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(54) Title: NON-HUMAN ANIMAL MODEL ENCODING A NON-FUNCTIONAL MANF GENE

(57) Abstract: The present invention provides a gene vector, or a host cell comprising said gene vector, expressing effective amount of a MANF or CDNF polypeptide for use in the prevention or treatment of type 1 or type 2 diabetes. The invention also provides genetically-modified non-human animal comprising a disrupted allele for the gene that naturally encodes and expresses a functional MANF gene.

Non-human animal model encoding a non-functional MANF gene

FIELD OF THE INVENTION

5 The present invention generally relates to the field of treatment of diabetes and more particularly to non-human animal models of diabetes. The present invention also relates to the fields of gene delivery vectors and gene therapy.

BACKGROUND OF THE INVENTION

10

Mesencephalic astrocyte-derived neuroptrophic factor (MANF; also known as arginine-rich protein ARP, or mutated in early stage tumors; ARMET) was first identified as a secreted factor with specific survival effects on cultured embryonic dopaminergic neurons and dorsal root ganglion neurons [1]. MANF and its paralogue CDNF (cerebral dopamine 15 neurotrophic factor) were shown to protect and rescue dopamine neurons in rodent models of Parkinson´s disease (PD) [2,3]. MANF was also shown to protect neurons and promote behavioural recovery in a rat brain ischemia model [4,5]. The mechanisms behind the neuronal protection for these factors have remained obscure but it has been suggested they activate pathways which tries to alleviate oxidative- and endoplasmic (ER) stress and 20 depress apoptotic cell death. Therefore MANF and CDNF are good candidates for the treatment of PD and other neurodegenerative diseases where ER stress is one of the causes leading to neuronal cell death [6].

The mouse Manf protein (179 aa, Id: MGI 1922090) is encoded by 4 small exons in 25 chromosome 9. MANF mRNA and protein are widely expressed in most human and mouse organs with high levels in glandular cells of secretory tissues such as pancreas and salivary gland [7]. Mouse Manf protein (179 amino acids) contains eight conserved cysteine residues with similar spacing forming four intramolecular disulphide bonds and a signal sequence (21 amino acids) for secretion and it is well conserved among species [7,8]. Although MANF 30 is a secreted protein it has been shown to be mostly localized to the luminal ER [7,9-11]. MANF secretion to the culture medium from cardiomyocytes and HeLa cells has been observed after thapsigargin treatment which depletes calcium from ER stores [11]. The same study revealed that MANF interacts with chaperone glucose regulated protein 78 (GRP78) (also known as Bip, a member of heat shock protein 70 kDa family, Hsp70) in a calcium-35 dependent manner. This suggests that MANF secretion is enhanced in pathological

situations involving reduced ER calcium concentrations such as in brain and heart ischemia thus providing a plausible mechanism whereby extracellular MANF could protect cells from death in response to ER calcium depletion.

5 MANF contains a C-terminal sequence (RTDL) closely resembling the classical ER retention signal (KDEL) [9,12]. Interestingly, recent indirect evidence suggest that MANF bind to the KDEL receptor intra-cellular but also on the cell surface after ER stress induction [13]. In addition, crystal structure of MANF revealed an amino-terminal saposin-like domain which may interact with lipids or membranes, and a carboxy-terminal domain 10 containing a CXXC cysteine bridge motif [14]. The C-terminal domain is similar to active site motif of thiol/disulfide oxidoreductases (that catalyze the formation of intramolecular disulphide bonds) suggesting that MANF may be involved in protein folding in the ER. Furthermore, three-dimensional NMR structure revealed that the C-terminal domain of MANF is homologous to the SAP domain of Ku70, a well-known inhibitor of proapototic 15 Bax [15]. MANF is an ER stress inducible protein and maintains the viability of many types of cells under stress [7,9,10,16]. In line with these results, MANF promoter has been found to contain an ER response element (ERSEII) that is recognized by cleaved ATF6 and sXBP1, which are important ER stress inducible transcription factors [10]. Thus, several lines of evidence suggest that MANF is an ER stress regulating protein.

