilized proinsulin as described herein and phosphate buffer, glycine and HSA.

[0132] In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein is stabilized when placed in buffered solutions having a pH between about 4 and 7.2. In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein is stabilized with an amino acid as a stabilizing agent and in some cases a salt (if the amino acid does not contain a charged side chain).

[0133] In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein is a liquid composition comprising a stabilizing agent at between about 0.3% and 5% by weight which is an amino acid.

[0134] In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein provides dosing accuracy and product safety. In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein provides a biologically active, stable liquid formulation for use in injectable applications. In another embodiment, the pharmaceutical composition comprises a non-lyophilized proinsulin as described herein.

[0135] In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein provides a liquid formulation permitting storage for a long period of time in a liquid state facilitating storage and shipping prior to administration.

[0136] In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein comprises solid lipids as matrix material. In another embodiment, the injectable pharmaceutical composition comprising proinsulin as described herein comprises solid lipids as matrix material. In another embodiment, the production of lipid microparticles by spray congealing was described by Speiser (Speiser and al., Pharm. Res. 8 (1991) 47-54) followed by lipid nanopellets for peroral administration (Speiser EP 0167825 (1990)). In another embodiment, lipids, which are used, are well tolerated by the body (e. g. glycerides composed of fatty acids which are present in the emulsions for parenteral nutrition).

[0137] In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein is in the form of liposomes (J. E. Diederichs and al., Pharm./nd. 56 (1994) 267- 275).

[0138] In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein comprises polymeric microparticles. In another embodiment, the injectable pharmaceutical composition comprising a proinsulin as described herein comprises polymeric microparticles. In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein comprises nanoparticles. In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein comprises liposomes. In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein comprises lipid emulsion. In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein comprises microspheres. In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein comprises lipid nanoparticles. In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein comprises lipid nanoparticles comprising amphiphilic lipids. In another embodiment, the pharmaceutical composition comprising a proinsulin as described herein comprises lipid nanoparticles comprising a drug, a lipid matrix and a surfactant. In another embodiment, the lipid matrix has a monoglyceride content which is at least 50% w/w.

[0139] In one embodiment, compositions of the present invention are presented in a pack or dispenser device, such as an FDA approved kit, which contain one or more unit dosage forms containing the active ingredient. In one embodiment, the pack , for example, comprise metal or plastic foil, such as a blister pack. In one embodiment, the pack or dispenser device is accompanied by instructions for administration. In one embodiment, the pack or dispenser is accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, in one embodiment, is labeling approved by the U.S. Food and Drug Administration for prescription drugs or an approved product insert.

[0140] In one embodiment, it will be appreciated that the proinsulins of the present invention can be provided to the individual with additional active agents to achieve an improved therapeutic effect as compared to treatment with each agent by itself. In another embodiment, measures (e.g., dosing and selection of the complementary agent) are taken to adverse side effects which are associated with combination therapies.

[0141] Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of

the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

[0142] Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference. Other general references are provided throughout this document.

EXAMPLE 1

Evaluation of Glucose-Lowering Efficiency of Human Proinsulin in Intact Rats

[0143] An ability of human proinsulin to decrease plasma glucose level in intact rats was investigated by determining the relationship between human proinsulin dose, plasma glucose concentration and the time after human proinsulin injection. Also, a short-term stability of human proinsulin in the rat bloodstream was evaluated.

[0144] Twenty one intact male autbred Wistar rats were used in this study. All animal studies where conducted upon approval of a local bioethical committee. Animals were housed under ambient temperature and light conditions and allowed free access to standard rodent chow and water. After 2 weeks of acclimatization to animal facility, rats were divided into three groups, comprising 7 rats each: vehicle group, low-dose proinsulin group and high-dose proinsulin group; the mean body weight of animals at that moment was 290 ± 16 g (mean ± SD).

