

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
28 December 2006 (28.12.2006)

PCT

(10) International Publication Number
WO 2006/136374 A2

- (51) International Patent Classification: Not classified
- (21) International Application Number: PCT/EP2006/005912
- (22) International Filing Date: 20 June 2006 (20.06.2006)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
05013255.4 20 June 2005 (20.06.2005) EP
05015366.7 14 July 2005 (14.07.2005) EP
05022528.3 14 October 2005 (14.10.2005) EP
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- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SY, 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, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— without international search report and to be republished upon receipt of that report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: USE OF GIP AND/OR VITAMIN D3 ANALOGUES THEREOF FOR ENHANCING STEM OR PROGENITOR CELL DIFFERENTIATION INTO INSULIN PRODUCING CELLS

(57) Abstract: This invention relates to the use of GIP (Gastric Inhibitory Polypeptide or Glucose Dependent Insulinotropic Polypeptide) and/or of vitamin D3 (cholecalciferol or 5,7-cholestadien-3- β -ol) and analogues thereof, to initiate or promote the differentiation of stem or progenitor cells into insulin producing cells. Such cells are useful for the therapy of pancreatic autoimmune disorders, e.g. type I diabetes or LADA, or diabetes type II.



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Use of GIP and/or vitamin D₃ analogues thereof for enhancing stem or progenitor cell differentiation into insulin producing cells

Description

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This invention relates to the use of GIP (Gastric Inhibitory Polypeptide or Glucose Dependent Insulinotropic Polypeptide) or of protease resistant GIP analogues and/or of vitamin D₃ (cholecalciferol or 5,7-cholestadien-3- β -ol) and analogues thereof, to promote differentiation of stem or progenitor cells into insulin producing cells. Cell preparations obtained by this method are useful for the treatment of diabetes, e.g. type I diabetes or LADA, or diabetes type II.

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Pancreatic beta-cells secrete insulin in response to elevated blood glucose levels. Insulin amongst other hormones plays a key role in the regulation of the fuel metabolism. For example, it leads to the storage of glycogen and triglycerides and to the synthesis of proteins. Furthermore, the entry of glucose into muscles and adipose cells is stimulated by insulin. In patients who suffer from diabetes mellitus type I or LADA (latent autoimmune diabetes in adults, Pozzilli & Di Mario, 2001, Diabetes Care. 8:1460-67) beta cells are being destroyed due to autoimmune attack. Consequently, the amount of insulin produced by the remaining pancreatic islets is too low, resulting in elevated blood glucose levels (hyperglycemia). In diabetes mellitus type II liver and muscle cells lose their ability to respond to normal blood insulin levels (insulin resistance). High blood glucose levels (and also high blood lipid levels) in turn lead to an impairment of beta cell function and to an increase in beta cell death. Interestingly, the rate of beta cell neogenesis and replication does not appear to increase in type II diabetics, thus causing a reduction in total beta cell mass over time. Thus, the application of exogenous insulin becomes necessary in many type II diabetics.

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In type I diabetics, where beta cells are being destroyed by autoimmune attack, treatments have been devised which modulate the immune system

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and may be able to stop or strongly reduce islet destruction (Raz et al., 2001, Lancet 358: 1749-1753; Chatenoud et al., 2003, Nat Rev Immunol. 3: 123-132; Homann et al., Immunity. 2002, 3:403-15). However, due to the relatively slow regeneration of human beta cells such treatments can be enhanced if they are combined with agents that can stimulate beta cell regeneration.

Diabetes is a very disabling disease, because today's forms of insulin therapy and common anti-diabetic drugs do not control blood sugar levels well enough to completely prevent the occurrence of high and low blood sugar levels. Frequently elevated blood sugar levels are toxic and cause long-term complications like for example nephropathy, retinopathy, neuropathy and peripheral vascular disease. Extensive loss of beta cells also leads to deregulation of glucagon secretion from pancreatic alpha cells which contributes to an increased risk of dangerous hypoglycemic episodes. There is also a host of related conditions, such as obesity, hypertension, heart disease and hyperlipidemia, for which persons with diabetes are substantially at risk.

Apart from the impaired quality of life for the patients, the treatment of diabetes and its long term complications presents an enormous financial burden to our healthcare systems with rising tendency. Thus, for the treatment of diabetes mellitus type I and LADA but also for the treatment of late stages of diabetes mellitus type II, there is a strong need in the art to identify factors that induce regeneration of pancreatic insulin producing beta cells. These factors could restore normal function of the endocrine pancreas and/or prevent or delay the development or progression of diabetes type I or LADA, but also of late stage diabetes type II.

The technical problem underlying the present invention was to provide for means and methods for treating insulin-dependent diabetes such as type I diabetes or LADA but also diabetes mellitus type II (particularly late stages). The solution to said technical problems is achieved by providing the

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embodiments characterized in the claims.

The differentiation of beta cells from embryonic stem cells can serve as a model for the stem cell-based regeneration of the same cell type in the adult organism. Being able to generate cells with beta cell character from stem cells also opens possibilities for improved cell based therapies of diabetes, e.g. to generate beta cell replacement material which could be transplanted into diabetic patients.

Beta cell replication has recently been described as the major mechanism of beta cell formation after birth in mice (Dor et al., Nature. 2004 429(6987):41-6). However, in similar experiments, very different results have been obtained by another group (Seymour et al., J Anat. 2004 204(2):103-16). Beta cell replication appears to be significantly less frequent in humans than in rodents, even in situations of increased need for beta cells such as obesity (Butler et al., Diabetes. 2003 Jan;52(1):102-10). A number of mechanisms of beta cell formation not based on beta cell replication has been described in the literature (Lechner and Habener, Am J Physiol Endocrinol Metab. 2003 284(2):E259-66; Bonner-Weir and Sharma, J Pathol. 2002 197(4):519-26).

From a practical point of view both replicative and stem or progenitor-based mechanisms of beta cell formation would be useful if means to stimulate them can be identified. Therefore, for the identification of regeneration factors different assays modelling both routes are required. While this is comparably straightforward for beta cell replication the situation is more difficult for the various adult stem or progenitor cell types as they are available only as primary cells in limiting quantities. This makes them unsuitable for drug screening even if the relevance of one of them could be confirmed (Kim et al., Dev Dyn. 2004 230(1):1-11; Suzuki et al., Diabetes. 2004 53(8):2143-52; Seaberg et al., Nat Biotechnol. 2004 22(9):1115-24; Hess et al., Nat Biotechnol. 2003 21(7):763-70; Guz et al., Endocrinology. 2001 142(11):4956-68; Rومان et al., Diabetes. 2002 51(3):686-90;

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Lechner and Habener, Am J Physiol Endocrinol Metab. 2003 284(2):E259-66).

5 Transdifferentiation of pancreatic duct cells into endocrine cells has played a major role in the literature about beta cell regeneration for many years. Unfortunately, the published *in vitro* procedures are lengthy, inefficient and difficult to reproduce (Bonner-Weir et al., PNAS 2000 97(14):7999-8004; Gao et al., Diabetes. 2003 52(8):2007-15) and lineage tracing experiments
10 remain to be published. Similarly, findings describing the use of human and rodent duct cell lines as model systems for the formation of beta cells from duct cells have turned out to be poorly reproducible (Palgi et al., Mol Cell Endocrinol. 2000 165(1-2):41-9; Hardikar et al., 2003 PNAS 100(12):7117-22). Therefore, because of these problems, there is a strong need in the art
15 for useful *in vitro* systems which can model the postnatal formation of beta cells from stem or progenitor cells *in vitro*.

It can be assumed that during the formation of beta cells from adult stem or progenitor cells and during embryogenesis similar control mechanisms are
20 used. Thus, factors which stimulate the formation of beta cells from an embryonic, undifferentiated cell type are likely to be able to stimulate beta cell formation from stem or progenitor cells in the adult. Mouse embryonic stem cells represent an attractive choice in this context since they are available in a non-variable, undifferentiated state in unlimited quantities. In
25 addition, due to the pluripotent nature of these cells generation of either desired cell type should be feasible if the conditions are optimized.

In this invention, we disclose that beta cells can be generated from progenitor cells, e.g. from embryonic stem (ES) cells, upon administration of
30 GIP (Glucose-dependent Insulinotropic Polypeptide) or a GIP analogue. The differentiated beta cells are useful for improved cell based therapies of diabetes, in particular e.g. for generating replacement material for destroyed beta cells of pancreatic patients which could be transplanted into these

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patients.