20

The ER is an organelle that contributes to the proper folding and processing of nascent translated proteins destined for secretion. A number of cellular insults including pharmacological perturbation, reduction in ER calcium stores, viral infections, altered protein glycosylation, increased or unbalanced protein expression, can disrupt protein 25 folding and cause accumulation and aggregation of unfolded proteins in the ER lumen causing ER stress and if prolonged, leading to ER stress induced apoptosis [17]. ER stress triggers the activation of the unfolded protein response (UPR), a cellular defence mechanism to alleviate the critical status of the cell by three different pathways; suppression of further protein translation, facilitation of the refolding of unfolded proteins 30 by the induction of ER chaperons and activation of ER-associated degradation of unfolded proteins by the ubiquitin-proteasome pathway (for a review see [18] et al.). This complex cellular response is mediated through three ER transmembrane receptors: pancreatic ER kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) [17,18]. Upon ER stress these transmembrane receptors are 35 activated by dissociating from an ER chaperone, GRP78 which has been sequestered by

unfolded or misfolded proteins [19]. Phosphorylated PERK blocks global mRNA translation by phosphorylating eukaryotic initiation factor 2 α (eIF2alpha) subunit. Transcription factor ATF4 escapes eIF2α translational regulation and in turn induces transcription of pro-survival genes such as redox-balance, amino-acid metabolism and protein folding to
5 restore ER homeostasis [20]. ATF4 mRNA expression has been shown to be induced in by various stress factors and upregulates the expression of transcription factor C/EBP homologous protein (CHOP) which have dual functions. It induces the expression of genes which functions to restore the activity of eIF2α. On the other hand it also promotes the apoptotic response by promoting the transcription of BCL2-interacting mediator of cell
10 death (BIM), and downregulation of BCL-2 expression in non-neuronal cells, leading to cell death.

ATF6 translocates to the Golgi, where it is cleaved by site 1 and site 2 proteases. Active ATF6 (ATF6f) translocates to the nucleus where it induces chaperone genes with ERSE
15 elements in their promoters such as GRP78 and X box-binding protein 1 (XBP1) [21]. It also controls directly genes encoding for components in the ER associated degradation (ERAD). IRE1 contains in its cytoplasmic domain a serine-threonine kinase domain and an endoribonuclease domain. The endoribonuclease activity of IRE1α removes by splicing an intron from XBP1, generating spliced XBP1 (XBP1s) which translates into an active
20 transcription factor activating genes for ERAD and chaperons for protein folding. IRE1α is also actively involved in the cleavage of other ER associated mRNAs such as insulin thus relieving the workload placed on the ER in pancreatic beta cells [22,23]. IRE1α can also activate apoptosis signalling cascade JUN N-terminal kinase (JNK) through apoptosis signal regulating kinase 1 (ASK1) [24].

25

Many pathophysiological conditions including diabetes mellitus and neurodegenerative diseases such as Parkinson´s disease, Alzheimer´s disease (AD) and amyotrophic lateral sclerosis (ALS) are associated with ER stress [6,17,25]. Diabetes mellitus is a group of metabolic disorders where type 1 (T1D, 5-10% of diabetes patients) is characterized by
30 auto-immune destruction of insulin producing pancreatic beta cells leading to insulin deficiency. Type 2 diabetes (T2D, 90-95% of diabetes patients) is characterized by insulin resistance in target cells which in later stages is combined with reduced insulin production. Pancreatic beta cells, producing large quantities of insulin, are especially sensitive to ER stress [26]. Currently increasing evidence indicates that ER stress and prolonged UPR is a
35 major cause of beta cell destruction in both T1D and T2D. Chronic high glucose, fatty

acid exposure and insulin resistance may contribute to unresolved ER stress and beta cell death in T2D whereas in T1D, pro-inflammatory cytokines might through nitric oxide production and ER calcium depletion induce ER stress upregulating UPR and proapoptotic genes [26-28].