[0145] Prior to beginning of experiment, rats were fasted for approximately 13 hr. Liophilyzed human proinsulin was dissolved in 0.9% NaCl solution (vehicle) to prepare working solutions with concentrations of human proinsulin of 3 μg/ml and 30 μg/ml. Rats of the vehicle group were injected subcutaneously with 1 ml of 0.9% NaCl; rats of the low-dose proinsulin group were injected with approximately 1 ml of 3 μg/ml human proinsulin solution to achieve a dose of human proinsulin equal to 10 μg/kg body weight; rats of the high-dose proinsulin group were injected with approximately 1 ml of 30 μg/ml human proinsulin solution to achieve a dose of human proinsulin equal to 100 μg/kg body weight.

[0146] Blood samples for measurement of glucose and human proinsulin concentrations in plasma were drawn from the tail vein into 1 ml untreated Eppendorf tubes just before injection of vehicle or human proinsulin solutions (0 minute) and at 45 and 180 minutes after injection. Immediately after drawing, blood samples were centrifuged at 12,000 rpm for 1 to 2 minutes, plasma was separated and pipetted into pre-labeled polypropylene tubes. Plasma samples were stored at –70 °C until analysis. Glucose concentrations in plasma samples were measured by in-house glucose oxidase method. Human proinsulin concentrations in plasma samples were measured using the Proinsulin ELISA [DRG Diagnostics, USA/Germany, Cat. # EIA-1560]. Results of measurements were subjected to multiple comparative analyses using one-sided Student's t-test with the Bonferroni correction. All statistical tests were performed with a significance level of 5% ($\alpha = 0.05$). The tests were declared statistically significant if the calculated P-value was ≤ 0.05.

[0147] Results of plasma glucose measurements are shown in FIG. 1 (Twenty one intact male Wistar rats were divided into the 3 groups, 7 animals each: vehicle group, low-dose group, and the high-dose group. After 13-hour fasting period rats from the vehicle group were injected

subcutaneously with 0.9% NaCl, rats from the low-dose group were injected with human proinsulin at a dose of 10 mg/kg body weight, rats from the high-dose group were injected with human proinsulin at a dose of 100 mg/kg body weight. At 0, 45, and 180 minutes after injection blood was drawn from the tail vein, plasma was immediately separated by centrifugation, and glucose concentrations were measured by glucose oxidase method.). As can be seen from the mean glucose concentration-time profiles in FIG. 1, human proinsulin at a dose of 10 μg/kg did not decrease plasma glucose concentration over a time period of 180 min, while human proinsulin at a dose of 100 μg/kg body weight caused statistically significant drop in glucose concentration by 180th minute. Thus, human proinsulin at a dose of 10 μg/kg does not exert glucose-lowering effect, while at a dose of 100 μg/kg it exerts prominent, albeit moderate, glucose-lowering effect in intact rats.

[0148] Results of human proinsulin measurements in the rat plasma are shown in FIG. 2 (Twenty one intact male Wistar rats were divided into the 3 groups, 7 animals each: vehicle group, low-dose group, and the high-dose group. After 13-hour fasting period rats from the vehicle group were injected subcutaneously with 0.9% NaCl, rats from the low-dose group were injected with human proinsulin at a dose of 10 mg/kg body weight, rats from the high-dose group were injected with human proinsulin at a dose of 100 mg/kg body weight. At 0, 45, and 180 minutes after injection blood was drawn from the tail vein, plasma was immediately separated by centrifugation, and human proinsulin concentrations were measured by ELISA.). As can be seen from the FIG. 2, maximal concentrations of human proinsulin are registered at 45th minute after injection; human proinsulin is not completely degraded in the rat bloodstream over a time period of at least 180 minutes after injection. From a physiological standpoint, this time window is sufficient for human proinsulin to exert its biological effects.