Further, we disclose a novel and so far unknown use for the vitamin D₃ to stimulate the formation or regeneration of insulin producing beta cells and thus, a use in the treatment and prevention of diabetes.

A protocol for the generation of insulin-producing cells from mouse ES-cells based on the selection of nestin-positive progenitors has recently been developed (described in WO 02/086107; Blyszczuk et al., 2003, PNAS 100 (3):998-1003). Cells generated using this protocol express insulin and are C-peptide positive, show glucose and tolbutamide stimulated insulin secretion and exhibit functionality in STZ-transplantation experiments (see, for example, WO 02/086107; Blyszczuk et al., supra; Int J Dev Biol. 2004 48 (10):1095-104).

In order to analyze the effect of various candidate proteins and peptides on stem cell based pancreatic regeneration processes we have studied their effects on the differentiation of mouse ES cells into insulin producing cells. At the end of the differentiation procedure the outcome of the experiment was determined by measuring insulin mRNA levels.

GIP (Gastric inhibitory peptide or Glucose-dependent Insulinotropic Polypeptide) is a 42 amino acid peptide made by K-cells in the gut epithelium. It is secreted into the circulation after meal ingestion. Together with the other major hormone, Glp-1, it stimulates insulin secretion significantly over levels obtained with an equimolar intravenous glucose infusion (see review by Meier & Nauck, 2004 Horm Metab Res. 36(11-12): 859-866).

GIP dose-dependently stimulates the replication of Ins-1 rat insulinoma cells under both low and high glucose conditions at GIP concentrations ≥ 10 nM (Trümper et al., Mol Endocrinol.2001 15:1559-70; Trümper et al., J Endocrinol. 2002 174:233-46; WO 03/082998).

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- GIP has also been described as having a suppressive effect on beta cell death (Wideman and Kieffer, Horm Metab res. 2004 36:782-6). More precisely, GIP suppresses cell death in Ins-1 insulinoma cells (Trümper et al., 2002; supra, WO 03/082898), in dispersed primary beta cells from
5 C57Bl/6 mice and Vancouver diabetic fatty (VDF) rats *in vivo* (Kim et al., J Biol. Chem. 2005 280:22297-307). However, an effect of GIP on embryonic beta cell development or stem cell differentiation into insulin producing cells has not been described.
- 10 Early addition of GIP during embryoid body stages was chosen to study the effects of GIP on stem cell differentiation as opposed to the previously described mitogenic effect on mature beta cells (Trümper et al., Mol Endocrinol. 2001 15(9):1559-70).
- 15 We have detected a strong stimulating effect of GIP on insulin mRNA expression in differentiated ES-cells at the end of the differentiation period, showing that GIP strongly stimulates stem cell differentiation into beta cells. Analysis of insulin expression the last day of GIP addition (at day 5+9, see Examples for more details) did not reveal any detectable levels of insulin
20 expression, which would have indicated the presence of immature or mature beta cells at this stage (see Figures for more details). Also, we have seen an effect of GIP-derived synthetic peptides on insulin secretion. Our finding supports the idea that the stimulation of ES-cell differentiation towards beta cells by GIP is not based on increased replication or improved survival of
25 beta cells but rather represents a novel, so far undescribed activity of GIP. Therefore, GIP or GIP analogues appear to be especially useful for stem cell or progenitor cell based therapy in patients with type I diabetes or LADA or insulin-dependent type II diabetes.
- 30 In an effort to identify protein factors able to stimulate beta cell regeneration we have screened embryonic cDNA libraries for genes that encode novel secreted proteins. During these experiments we have found that GIP is expressed in mouse pancreatic buds at the end of the differentiation period

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(between day e9.5 and day e15). Melton and colleagues found that GIP is specifically expressed in Neurogenin 3+(Ngn3+) cells in the developing mouse pancreas (Gu et al., Development. 2004 131:165-79). In this invention, we disclose the effect of GIP on the *in vitro* differentiation of beta cells from embryonic stem cells. The inventors found that GIP, in contrast to many other factors that are expressed during this developmental time, can strongly stimulate the differentiation into cells with beta cell character. Beta cell formation from stem or progenitor cells has been previously proposed as an important mechanism of postnatal beta cell regeneration in humans (Butler et al., Diabetes. 2003 52:102-10; Bonner-Weir et al., Pediatr Diabetes 2004; 5 Suppl 2:16-22; Lechner and Habener, Am J Physiol Endocrinol Metab. 2003 284:E259-66).

Native GIP has a very short serum half life of 5 minutes in humans, and is therefore not preferred for therapeutic use (Meier and Nauck, Horm Metab Res. 2004 36:859-66). The ubiquitously expressed protease DPP-IV cleaves the peptide bond between amino acids two and three of GIP, resulting in a truncated peptide which can no longer activate the receptor. However, the remaining fragment can still bind to the receptor and thus can act as an antagonist to full length GIP (Deacon, Horm Metab Res. 2004 36(11-12): 761-5; Meier and Nauck, Horm Metab Res. 2004 36:859-66). Modified variants of GIP have been designed which are less prone to DPP-IV-mediated degradation. Examples for such modifications are alterations of amino acids two and three in native GIP or N-terminal modifications of GIP.

Further improvements of the half life can be achieved by attaching fatty acids, poly-ethylene-glycol (PEG) or other moieties to the peptide which reduce renal clearance and/or stabilize against proteolytic degradation (see, for example, Irwine et al., J Med Chem. 2005 48:1244-50; Deacon, Horm Metab Res 2004, 36: 761-5; Meier et al., Diabetes 2004 53:654-62; Harris and Chess, Nat Rev Drug Discov 2003 2:214-21).

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The present invention relates to the use of GIP and analogues thereof, to stimulate or induce differentiation of stem or progenitor cells into insulin producing cells, which can then be used for the treatment of diabetes, more preferably for the treatment of type I diabetes or LADA or insulin-dependent type II diabetes.

The present invention also relates to new methods for stimulating and/or inducing the differentiation of progenitor cells, e.g. stem cells into insulin-producing cells using vitamin D₃.

Vitamin D, a fat soluble vitamin, is well-known for its role in mineral (calcium and phosphorus) homeostasis and maintenance of normal skeletal architecture. It is now becoming increasingly clear that vitamin D is involved in a diverse range of cellular functions including cell differentiation, cell proliferation, immune function etc. Vitamin D, the biological inert moiety, is converted into the hormonally active form, 1 α ,25-dihydroxy-vitamin D₃, (referred to as 1,25(OH)₂D₃, by successive hydroxylation reactions in liver and kidney. This activated form of vitamin D₃ not only plays a central role in bone and calcium metabolism, but also modulates the immune response via specific receptors expressed in APCs and activated T-cells (Bouillon, R., et al., Eur. J. Endocrinol. 1995, 133:7-16). 1,25(OH)₂D₃ and its analogues have been shown to inhibit autoimmune diseases in animal models, such as experimental allergic encephalomyelitis, murine lupus, collagen-induced arthritis and type I diabetes mellitus (Mathieu, C., M. et al., Diabetologia. 1994, 37:552-558). Further, polymorphisms of the vitamin D receptor gene have been associated with type I diabetes mellitus in different populations (Pani, M. A., et al., Diabetes 2000, 49:504-507).

Vitamin D₃ has been shown to preserve the function of *in vitro* cultured pancreatic islet cells for significantly longer periods of time than control cells that have not been treated with vitamin D₃. Based on these results, it is suggested to use vitamin D₃ for the treatment of diabetes (Luca G. et al., Diabetes, nutrition and metabolism, 2000 Dec, 13(6), 301-7). The use of

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vitamin D₃ analogues for the treatment of autoimmune diabetes is also disclosed in WO 02/094247 and in a publication of Nagpal, S. et al. (Vitamin D analogs: Mechanism of action and therapeutic applications, Current Medicinal Chemistry 2001, 8, 1661-1679).

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In a prevention study of autoimmune diabetes in NOD mice, 1,25(OH)₂D₃ reduced the incidence of disease by greater than 90% (Mathieu, C. et al.; Diabetologica, 1994, 37, 552). NOD mouse is an animal model for human autoimmune diabetes, characterized by destruction of the beta-Langerhans cells of the pancreas. A synthetic analog of vitamin D, 20-epi-22-oxa-24,26,27-trishomo-1 α ,25-dihydroxy-vitamin D₃ (kh 1060), also prevented the onset of type I diabetes in NOD mice at non-hypercalcemic doses (Mathieu, C. et al.; Endocrinol., 1995, 136, 866). 1 α ,25-dihydroxy-vitamin D₃ induced a shift in the autoantigen-specific TH (T-helper) cells from a Th1 to a Th2 phenotype. This immune shift appears to be the mechanism underlying the therapeutic action of VDR ligands in NOD mice (Overbergh, L. et al.; Diabetes, 2000, 49, 1301).