5

Importantly, mutations in the PERK gene associated with Wolcott-Rallison syndrome (WRS) result in permanent neonatal diabetes in humans [29] Similarly, *Perk* mutations in mouse recapitulates many of the defects found in human WRS patients including hyperglycemia and growth retardation [30]. Furthermore, gene knockout studies for many of

10 the UPR genes (including *eIF2α, p58*^{IPK}, *Wfs1, Ire1α, Atf4, Atf6α and Xbp1*) result in beta cell loss and subsequent diabetes in mice [18,31]. Interestingly, MANF is up-regulated in the pancreatic beta cells of diabetic Akita mice, where ER stress is caused by the accumulation of proinsulin in the ER [10]. Taken together, functional ER homeotasis has important physiological relevance for beta cells in both rodents and humans.

15

To understand the biological role of MANF we have developed MANF knockout mice (MANF KO). Surprisingly, MANF KO mice develop severe diabetes due to progressive postnatal reduction of beta cell mass caused by decreased beta cell proliferation and increased beta cell apoptosis. UPR genes were up-regulated and proteins were activated in

20 pancreatic islets isolated from MANF knockout mice (MANF KO) suggesting that unresolved ER stress is one cause of the beta cell failure in the MANF KO mice. Importantly, we show that cytokine-induced stress in human islet cells upregulates MANF-expression, suggesting that MANF is involved in ER stress pathways also in human beta cells. Additionally, delivery of MANF by adeno-associated virus serotype 6 into the

25 pancreas specifically enhances beta cell proliferation and protects against beta cell death in a multiple low dose streptozotocin mouse model of diabetes. Furthermore, direct *in vitro* evidence reveals that MANF protein stimulates mouse islet beta cell proliferation. Our results strongly suggest that MANF is a novel growth factor for promoting beta cell proliferation and survival, as well as a novel regulator of UPR *in vivo*. Therefore, MANF

30 will be a promising candidate as a therapeutic agent to be validated for the treatment of diabetes mellitus.

No scientific evidence linking MANF to beta cell apoptosis in pancreatic islets has been previously disclosed. However, biotech companies Generex Biotechnology and Amarantus

35 BioSciences announced in August, 2011, their programs for developing MANF as a

biomarker for beta cell stress and also as a therapeutic agent to be administered by a nasal delivery system for the treatment of diabetes and impaired glucose tolerance.

SUMMARY OF THE INVENTION

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Object of the present invention is to provide a genetically-modified non-human animal comprising a disrupted allele for the gene that naturally encodes and expresses a functional MANF gene, wherein the disrupted allele is a conditionally or non-conditionally non-functional MANF gene and said animal displays progressive postnatal reduction of
10 pancreatic beta cell mass due to the disrupted non-functional MANF gene.

Another object of the present invention is to provide a gene therapy vector expressing effective amount of a MANF polypeptide comprising the sequence of SEQ ID NO:1 or a CDNF polypeptide polypeptide comprising the sequence of SEQ ID NO:3 for use in the
15 intrapancreatic treatment of pancreatic beta cells.

Another object of the invention is to provide an isolated host cell transfected with the gene therapy vector as defined in the invention, wherein said host cell secrets a MANF or CDNF polypeptide, for use in the intrapancreatic treatment of pancreatic beta cells.

20

Another object of the invention is to provide a pharmaceutical composition comprising a gene therapy vector or a host cell as defined in the invention for use in the intrapancreatic treatment of pancreatic beta cells.