EXAMPLE 2

Evaluation of Therapeutic Effect of Human Proinsulin in Rats with Chemically Induced Insulin-Dependent Diabetes

[0149] Study design is shown in FIG. 3. Eighty two intact male autbred Wistar rats were initially included in the study. Body weight of animals at the beginning of the study was 200—250 g. Animals were housed as described above in Example 1. After acclimatization, rats were divided into two groups: the streptozotocin (STZ) group (N = 74) and the control group (N = 8). Before the beginning of experiment, both groups were fasted for 18 hr. In STZ group, diabetes was induced by a single intraperitoneal injection of STZ (Sigma-Aldrich, USA, Cat. # S0130) at a dose of 50 mg/kg body weight. STZ was dissolved in citrate buffer at pH 5.0 just

prior to injection. This induction protocol is widely used in rodent studies to induce mild to moderate insulin-dependent diabetes resembling type 1 diabetes mellitus in humans. Control rats received injections of citrate buffer. To assess diabetes development, capillary blood glucose levels were monitored. Capillary blood was taken from foot pad. Glucose concentrations were measured using a glucometer One-Touch Select (Lifescan Inc., USA). Diabetes was diagnosed when random blood glucose concentration was equal to or exceeded 7 mmol/l.

[0150] In rats who developed diabetes, insulin therapy was initiated and adjusted so as to prevent severe hyperglycemia. Several types of human recombinant insulin and human insulin analogs were tried. Finally, human recombinant NPH insulin (Protaphane HM, Novo Nordisk) was selected. Typically, animals in the STZ group received 1—2 injections of NPH insulin daily (at approximately 9:00 a. m. and 9:00 p. m.). Total daily dose of NPH insulin ranged between 2 and 10 IU/kg body weight as indicated by blood glucose concentrations. Insulin was injected subcutaneously into the left subscapular region using pen injector (NovoPen 3, Novo Nordisk). The control group received injections of 0.9% NaCl. Diabetes induction period lasted 37 days. During the diabetes induction period, rats were allowed free access to chow and water. Blood glucose concentrations were periodically measured in the morning and/or in the evening. Over the diabetes induction period, 13 rats died of severe hyperglycemia or of acute hypoglycemia. Thus, 61 rats were available for further treatment.

[0151] These 61 rats were randomly distributed into three treatment groups: insulin alone group (IA group, N = 8), insulin + low-dose proinsulin group (I/LpI group, N = 26) and insulin + high-dose proinsulin group (I/HpI group, N = 27). IA group received monotherapy with NPH insulin as described above. I/LpI group received combination therapy with NPH insulin plus one daily injection of human proinsulin at a dose of 10 μg/kg. I/HpI group received combination therapy with NPH insulin plus one daily injection of human proinsulin at a dose of 100 μg/kg. Human proinsulin was dissolved in 0.9% NaCl as described in Example 1 and injected subcutaneously into the right subscapular region using 1-ml syringe between 5:30 p. m. and 9:30 p.m. Control group received injections of 0.9% NaCl. The treatment period lasted 24 days. During the treatment period, rats were allowed free access to chow and water, unless experimental goals dictated otherwise. The treatment regimens described above were temporary changed when necessary. During the treatment period, blood glucose concentrations were measured in the morning or in the evening. Over the treatment period, 5 rats died of acute hypoglycemia. The treatment period was followed by the washout period of 14 days. During the washout period, all groups received no treatment and were allowed free access to chow and

water. Non-fasting blood glucose concentrations were measured on the last day of the washout period. Upon completion of the washout period, animals were sacrificed.

[0152] After completion of experiment, the data obtained from blood glucose measurements, performed during the treatment and the washout periods, were subjected to descriptive analysis. Descriptive analysis included calculation of individual mean ± SD blood glucose concentrations over the treatment and the washout periods (not shown) and plotting individual blood glucose concentration versus time charts for each rat (not shown). On the basis of the descriptive analysis results, we performed a *post hoc* selection of subgroups of rats in each treatment group, in whom mean blood glucose concentrations were equal to or exceeded 8 mmol/l and random blood glucose concentrations measured over the treatment and the washout periods, never exceeded 26 mmol/l. This interval was chosen on the assumptions that (i) in rats with mean blood glucose concentrations less than 8 mmol/l diabetes may have been spontaneously resolved; (ii) severe hyperglycemia (> 26 mmol/l) completely suppresses compensatory mechanisms in endocrine pancreas, including beta-cell regeneration. Numbers of animals in IA, I/LpI and I/HpI treatment subgroups were 4, 12 and 10, respectively. Data from blood glucose measurements in the treatment subgroups and in the control group were subjected to statistical analysis as described above in Example 1. Results are presented in figures 4-9.