EP 0 707 566 describes the use of vitamin D₃ analogues for the preparation of a medicament for therapy and/or prevention of immune disorders, e.g. diabetes mellitus type I, optionally in combination with an immune system interfering drug. Further, the use of said medicament for induction of cell differentiation or any induction of cell proliferation is mentioned. There is, however, no disclosure that vitamin D₃ analogues may stimulate beta cell formation from stem cells.

EP 0 972 762 discloses the use of vitamin D₃ analogues for the preparation of a medicament for inhibiting cell proliferation and/or inducing cell differentiation. Said medicament is intended for treatment and/or prevention of immune disorders such as diabetes mellitus type I, among others. Further, said medicament can be used in combination with other drugs known to interfere with the immune system. There is no indication that treatment with vitamin D₃ analogues stimulates beta cell formation from stem cells.

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US Patent 5,856,189 relates to a method of treating cells by administration of vitamin D₃ analogues and an extracellular matrix. However, there is no hint relating that treatment with vitamin D₃ analogues stimulates beta cell
5 formation from stem cells neither to the treatment of autoimmune or pancreatic disorders by using this method.

WO 2004/044146 describes a method for the *in vitro* induction of cells produced or modified into trans-differentiated organ-specific cells, for
10 example pancreatic islet β -cells, wherein the administration of vitamin D₃ is mentioned. However, the use of vitamin D₃ or vitamin D₃ analogue for the manufacture of a medicament for the prevention and/or treatment of pancreatic and/or autoimmune disorders is not mentioned.

15 The present invention also relates to the use of vitamin D₃ or analogues thereof, to stimulate or induce differentiation of stem or progenitor cells into insulin producing cells, which can then be used for the treatment of diabetes, more preferably for the treatment of type I diabetes or LADA or insulin-dependent type II diabetes.

20 In the present invention, the term „GIP analogue“ preferably relates to linear or cyclic peptidic compounds which comprise at least the 14 N-terminal amino acids of GIP, i.e. Tyr-Ala-Glu-Gly-Thr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met having at least one modification compared to the native sequence.
25 Preferably, the modifications are selected from amino acid substitutions, N-terminal modifications, C-terminal modifications, cyclizations, peptide bond modifications and combinations thereof.

Amino acid substitutions preferably comprise substitutions of at least one
30 amino acid in the N-terminal amino acid sequence of GIP as indicated above by a D-amino acid, and/or by a naturally occurring or non-naturally occurring amino acid having a different side chain, and/or by a non-amino acid chemical moiety which preserves or enhances activity. Especially preferred

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amino acid substitutions are on positions 1, 2, and/or 3 of GIP. Preferably, the GIP analogue contains 1-4 amino acid substitutions.

5 N-terminal and C-terminal modifications preferably comprise modifications of the N- and C-terminal amino group with a modifying group, e.g. an alkyl, acyl, carbohydrate, cholesteryl, lipid or vitamin group, particularly with a hydrophobic, hydrophilic and/or bulky group. N-terminal modifications can also include the following: alkylation, sulphonylation, glycation, homoserine formation, pyroglutamic acid formation, disulphide bond formation,
10 deamidation of asparagine or glutamine residues, methylation, t-butylation, t-butyloxycarbonylation, 4-methylbenzylation, thioanysilation, thiocresylation, benzyloxymethylation, 4-nitrophenylation, benzyloxycarbonylation, 2-nitrobenzoylation, 2-nitrosulphenylation, 4-toluenesulphonylation, pentafluorophenylation, diphenylmethylation, 2-chlorobenzyloxycarbonylation, 2,4,5-trichlorophenylation, 2-bromobenzyloxycarbonylation, 9-fluorenylmethyloxycarbonylation, triphenylmethylation, 2,2,5,7,8-pentamethylchroman-6-sulphonylation, hydroxylation, oxidation of methionine, formylation, acetylation, anisylation,
20 or glutamic acid, phosphorylation, sulphation, cysteinylation, glycolysation with pentoses, deoxyhexoses, hexosamines, hexoses or N-acetylhexosamines, farnesylation, myristoylation, biotinylation, palmitoylation, stearylation, geranylgeranylation, glutathionylation, 5'-adenosylation, ADP-ribosylation, modification with N-glycolylneuraminic acid, N-acetylneuraminic acid, pyridoxal phosphate, lipoic acid,
25 4'-phosphopantetheine, and N-hydroxysuccinimide.

N- and C-terminal modifications may also comprise the addition of hydrophilic groups, basic groups and preferably amino acids and analogues thereof. Preferably, the amino acids and analogues are hydrophilic,
30 preferably basic. Particularly preferred is the amino acid lysine. One or more such groups may be added, preferably 1 to 5, preferably 1 to 4, preferably 1 to 3, preferably 2 to 3, preferably 3. The addition of three lysine residues is

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particularly preferred.

Further, N- and C-terminal modifications include steroids and steroid analogues, e.g. lithocholic acid.

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One or more modifications may be added.

10

Peptide bond modifications preferably comprise introduction of reduced peptide bonds and/or other modifications, e.g. alkylation of the N-atom that is part of the peptide bond. Peptide bond modifications preferably comprise the formation of a carbamate bond, preferably between any two adjacent amino acid positions of GIP(1-14) or between any other two adjacent amino acid positions in the GIP analogue of the invention, preferably any two adjacent amino acid positions 2-5, more preferably between amino acid positions 2 and 3. The number of such modified peptide bonds, in particular carbamate bonds, is preferably from 1-5, more preferably 1-4, more preferably 1-3, more preferably 2, and most preferably 1.

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C-terminal modifications comprise any of the modifying groups mentioned above for N-terminal modifications. Preferably, C-terminal modifications comprise the addition of at least one heterologous amino acid at the C-terminus. For the purposes of the present invention, a heterologous amino acid is one which is not present at the corresponding position in native GIP.

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Preferably, the number of heterologous amino acids or analogues thereof at the C-terminal end is from 1-5, preferably 1-4, more preferably 1-3, in particular 2-3 and most preferably 3. For these additional heterologous amino acids at the C-terminal end, basic and hydrophilic amino acids are preferred, in particular lysine. In particular, 3 lysine residues are preferred. In the case of GIP analogues with C-terminal amino acid modifications, it is preferred that the peptide contains no more than 14 native amino acids from the N-terminus of native GIP. Preferably, the C-terminal modifications are added to the C-terminal position 14 amino acid residue of the GIP analogue.

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Two particularly preferred GIP analogues which are subject to the present invention are the following: GIP(1-14)-KKK-NH₂ (SEQ ID NO: 1) and Ala2-carbamate-GIP(1-14)-NH₂ (SEQ ID NO: 2).

5

Preferably, the GIP analogues have improved resistance against degradation by proteases, particularly against degradation by dipeptidyl-peptidase IV (DP IV).

10 In a second embodiment, the therapeutic agent of the present invention may be a preparation of insulin producing cells generated in the presence of vitamin D₃ or an analogue thereof. Suitable vitamin D₃ analogues are e.g. described by Nagpal, S. et al. (Vitamin D analogs: Mechanism of action and therapeutic applications, Current Medicinal Chemistry 2001, 8, 1661-1679),
15 EP 0 707 566, US Patent No. 5,856,189, WO 04/098522, WO 04/098507, by Qiao, G. et al. (Analogues of 1 α ,25-dihydroxyvitamin D₃ as novel inhibitors of renin biosynthesis, J. Steroid. Biochem. Mol. Biol. 2005, May 5), by Posner, G. H. et al., Bioorg. Med. Chem. 2005 Apr 15; 13(8):2959-66), by van Etten, E. et al. (NOD bone marrow - derived dendritic cells are modulated by analogues of 1,25-dihydroxyvitamin D₃, J. Steroid. Biochem. Mol. Biol. 2004, May, 89-90 (1-5); 457-9) and by Dusso, A. S. et al. (Vitamin D receptor and analogues, Semin. Nephrol. 2004, Jan; 24(1): 10-6), which are
20 herein incorporated by reference. Vitamin D₃ analogues according to the invention are capable of binding the vitamin D receptor (VDR) (Nagpal, S. et al., Current Medicinal Chemistry 2001, 8, 1661-1679) and have vitamin D₃ agonistic activity, e.g. as determined by binding to the vitamin D₃ nuclear hormone receptor and eliciting a transcriptional response corresponding to a partial or full agonistic activity regarding this receptor.

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30 In this invention we show that vitamin D₃ and analogues thereof are capable of promoting the differentiation of insulin-producing cells, particularly pancreatic beta cells.

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The agent could be used in stem cell differentiation protocols aiming to the production of beta cell-like cells in culture. The agent can act as a maturation factor promoting the differentiation of stem cells towards the pancreatic lineage or promoting the growth of differentiated cells.

Thus, the present invention provides methods for treating patients suffering from a pancreatic autoimmune disease caused by, associated with, and/or accompanied by functionally impaired and/or reduced numbers of pancreatic islet cells, particularly insulin producing beta cells, by administering a therapeutically effective amount of differentiated cells as indicated above. Functional impairment or loss of pancreatic islet cells may be due to e.g. autoimmune attack such as in diabetes type I or LADA and/or due to cell degeneration such as in progressed diabetes type II.

Numerous additional aspects and advantages of the invention will become apparent to those skilled in the art upon consideration of the following detailed description of the invention which describes presently preferred embodiments thereof.

In connection with the present invention, the term "progenitor cells" relates to undifferentiated cells capable of being differentiated into insulin producing cells. The term particularly includes stem cells, i.e. undifferentiated or immature embryonic, adult, or somatic cells that can give rise to various specialized cell types. The term "stem cells" can include embryonic stem cells (ES) and primordial germ (EG) cells of mammalian, e.g. human or animal origin. Isolation and culture of such cells is well known to those skilled in the art (see, for example, Thomson et al., (1998) Science 282: 1145-1147; Shambloott et al., (1998) Proc. Natl. Acad. Sci. USA 95: 13726-13731; US 6,090,622; US 5,914,268; WO 00/27995; Notarianni et al., (1990) J. Reprod. Fert. 41: 51-56; Vassilieva et al., (2000) Exp. Cell. Res. 258: 361-373; Guan et al., (2006) Nature 440:1199-1203). Adult or somatic stem cells have been identified in numerous different tissues such as intestine, muscle, bone marrow, liver, and brain. WO 03/023018 describes a novel method for

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isolating, culturing, and differentiating intestinal stem cells for therapeutic use. In the pancreas, numerous reports suggest that stem cells are also present within the adult tissue (Gu and Sarvetnick, (1993) Development 118: 33-46; Bouwens, (1998) Microsc Res Tech 43: 332-336; Bonner-Weir, (2000) J. Mol. Endocr. 24: 297-302).

Embryonic stem cells can be isolated from the inner cell mass of pre-implantation embryos (ES cells) or from the primordial germ cells found in the genital ridges of post-implanted embryos (EG cells). When grown under special culture conditions such as static or rotating culture or hanging drops, both ES and EG cells aggregate to form embryoid bodies (EB). EBs are composed of various cell types similar to those present during embryogenesis. When cultured in appropriate media, EB can be used to generate *in vitro* differentiated phenotypes, such as extraembryonic endoderm, hematopoietic cells, neurons, cardiomyocytes, skeletal muscle, and vascular cells. We have previously described a method that allows EB to efficiently differentiate into insulin-producing cells (as described in WO 02/086107 and by Blyszczuk et al., (2003) Proc Natl Acad Sci USA 100: 998-1003, which are incorporated herein by reference).

In the present invention the term "beta cell regeneration" refers to an at least partial restoration of normal beta cell function by increasing the number of functional insulin secreting beta cells and/or by restoring normal function in functionally impaired beta cells.

Before the present invention is described in detail, it is understood that all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

The data disclosed in this invention shows that the compositions of the invention are useful in diagnostic and therapeutic applications implicated, for example, but not limited to, pancreatic autoimmune disorders. Hence,

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diagnostic and therapeutic uses for the compositions of the invention are, for example but not limited to, the following: (i) tissue regeneration *in vitro* (regeneration for all these tissues and cell types composing these tissues and cell types derived from these tissues), and (ii) research tools.

5

According to this invention the composition may be administered via implantation of treated cells.

10 The compositions of the invention may be administered alone or in combination with another medicament useful to prevent or treat pancreatic disorders or metabolic syndrome, particularly beta cell degeneration. Examples for factors that could be used in a combination therapy are: hormones, growth factors or antioxidants such as GLP-1 and stabilized forms of GLP-1, GLP-1 analogues, DPP-IV inhibitors, nicotinamide, vitamin
15 C, INGAP peptide, TGF-alpha, gastrin, prolactin, members of the EGF-family, or immune modulating agents such as anti-CD3 antibodies, DiaPep277 or anti-inflammatory agents such as Cox2 inhibitors, acetyl-salicylic acid, or acetaminophen. The compositions may be administered in combination with the beta cell regenerating proteins, nucleic acids and effectors/modulators
20 thereof described in PCT/EP2004/007917, e.g. pleiotrophin and agonists thereof, or in PCT/EP2004/013175, PCT/EP2004/013535, PCT/EP 2005/000545, PCT/EP 2005/0017111 and EP 04018751.0, which are herein incorporated by reference.

25 More particularly, the compositions may be administered together with factors with beneficial effects on beta cells such as GLP-1 or derivatives thereof, e.g. GLP-1 (7-36 amide), exendin-4, prolactin or a neurotrophins such as NGF.

30 The compositions are preferably administered together with pharmaceutical agents which have an immunosuppressive activity, e.g. antibodies, polypeptides and/or peptidic or non-peptidic low molecular weight substances.

Preferred examples of immunosuppressive agents are listed in the following Table 1.

5 Table 1: Exemplary agents for immune suppression

Names	Mechanisms
2-amino-1,3-propanediol derivatives	Used for preventing or treating chronic rejection in a patient receiving an organ or tissue allo- or xenotransplant
2-amino-2[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride	Immunosuppression, from accelerated lymphocyte homing
4-thiophenoxy-n-(3,4,5-trialkoxyphenyl) pyrimidine-2-amines	Lck inhibitors
40-O-(2-hydroxyethyl)-rapamycin, SDZ-RAD, Everolimus	Sirolimus (rapamycin) derivative, used for acute kidney rejection; reduces rejection and graft vasculopathy following heart transplantation by inhibiting cell proliferation
6-(3-dimethyl-aminopropionyl) forskolin	Immunosuppressing action useful also for treating autoimmune disease
6-mercaptopurine (6-MP)	Used to treat Crohn's disease, inflammatory bowel disease and for organ transplant therapy
A-420983	Lck-inhibitor
ABX-CBL (CBL-1)	Mouse monoclonal AB targeted against human T-cell, B-cells, NK-cells and monocytes, for treatment of steroid-resistant graft-vs-host diseases, potential use in treatment of inflammatory and autoimmune disorders
Alefacept (human LFA-3 IgG1 fusion protein)	Knocks out causative memory T-lymphocytes; used to treat psoriasis, a T-cell mediated inflammatory disorder
Antisense ICAM-1 inhibitor (ISIS 2302), Enlimomab, BIRR1, Alicaforsen	Mouse monoclonal AB blocks white blood cell adhesion to T-cell surface molecule (ICAM-1r); treatment of kidney transplant rejection
Antithymocyte immunoglobulin (ATGAM)	Anti-human thymocyte, immunoglobulin; used in reversal of acute kidney transplant rejection and will likely be used off-label for transplant induction therapy
Azathioprine	Treatment of rheumatoid arthritis and prevention of kidney transplant rejection, and other autoimmune or inflammatory disorders such as inflammatory bowel disease
Baohuoside-1	Flavonoid; inhibits lymphocyte activation; Ma et al., Transplantation 78:831-838, (2004)