[0153] Results of non-fasting blood glucose measurements on the 1st day of the treatment period are shown in FIG. 4. As can be seen from FIG. 4, rats in all three treatment subgroups have developed marked hyperglycemia. There were no significant differences among all three treatment subgroups. At the same time, blood glucose concentrations in each of the treatment subgroups differed significantly from the blood glucose concentration in the control group ($P < 0.05$).

[0154] Results of non-fasting blood glucose measurements on the 8th day of the treatment period are shown in FIG. 5. As can be seen from FIG. 5, blood glucose concentration in the IA subgroup was clearly higher than in I/LpI subgroup and I/HpI subgroup ($P < 0.05$). Blood glucose concentrations in I/LpI subgroup and I/HpI subgroup were very similar and differed significantly from the blood glucose concentration in the control group ($P < 0.05$).

[0155] Results of fasting blood glucose measurements on the 10th day of the treatment period are shown in FIG. 6. Rats were fasted for 30 hr before blood glucose measurements. All treatment subgroups did not receive either insulin or proinsulin injections on a previous day. As can be seen from FIG. 6, blood glucose concentration in the IA subgroup was significantly

higher than in I/LpI subgroup, I/HpI subgroup and control group ($P < 0.05$). Blood glucose concentrations in I/LpI subgroup and I/HpI subgroup were similar and did not differ significantly from the blood glucose concentration in the control group ($P > 0.05$).

[0156] Results of fasting blood glucose measurements on the 18th day of the treatment period are shown in FIG. 7. Rats were fasted for 30 hr before blood glucose measurements. All treatment subgroups did not receive either insulin or proinsulin injections on a previous day. As can be seen from FIG. 7, blood glucose concentration in the IA subgroup was significantly higher than in I/LpI subgroup, I/HpI subgroup and control group ($P < 0.05$). Blood glucose concentrations in I/LpI subgroup and I/HpI subgroup were similar and did not differ significantly from the blood glucose concentration in the control group ($P > 0.05$).

[0157] Results of fasting blood glucose measurements on the 24th day of the treatment period are shown in FIG. 8. Rats were fasted for 30 hr before blood glucose measurements. All treatment subgroups did not receive either insulin or proinsulin injections over 2 previous days. As can be seen from FIG. 8, blood glucose concentration in the IA subgroup was significantly higher than in I/LpI subgroup, I/HpI subgroup and control group ($P < 0.05$). Blood glucose concentrations in I/LpI subgroup and I/HpI subgroup were similar and did not differ significantly from the blood glucose concentration in the control group ($P > 0.05$).

[0158] Results of non-fasting blood glucose measurements on the 14th day of the washout period are shown in FIG. 9. As can be seen from FIG. 9, blood glucose concentration in the IA subgroup was significantly higher than in I/LpI subgroup, I/HpI subgroup and control group ($P < 0.05$). Blood glucose concentrations in I/LpI subgroup and I/HpI subgroup were similar and did not differ significantly from the blood glucose concentration in the control group ($P > 0.05$).

EXAMPLE 3

Proinsulin induces the differentiation of Pancreatic Pluripotent cells into pancreatic Beta Cells of the Langerhans Islets

Induction of loss of beta cells

[0159] Streptozocin (Zanosar®), is a naturally occurring chemical produced by the bacterium Streptomyces achromogenes, that is specifically toxic to the insulin-producing beta cells of the pancreas in mammals. Streptozocin was used to produce an animal model for type 1 diabetes in a group of rats.

Animals

[0160] 100 male rats of the Winstar and Sprague-Dawley breeds weighing 150 to 180 g were fed with mixed fodder and water ad libitum and kept under controlled temperature (20-25 °C) and light cycle of 12 hrs.

Treatment

[0161] 75mg/ kg body weight of Streptozocin in a single or multiple injection dose was used to induce the insulin-dependent diabetes in rats. This type of induced diabetes provides an accurate model to diabetes type 1 in humans.