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basiliximab	Monoclonal AB that binds to receptor sites on T-cells, preventing activation by transplanted tissue (renal transplant)
BMS-279700	Lck-inhibitor
BTI-322	Mouse derived monoclonal AB targeted to CD2 receptor; used for prevention of first-time kidney rejection, and treatment of resistant rejection
Cladribine	Antimetabolite and immunosuppressive agent that is relatively selective for lymphocytes; used to treat lymphoid malignancies, e.g. hairy-cell leukemia
CP-690550	JAK-3 inhibitor
Cyclophosphamide (CTX)	Immunosuppressant for treatment of arthritis and other auto-immune disorders and cancers
Cyclosporine (cyclosporin A, cyclosporin)	11 amino acid cyclic peptide; blocks helper T-cell, immunosuppressant used in organ transplant therapy and other immune diseases
Daclizumab, HAT (Humanized Anti-Tac), SMART anti-Tac, anti-CD25, and humanized anti-IL2-receptor	Monoclonal AB inhibits binding of IL-2 to IL-2 receptor by binding to IL-2 receptor; suppresses T-cell activity against allografts (renal transplant)
Dexamethasone (Decadron, Dexone, Dexasone)	An adrenocorticoid, effective immunosuppressant in various disorders
DIAPEP-277	Immunomodulatory properties
DiaMyd peptide	GAD-derived immunomodulatory peptide
Dipeptide Boronic Acid (DPBA)	Proteasome inhibitor; Wu et al., Transplantation 78: 360-366, (2004)
Docosahexaenoic acid (DHA)	Immunosuppressant that lowers the proportion of T-cells expressing CD4 or CD8, blocks antigen recognition process; Taku et al., Journal of Agricultural and Food Chemistry 48: 1047, (2000)
efalizumab	T-cell modulator that target T-cells through interactions with adhesion molecules on endothelial cell surface, target migration of T-cells into the skin and target activation of T-cells; used to treat Psoriasis
Efomycine M	Leukocyte adhesion inhibitor, Anti-inflammatory
FTY720 (oral myriocin derivative)	Alters lymphocyte infiltration into grafted tissues; used for prevention of organ rejection in kidney transplants
GAD-based vaccine/immunomodulator, e.g. from Diamyd company	Prevention and treatment of insulin-dependent diabetes

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Glatiramer acetate (co-polymer-1)	Synthetic peptide copolymer; decoy that mimics structure of myelin so immune cells bind Copaxone instead of myelin; for multiple sclerosis
Glial fibrillary acidic protein (GFAP)	Possesses immunosuppressive activities in diabetic animal models; Winer et al., Nature Medicine 9: 198, (2003)
Gusperimus (15-deoxyspergualin)	Intravenous immunosuppressant; suppresses production of cytotoxic T-cells, neutrophils and macrophages
HLA-B2702 peptide	Human peptide, blocks action of NK cells and T-cell mediated toxicities, used for prevention of first kidney allograft rejection
hu1124(anti-CD11a)	Humanized monoclonal antibody; targets CD11a receptor on surface of T-cells to selectively inhibit immune system rejection of transplanted organs
hOKT31gamma (Ala-Ala)	Non Fc-binding humanized anti CD3 antibody
IBC-VSO1	A synthetic, metabolically inactive form of insulin designed to prevent pancreatic beta cell destruction (vaccine)
IGRP-derived peptides	T-cell modulator
Imatinib (STI571, Glivec or Gleevec)	Lck inhibitor
Infliximab	Monoclonal AB, binds and inactivates human TNFalpha; used to treat Crohn's disease and rheumatoid arthritis
Interferon	Immunomodulatory properties
ISAtx247	Used to treat autoimmune diseases such as rheumatoid arthritis and psoriasis
Isotretinoin	Immunosuppressant, reduces ability of T-cells to proliferate in response to immune challenge. Vergelli et al., Immunopharmacology, 31:191, (1997)
L-683,742: also described as 31-desmethoxy-31-hydroxy-L-683,590	Treatment of autoimmune diseases, infectious diseases and/or prevention of organ transplant rejections
Leflunomide (ARAVA)	Antiinflammatory agent
Medi-500 (T10B9)	Intravenous monoclonal AB that targets human T-cells; treats acute kidney rejection and graft-vs-host disease
Medi-507	Intravenous humanized AB directed against CD2 T-cell; used to treat corticosteroidresistant graft-vs-host disease and prevention of kidney rejection
Methotrexate	Antimetabolite used to treat Crohn's disease, severe psoriasis, and adult rheumatoid arthritis (and as an anti-cancer drug)

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Mitoxantrone	Antiproliferative effect on cellular immune system including T-cells, B-cells and macrophages; used to treat hormone-refractory prostate cancer, acute myelogenous leukemia and multiple sclerosis
Mycophenolate mofetil	Proliferation of T and B lymphocytes by blocking the synthesis of purine nucleotides; used in organ transplant therapy and inflammatory bowel disease
OKT4A	Mouse monoclonal AB targeted against human CD4 T-cell; used for prevention of kidney transplant rejection when used in combination with other immunosuppressant drugs
Oral interferon-alpha (IFN-alpha)	Early onset type 1 diabetes
Muromonab-CD3	Monoclonal AB that binds to receptor sites on T-cells, preventing activation by transplanted tissue
Prednisolone	Corticosteroid, suppresses inflammation associated with transplant rejection
Psora-4	Kv1.3-blocker
Rifampicin	Antibiotic; has immunomodulatory properties
Rituximab	CD20 antibody
S100beta	Possesses immunosuppressive activities in diabetic animal models
Sirolimus, Rapamycin	Immunosuppressant and potent inhibitor of cytokine (e.g. IL-2)-dependent T-cell proliferation (kidney transplant)
Tacrolimus (Prograf, FK-506)	Interferes with IL-2 TCR communication

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Preferred immunosuppressive agents are DiaPep-277, anti-CD3-antibodies such as hOKT31 gamma (Ala-Ala) and GAD peptides such as DiaMyd GAD peptides if stem or progenitor cells not subject of allograft rejection are used (e.g. autologous cells or cells with reduced visibility to the recipients immune system) in patients with autoimmune diabetes. Use of cells derived from non-autologous stem or progenitor cells preferably requires the use of more potent immunosuppressive agents such as mycophenolate mofetil or tacrolimus or other agents appropriate for a use in an organ transplant setting (see table 1) both in autoimmune diabetes or type II diabetes patients. No additional immune suppression or immune modulation is needed if differentiated cells derived from autologous stem or progenitor cells or cells with reduced visibility to the recipients' immune system are administered in patients with type II diabetes.

The combination therapy may comprise coadministration of the medicaments during the treatment period and/or separate administration of single medicaments during different time intervals in the treatment period.

The compositions are also useful for *in vitro* applications for which a pro-differentiation effect on pancreatic beta cells and precursors thereof would be beneficial:

- *In vitro* differentiation of stem cells into beta cells
- *In vitro* transdifferentiation of duct or exocrine cells into beta cells

25

The present invention also relates to methods for differentiating progenitor cells into insulin-producing cells *in vitro* comprising

- (a) activating one or more pancreatic genes in a progenitor, e.g. stem cell (optional step, particularly if embryonic stem cells are used)
- 30 (b) aggregating said cells to form embryoid bodies (optional step, particularly if embryonic stem cells are used)
- (c) cultivating embryoid bodies or cultivating adult stem cells (e.g., duct cells, duct-associated cells, nestin-positive cells) in specific

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differentiation media containing a composition as indicated above under conditions wherein beta cell differentiation is significantly enhanced, and

(d) identifying and selecting insulin-producing cells.

5

Activation of pancreatic genes may comprise transfection of a cell with pancreatic gene operatively linked to an expression control sequence, e.g. on a suitable transfection vector, as described in WO 03/023018, which is herein incorporated by reference. Examples of preferred pancreatic genes
10 are Pdx1, Pax4, Pax6, neurogenin 3 (Ngn3), Nkx 6.1, Nkx 6.2, Nkx 2.2, HB 9, BETA2/Neuro D, Isl 1, HNF1-alpha, HNF1-beta and HNF3 of human or animal origin. Each gene can be used individually or in combination with at least one other gene. Pax4 is especially preferred.

15 Further, the compositions are useful for the modulation, e.g. stimulation, of pancreatic development and/or for the regeneration of pancreatic cells or tissues, e.g. cells having exocrine functions such as acinar cells, centroacinar cells and/or ductal cells, and/or cells having endocrine functions, particularly cells in Langerhans islets such as alpha-, beta-, delta- and/or PP-cells, more
20 particularly beta cells.

Further, the invention relates to a cell preparation comprising differentiated progenitor cells, e.g. stem cells exhibiting insulin production, particularly an insulin-producing cell line obtainable by the method described above. The
25 insulin-producing cells may exhibit a stable or a transient expression of at least one pancreatic gene involved in beta cell differentiation. The cells are preferably human cells that are derived from human stem cells. For therapeutic applications the production of autologous human cells from adult stem cells of a patient is especially preferred. However, the insulin producing
30 cells may also be derived from non-autologous cells. If necessary, undesired immune reactions may be avoided by encapsulation, immunosuppression and/or modulation or due to non-immunogenic properties of the cells.

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The insulin producing cells of the invention preferably exhibit characteristics that closely resemble naturally occurring beta cells. Further, the cells of the invention preferably are capable of a fast response to glucose. After addition of 27.7 mM glucose, the insulin production is enhanced by a factor of at least 2, preferably by a factor of at least 3. Further, the cells of the invention are capable of normalizing blood glucose levels after transplantation into mice.

The invention further encompasses functional pancreatic cells obtainable or obtained by the method according to the invention. The cells are preferably of mammalian, e.g. human origin. Preferably, said cells are pancreatic beta cells, e.g. mature pancreatic beta cells or stem cells differentiated into pancreatic beta cells. Such pancreatic beta cells preferably secrete insulin in response to glucose. Moreover, the present invention may provide functional pancreatic cells that secrete glucagon in response to hypoglycemia. A preparation comprising the cells of the invention may additionally contain cells with properties of other endocrine cell types such as delta-cells and/or PP-cells. These cells are preferably human cells.

The cell preparation of the invention is preferably a pharmaceutical composition comprising the cells together with pharmacologically acceptable carriers, diluents and/or adjuvants. The pharmaceutical composition is preferably used for the treatment or prevention of pancreatic autoimmune diseases, e.g. diabetes mellitus type I or LADA, or diabetes mellitus type II.

According to the present invention, the functional insulin producing cells treated with compositions of the invention may be transplanted preferably intrahepatically, directly into the pancreas of an individual in need, or by other methods. Alternatively, such cells may be enclosed into implantable capsules that can be introduced into the body of an individual, at any location, more preferably in the vicinity of the pancreas, or the bladder, or the liver, or under the skin. Methods of introducing cells into individuals are well known to those of skill in the art and include, but are not limited to, injection, intravenous or parenteral administration. Single, multiple,

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continuous or intermittent administration can be effected. The cells can be introduced into any of several different sites, including but not limited to the pancreas, the abdominal cavity, the kidney, the liver, the celiac artery, the portal vein or the spleen. The cells may also be deposited in the pancreas of the individual.

The methodology for the membrane encapsulation of living cells is familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished without undue experimentation. See, e.g., U.S. Patent Numbers 4,892,538, 5,011,472, and 5,106,627, each of which is specifically incorporated herein by reference. A system for encapsulating living cells is described in PCT Application WO 91/10425 of Aebischer et al., specifically incorporated herein by reference. See also PCT Application WO 91/10470 of Aebischer et al., Winn et al., Exper. Neurol., 113:322-329, 1991, Aebischer et al., Exper. Neurol., 111:269-275, 1991; Tresco et al., ASAIO, 38: 17-23, 1992, each of which is specifically incorporated herein by reference. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible particles or beads and depot injections, are also known to those skilled in the art.

Immunomodulating medicaments, e.g. immunosuppressive drugs, such as the agents listed in Table 1, are preferably administered to the patient in need to reduce the host reaction versus graft. Allografts using the cells obtained by the methods of the present invention are also useful because a single healthy donor could supply enough cells to regenerate at least partial pancreas function in multiple recipients.

The effect of the transplanted insulin producing cells upon the body reverses the condition of diabetes partially or completely. As the subject's blood glucose homeostasis improves, the dosage of insulin administered may be reduced in strength. In at least some cases further administration can be discontinued entirely and the subject continues to produce a normal amount of insulin

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without further treatment. The subject is thereby not only treated but could be cured entirely of a diabetic condition. However, even moderate improvements in beta cell mass can lead to a reduced requirement for exogenous insulin, improved glycemic control and a subsequent reduction in diabetic complications.

Fig. 1 shows the GIP dependent induction of the differentiation of insulin producing cells.

Mouse embryonic stem (ES) cells were differentiated into insulin producing cells as described previously (patent application PCT/EP02/04362, published as WO 02/086107, which is incorporated herein by reference). In differentiated cells, the abundance of insulin mRNA (Fig. 1) was determined using quantitative RT-PCR in two independent experiments. Levels were normalized using 18S RNA and a cycle number of 36 as references. The numbers on the vertical line refer to the abundance of the indicated transcripts relative to an abundance for which 36 cycles are necessary for detection. ,fold expression rel. to delta Ct36' refers to expression of insulin; ,undiff. ES' refers to R1 mouse embryonic stem (ES) cells stably transfected with a CMV-Pax4 expression construct as described in Example 1; ,control' refers to the differentiation protocol as described in Example 2, without any addition of GIP; + GIP' refers to the differentiation protocol as described in Example 4, with the addition of GIP to embryoid bodies. The expression of insulin is shown at different time points: EBs day 5 (end of embryoid body formation), EBs day 5+9 (spontaneously differentiated embryoid bodies shortly before dissociation), and differentiated cells at day 5+33.

Fig. 2 and Fig. 3 show the vitamin D₃ dependent induction of the differentiation of insulin producing cells.

Mouse embryonic stem (ES) cells were differentiated to insulin producing cells as described previously (patent application PCT/EP02/04362, published as WO 02/086107, which is incorporated herein by reference). In

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differentiated cells, the abundance of insulin mRNA (Fig.2) and of beta cell glucose transporter Glut2 mRNA (Fig. 3) was determined using quantitative RT-PCR in two independent experiments. Levels were normalized using 18S RNA as control and a cycle number of 36 as reference. The numbers on the vertical line refer to the abundance of the indicated transcripts relative to an abundance for which 36 cycles are necessary for detection. ,fold expression rel. to delta Ct36' refers to expression of insulin (Fig. 2) and Glut2 (Fig. 3), respectively; ,undiff. ES' refers to R1 mouse embryonic stem (ES) cells stably transfected with a CMV-Pax4 expression construct; ,control' refers to the differentiation protocol as described in Example 2, without any addition of vitamin D₃; ,+ VD3' refers to the differentiation protocol as described in Example 5, with the addition of vitamin D₃ to embryoid bodies. The expression of insulin (Fig. 2) and Glut2 (Fig. 3) is shown at different time points: undifferentiated ES cells, EBs day 5 (end of embryoid body formation), EBs day 5+9 (spontaneously differentiated embryoid bodies shortly before dissociation), and differentiated cells at day 5+33.

The examples illustrate the invention:

Example 1: Generation of ES cells expressing the Pax4 gene.

Mouse R1 ES cells (Nagy et al. (1993) Proc. Natl. Acad. Sci. U S A. 90: 8424-8428) were electroporated with a plasmid expressing the Pax4 gene under the control of the CMV promoter and the neomycin resistance gene under the control of the phosphoglycerate kinase I promoter (pGK-1).

ES cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, 10⁻⁴ M beta-mercaptoethanol, 2 nM glutamine, 1% non-essential amino acids, 1 nM Na-pyruvate, 15% fetal calf serum (FCS) and 500 U/ml leukemia inhibitory factor (LIF). Briefly, approximately 10⁷ ES cells resuspended in 0.8 ml phosphate buffered saline (PBS) were subjected to electroporation with 25 µg/ml of linearized expression vector (Joyner, Gene Targeting: A Practical Approach, Oxford University Press, New York, 1993).

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Five minutes after electroporation, ES cells were plated on petri dishes containing fibroblastic feeder cells previously inactivated by treatment with 100 µg/ml mitomycin C. One day after electroporation, culture medium was changed to medium containing 450 µg/ml G418. Resistant clones were separately isolated and cultured 14 days after applying the selection medium. Cells were always cultured at 37°C, 5% CO₂. These untreated and undifferentiated ES cells were used as control for the experiment shown in Fig. 1, 2 and 3 (referred to as , undiff. ES' in Fig. 1, 2 and 3).

Example 2: Differentiation of ES cells into insulin-producing cells (referred to as ,control' in Fig. 1, 3 and 4)

The ES cell line R1 constitutively expressing Pax4 (Pax4 ES cells) (,undiff. ES' in Fig. 1, 3 and 4) were cultivated as embryoid bodies (EB) by the hanging drop method, as described in patent application PCT/EP02/04362, published as WO 02/086107 and by Blyszczuk et al., 2004, Int. J. Dev. Biol. 48: 1095-1104, which are incorporated herein by reference, with media as described below and in Table 2. The embryoid bodies were allowed to form in hanging drop cultures for 2 days and then transferred for three days to suspension cultures in petri dishes. At day 5, EBs were plated separately onto gelatin-coated 6 cm cell culture dishes containing a differentiation medium prepared with a base of Iscove modified Dulbecco's medium. After dissociation and replating at day 14, cells were cultured up to 40 days in the differentiation medium prepared with a base of Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F12).