[0162] Single intravenous or intraperitoneal introduction of such dose of Streptozocin causes insulin-depended diabetes in rats, by clinical and laboratory signs similar to human type 1 diabetes. Proof hyperglycemia (glucose level in blood from a tail vein -15mmol/l during the period since the 4-th to the 9-th day after the introduction of Streptozocin was assessed using a glucometer) serves as criterion of the diagnosis of a diabetes.

[0163] Stable hyperglycemia was confirmed by measurements of glucose levels in blood, taken from the tail vein and measured with a glucometer, being higher than or equal to 15 mmol/L for the period of 4-9 days after injection of Streptozocin- indication of diabetes type 1.

[0164] A group of 72 rats was induced with insulin-dependent diabetes and subdivided into two groups of 36 rats each, as follows: Group I: having glucose level in the range of 15-25 mmol/L; Group II: having glucose level higher than 25 mmol/L.

[0165] Starting from the day on which the diabetes type 1 was confirmed, the rats of both groups received the replacement insulin-therapy in a dose of 0.5 Unit per day of the human medium-term insulin.

Randomization

[0166] Groups 1 and 2 were further subdivided into three groups, each consisting 12 animals: Subgroups 1-LD and 2-LD – treated with low dose of proinsulin; Subgroups 1-HD and 2-HD treated with high dose of proinsulin; Subgroups 1-C and 2-C control subgroups.

[0167] After randomization, the rats of subgroups 1-LD, 2-LD, 1-HD and 2-HD received an injection of proinsulin (synthetic preparation) for 4 weeks. Subgroups 1-LD and 2-LD received 10 ng of proinsulin/day. Subgroups 1-HD and 2-HD received 50 ng of proinsulin/day. Subgroups 1-C and 2-C were injected with a physiological solution (control).

[0168] Clinical conditions of diabetes, such as weight loss, water consumption and diuresis, and the level of blood glucose at 1, 2, 3 and 4 weeks after the initial injection of proinsulin were compared between the subgroups.

[0169] The level of endogenic insulin in blood serum was measured at 2 and 4 weeks after the initial injection of proinsulin in all the subgroups.

Proinsulin effects

[0170] In group 1: no insulin dependence with preservation of normal glycemia in 40% of rats which received 10ng proinsulin a day.

[0171] In group 2: no insulin dependence with preservation of normal glycemia in 10% of rats which received 10ng proinsulin a day.

[0172] In group 1: no insulin dependence with preservation of normal glycemia in 90 % of rats which received 50 ng proinsulin a day.

[0173] In group 2: no insulin dependence with preservation of normal glycemia in 70 % of rats which received 50 ng proinsulin a day.

[0174] In the control group: Rats have not recovered. Once insulin treatment was ended all rats died.

Evaluation of beta cells content in pancreas

[0175] At 2 and 4 weeks after initial injection of proinsulin, 4-6 rats from each subgroup were sacrificed and their pancreas was extracted.

[0176] Segments of pancreatic tissue were treated with collagenase and exposed to protease. Beta cells content in each cell suspension was evaluated using flow cytofluorometry, measuring auto-fluorescence of beta cells. The rest of the extracted pancreas tissue has been used for morphologic immuno-histochemical (IHC) research, particularly for revealing the insulin containing cells, and the cells bearing the markers for differentiating the beta-cells antecedents.

Pro-insulin toxicology

[0177] Proinsulin toxicology, immunologic and allergenic features according to international standard has been performed on male rats of the Winstar and Sprague-Dawley breeds of 150-180 g. Also, the mortal dose of proinsulin has been determined.

[0178] These examples demonstrate the ability of human proinsulin at a doses of 10 μg/kg to 100 μg/kg to improve the course of streptozotocin-induced diabetes in rats with mild to moderate hyperglycemia receiving insulin therapy, and document sustained decrease in glucose levels caused by addition of human proinsulin to treatment regimen.

[0179] Antidiabetic effects of human proinsulin cannot be attributed solely to its direct glucose-lowering action, given the facts that (i) human proinsulin at a dose of 10 μg/kg does not exert glucose-lowering action in intact rats, while human proinsulin at a dose of 100 μg/kg exerts only moderate glucose-lowering action in intact rats (see Example 1); (ii) glucose-lowering effect of human proinsulin in diabetic rats is not dose-dependent; (iii) antidiabetic effect of human proinsulin persists for 2 weeks after discontinuation of proinsulin treatment.