Example 3: Expression of pancreas specific genes after differentiation of ES cells into insulin-producing cells

Total RNA from 8x10⁴ cells growing on 4 cm² surface area of a tissue culture dish was extracted using Qiagen RNAeasy kit according to the instructions of the manufacturer (Qiagen) and 2 µg was converted into cDNA. Primers for insulin and 18S RNA were designed using the Primer Express 1.5 Software

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from Applied Biosystems and sequences can be obtained upon request. Quantitative real-time PCR was performed using Applied Biosystems SDS 7000 detection system. Amplifications from 2 independent experiments were performed in duplicate for each transcript and mean values were normalized to the mean value of the reference RNA 18S RNA.

Example 4: Induction of differentiation of insulin-producing cells by GIP (referred to as ,+ GIP' in Fig. 1)

In order to study the effect of GIP to induce beta cell differentiation *in vitro*, we have generated stable mouse embryonic stem (ES) cells expressing Pax4 under control of the cytomegalovirus (CMV) early promoter/enhancer region. Pax4 ES cells were then cultured in hanging drops to allow the formation of embryoid bodies. Embryoid bodies were formed in the presence of 100 nM of GIP. Embryoid bodies were subsequently plated, again GIP was added every other day until day 14 and afterwards enzymatically dissociated, and replated. After dissociation, cells were cultured without GIP in a differentiation medium containing various growth factors (see Table 2 for more detail). GIP was obtained from SIGMA, USA, Order Number G2269. Under such conditions, the expression of insulin was significantly induced by GIP. These data demonstrate that GIP can significantly promote and enhance differentiation of ES cells into insulin-producing cells.

Table 2. Protocol for the induction of differentiation of insulin-producing cells by GIP

Media B2 and B27 supplement, (NA-Niacinamide) are described in Rolletschek et al., 2001, Mech. Dev. 105: 93-104.

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Day	Stage of Cultivation	Medium	Coating and Analysis
0	Hanging drops (600 cells/drop)	Iscove + 20% FCS addition of Glucose- dependent insulino- tropic polypeptide (GIP) 100 µM)	RNA (ES cells)
1			
2	EBs in suspension	Iscove + 20% FCS + GIP, 100 µM	
3			
4			
5	plating of EBs	Iscove + 20% FCS + GIP, 100 µM	gelatin coating RNA (Ebs)
+1	medium change		ornithine/laminin coating
+2			
+3	medium change		
+4			
+5	medium change		Poly-L-Ornithine coating
+6			
+7	medium change		Laminin coating
+8			
+9	dissociation	B2+B27+NA+10% FCS	RNA (1x6 cm dish)
+10	medium change	see above, withdraw FCS	
+11	medium change		
+12			
+13	medium change		
+14			
+15	medium change		
+16			
+17	medium change		
+18			
+19	medium change		
+20			

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+21	medium change		
+22			
+23	medium change		
+24			
+25	medium change		RNA
+26			
+27	medium change		
+28			RNA
+29	medium change		
+30			
+31	medium change		
+32			RNA

Example 5: Induction of differentiation of insulin-producing cells by Vitamin D₃

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In order to study the effect of vitamin D₃ to induce beta cell differentiation *in vitro*, we have generated stable mouse embryonic stem (ES) cells expressing Pax4 under control of the cytomegalovirus (CMV) early promoter/enhancer region (Example 1). Pax4 and wild type ES cells were then cultured in hanging drops or spinner cultures to allow the formation of embryoid bodies. Embryoid bodies were formed in the presence of 10 nM of 1 α -25-Dihydroxycholecalciferol D₃ (vitamin D₃) solution in 95% Ethanol. Embryoid bodies were subsequently plated, again vitamin D₃ was added every second day until day 14 and afterwards enzymatically dissociated, and replated. After dissociation, cells were cultured in a differentiation medium containing various growth factors (see Table 2 for more detail). 1 α -25-Dihydroxycholecalciferol was obtained from SIGMA, USA, Order Number D1530. Under such conditions, the expression of insulin was significantly induced by vitamin D₃ (see Fig. 2). In addition, the addition of vitamin D₃ to the differentiation medium did enhance the expression of the glucose transporter Glut-2 (see Fig. 3). By comparison, wild type ES cells did not contain any insulin-producing cell or Glut-2 expressing cells at the same

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stage. These data demonstrate that vitamin D₃ can significantly promote and enhance differentiation of Pax4-ES cells.

Claims

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1. Use of GIP or a GIP analog and/or vitamin D₃ or a vitamin D₃ analogue and optionally an immunosuppressive agent for the manufacture of a medicament for the protection, survival and/or regeneration of insulin-producing cells, particularly insulin-producing beta cells.

10

2. The use of claim 1 in the prevention and/or treatment of autoimmune diabetes and/or insulin-dependent diabetes type II.

15

3. The use of claim 2, wherein the autoimmune diabetes is type I diabetes and/or LADA (latent autoimmune diabetes in adults).

4. The use of any one of claims 1-3, wherein the medicament comprises a vitamin D₃ or a vitamin D₃ analogue.

20

5. The use of any one of claims 1-3, wherein the medicament comprises GIP or a GIP analogue having an increased in vivo half life.

25

6. The use of any one of claims 1-5 wherein the immunosuppressive agent is an agent as indicated in Table 1, particularly selected from DiaPep-277, anti-CD3-antibodies and/or GAD peptides.

7. The use of any one of claims 1-6 for the promotion of the differentiation of insulin-producing cells, particularly insulin-producing beta cells.

30

8. The use of claim 7 for the generation of beta cells from stem cells, particularly from embryonic stem cells.

9. The use of claim 6 for the generation of beta cells from duct or exocrine pancreatic cells.

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10. The use of any one of claims 1-9, wherein the medicament is for administration to a patient in need thereof.
- 5 11. The use of claim 10 wherein the medicament is for administration to a patient who is to receive or has received a transplantation of pancreatic tissue.
- 10 12. The use of any one of claims 1-9, wherein the medicament is for administration to beta cells or progenitor cells thereof or of islets and/or non-islet pancreatic tissue *ex vivo*.
13. The use of claim 12 for generating replacement material for dysfunctional and/or destroyed beta cells.
- 15 14. The use of claim 12 or 13 for the manufacture of a transplantable beta cell preparation.
15. A GIP analogue selected from the group consisting of synthetic peptides comprising amino acids 1-14 of GIP and one or more of the following:
- 20 (a) at least one carbamate bond,
(b) at least one heterologous amino acid or analogue thereof at the N- and/or C-terminus,
(c) at least one C-terminal modification.
- 25 16. The GIP analogue of claim 15, additionally comprising one or more N-terminal modifications.
- 30 17. The GIP analogue of claim 16, wherein the C- and N-terminal modifications comprise the addition of a modifying group selected from alkyl, acyl, carbohydrate, cholesteryl, lipid or vitamin group, hydrophobic groups, hydrophilic groups, bulky groups, amino acids and analogues thereof, steroids and analogues thereof.

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18. The GIP analogue of claims 15-17, wherein the at least one carbamate bond is between any two adjacent amino acid positions 1-14 of GIP.
19. The GIP analogue of claim 18, wherein the at least one carbamate bond
5 is between any two adjacent amino acid positions 2-5.
20. The GIP analogue of claim 18 or 19, wherein it is Ala2-carbamate-GIP (1-14)-NH₂ (SEQ ID NO: 2).
- 10 21. The GIP analogue of any one of claims 15-20, wherein the at least one heterologous amino acid at the N-and/or C-terminus is selected from basic and hydrophilic amino acids.
22. The GIP analogue of claim 21, wherein the at least one heterologous
15 amino acid at the N- and/or C-terminus is lysine.
23. The GIP analogue of claim 21 or 22, wherein it comprises 1-5 heterologous amino acids at the N- and/or C-terminus.
- 20 24. The GIP analogue of claim 21, 22 or 23, wherein it is GIP(1-14)-KKK-NH₂ (SEQ ID NO: 2).
25. A pharmaceutical composition comprising as the active ingredient a GIP
analogue of any one of claims 15-24.
- 25 26. Use of a GIP or GIP analogue, particularly of a GIP analogue or a composition of any of claims 15-25 and optionally an immunosuppressive agent for the manufacture of a medicament for the prevention and/or treatment of pancreatic autoimmune disorders.
- 30 27. The use of claim 26 for the prevention and/or treatment of autoimmune diabetes.

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28. The use of claim 26 or 27 for the prevention and/or treatment of type I diabetes or LADA (latent autoimmune diabetes in adults) or insulin-dependent type II diabetes.
- 5 29. The use of any one of claims 26-28 wherein the medicament comprises a GIP analogue having an increased *in vivo* half life.
30. The use of claim 29 wherein the GIP analogue has a lowered kidney clearance rate and/or an increased resistance against degradation by
10 proteases.
31. The use of any one of claims 26-30 wherein the GIP analogue has a modification selected from amino acid substitutions, N-terminal modifications, C-terminal modifications, peptide bond modifications and
15 combinations thereof.
32. The use of any one of claims 26-31, wherein the GIP analogue is selected from any one of claims 15-24.
- 20 33. The use of any one of claims 26-32 for the protection, survival and/or regeneration of insulin producing cells, particularly insulin producing beta cells.
- 25 34. The use of any one of claims 26-33 for the promotion of the differentiation of insulin producing cells, particularly insulin producing beta cells.
35. The use of claim 33 or 34 for the generation of beta cells from stem cells, particularly from embryonic stem cells.
30
36. The use of claim 33 or 34 for the generation of beta cells from duct or exocrine pancreatic cells.

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37. The use of any one of claims 26-36 wherein the medicament is for administration to a patient in need thereof.
- 5 38. The use of claim 37 wherein the medicament is for administration to a patient who has received a transplantation of pancreatic tissue.
39. The use of any one of claims 26-38 wherein the medicament is for administration to beta cells or progenitor cells thereof or of islets and/or
10 non-islet pancreatic tissue *ex vivo*.
40. The use of claim 39 for generating replacement material for dysfunctional and/or destroyed beta cells.
- 15 41. The use of any one of claim 39 or 40 for the manufacture of a transplantable beta cell preparation.

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FIG. 1. GIP dependent induction of the differentiation of insulin producing cells

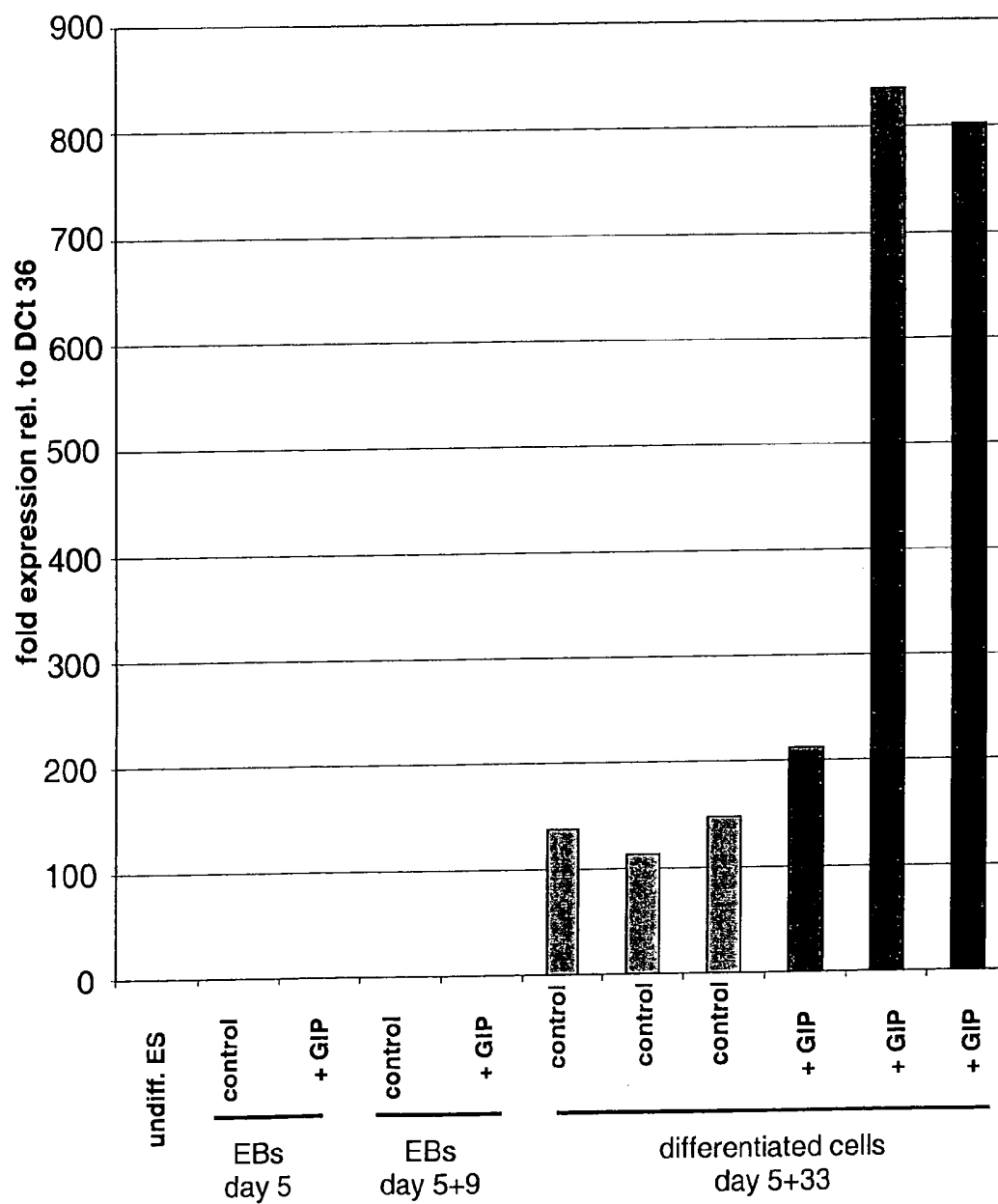


Fig. 2

GLUT 2 expression (qRT-PCR)

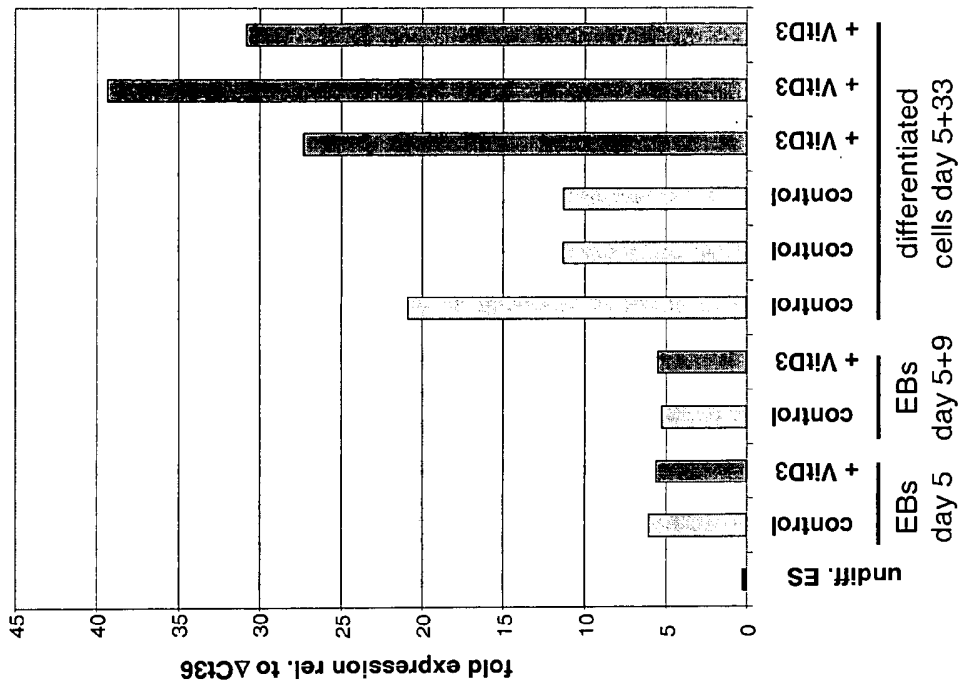


Fig. 3

Insulin expression (qRT-PCR)