[0180] This data provide evidence that proinsulin induces pancreatic beta-cell regeneration, increasing the beta-cell mass enough to achieve normoglycemia without the replacement insulinotherapy.

[0181] Additional results indicated that proinsulin induces the differentiation of the primary embryonic pancreatic cells into beta-cells (insulin secreting cells).

What is claimed is:

1. A method of inducing the generation of insulin secreting cells in a subject, comprising the step of administering to said subject from 0.05 to 1.00 unit of proinsulin per kg body weight in a pharmaceutical composition, thereby inducing the generation of beta cells.

2. The method of claim 1, wherein said subject is suffering from a deficiency in insulin secretion.

3. The method of claim 1, whereby said inducing the generation of insulin secreting cells is in a pancreas.

4. The method of claim 1, whereby said inducing the generation of insulin secreting cells is inducing the differentiation of pancreatic pluripotent cells into beta cells.

5. The method of claim 1, whereby said insulin secreting cells further secrete C-peptide, produce Amylin, or any combination thereof.

6. The method of claim 1, wherein said proinsulin is a recombinant proinsulin.

7. The method of claim 1, whereby said subject is afflicted with insulin dependent diabetes mellitus.

8. The method of claim 1, whereby said subject is a human subject and said proinsulin is a human proinsulin.

9. The method of claim 1, whereby said administering to said subject from 0.05 to1.00 unit of proinsulin per kg body weight in a pharmaceutical composition comprises administering to said subject from 0.05 to1.00 unit of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to breakfast, administering to said subject from 0.05 to1.00 unit of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to lunch, administering to said subject from 0.05 to1.00 unit of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to dinner, and administering to said subject from 0.05 to1.00 unit of proinsulin per kg body weight in a pharmaceutical composition about 1 hour prior to bedtime.

10. A method of treating insulin dependent diabetes mellitus in a subject, comprising the step of administering to said subject from 0.05 to1.00 unit of proinsulin per kg body weight in a

pharmaceutical composition, thereby treating insulin dependent diabetes mellitus in a subject.

11. The method of claim 10, whereby said treating insulin dependent diabetes mellitus is inducing the generation of insulin secreting cells in a pancreas.

12. The method of claim 11, whereby said inducing the generation of insulin secreting cells is inducing the differentiation of pancreatic pluripotent cells into beta cells.

13. The method of claim 11, whereby said insulin secreting cells further secrete C-peptide, produce Amylin, or any combination thereof.

14. The method of claim 10, wherein said proinsulin is a recombinant proinsulin.

15. The method of claim 10, whereby said subject is a human subject and said proinsulin is a human proinsulin.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 10/00877

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/28; C07K 14/62 (2011.01)
USPC - 514/5.9
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)
USPC: 514/5.9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/336; 514/6.1-6.7 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic Database Searched: PUBWEST (USPT,PGPUB,EPAB,JPAB), Google. Search Terms Used induc$, insulin secreting cell, proinsulin, adminis$ proinsulin, Amylinm diabet$, adminis$, beta cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2003/0113305 A1 (Osborne et al.) 19 June 2003 (19.06.2003) entire document esp. Fig 4; para [0008], [0024]-[0025], [0033], [0046]-[0047], [0049], [0059], [0063], [0108], [0131], [0134]	1-15
Y	US 2002/0107198 A1 (Thule) 8 August 2002 (08.08.2002) entire document	1-15
Y	US 2007/0020237 A1 (Yoon et al.) 25 January 2007 (25.01.2007) entire document	1-15
Y	Solvason et al. Improved Efficacy of a Tolerizing DNA Vaccine for Reversal of Hyperglycemia through Enhancement of Gene Expression and Localization to Intracellular Sites. The Journal of Immunology, 2008, pp 8298-8307	1-15

☐ Further documents are listed in the continuation of Box C. ☐

* 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	Date of mailing of the international search report
29 January 2011 (29.01.2011)	14 FEB 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)