25 Another object of the present invention is to provide a method of screening a compound for preventing pancreatic beta cell apoptosis and dysfunction, the method comprising: (a) administering said compound to a non-human animal comprising a disrupted non-functional allele for the gene that naturally encodes and expresses a functional MANF gene; (b) observing the effect of said compound on said animal; and (c) relating the
30 observed effect of said compound on said animal to the beta cell preservation and/or regeneration activity of said compound, wherein a compound showing said preservation and/or regeneration activity is a candidate compound for the treatment of type 1 diabetes.

Another object of the present invention is to provide a method of producing a genetically-
35 modified non-human animal comprising a disrupted non-functional allele for the gene that

naturally encodes and expresses a functional MANF gene, the method comprising: mating non-human animals heterozygous for disruption of the MANF gene to produce a non-human animal homozygous for said MANF disrupted allele and identifying progeny displaying progressive postnatal reduction of pancreatic beta cell mass and in which both
5 alleles of the endogenous MANF gene are disrupted

Another object of the present invention is to provide a method of treating a subject suffering from type 1 or type 2 diabetes, the method comprising: administering by intrapancreatic delivery to the subject a gene vector expressing effective amount of a
10 MANF polypeptide.

Another object of the present invention is to provide a method of treating a subject suffering from type 1 or type 2 diabetes, the method comprising: administering by intrapancreatic delivery to the subject a gene vector expressing effective amount of a
15 CDNF polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D. Generation of *Manf*-knockout mice and characterization of the
20 **mutant allele product.** (A) Schematic illustration of the wild-type and mutant *Manf*-targeted allele. A β-galactosidase reporter cassette (β-gal) with a preceding strong splicing acceptor site (En2SA) was targeted between *Manf*- encoding exon (E) 2 and exon 3 in the *Manf* locus generating a constitutive null *Manf* allele. Frt (F)-sites enable Flp-recombinase excision of the reporter cassette, whereas LoxP (L)-sites enable Cre-excision for
25 conditional removal of exon 3. bAct::Neo; human β-actin promoter driven neomycin resistance gene. Arrowheads indicate priming sites used in genotyping by PCR and in RT-PCR. (B) Example of genotyping result. Genotyping of mice was performed by PCR using primers forward 2 (f2), reverse 2 (r2) and reverse 4 (r4) on genomic DNA isolated from ear-marks. Amplified bands of 547 bp represents wt allele (WT), bands of 323 bp
30 represents the targeted (KO) allele. MWM; molecular weight marker. (C) RT-PCR analysis for *Manf* and *β-galactosidase* mRNA expression in organs from adult WT and KO mice. Primers for *Gapdh* were used to normalize mRNA levels in each sample (D) Western blot analysis of tissue-lysates confirms that Manf protein is not expressed in organs of KO mice. Recombinant human MANF protein (rhMANF) was used as positive
35 control and anti-actin antibody for normalization of total protein content.

Figures 2A-2E. Retarded growth, hyperglycemia and hypoinsulinemia in *Manf*-deficient mice. (**A**) Growth curve of WT and KO littermates. KO mice gain significantly less weight compared to WT mice. The value of each time-point is the average body weight (+/- SEM) of 5-41 mice. Weights for both genders were included from embryonic day (E) 18.5 to postnatal day (P) 2 (enlargement in inset). From P14 to P56 only weights for males are shown. (**B**) *Ad libitum* fed blood glucose levels at P1 and P14 is similar between genotypes. At P56, KO mice display hyperclycemia in contrast to WT animals (n=16-34/group). (**C**) Serum insulin levels from *ad libitum* fed mice indicate hypoinsulinemia in KO animals at P28 and P56 (n=8-21/group). (**D**) In contrast to WT mice, KO mice show impaired capacity to reduce blood glucose levels after glucose (2g/kg) bolus injection (n=4-12/group). (**E**) Serum insulin levels measured from P14 and P56 mice after glucose (2g/kg) bolus injection (n=4-5/group). Data are expressed as average +/- SEM, evaluated using two-tailed Student´s t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Figures 3A-3F. Polydipsia and decreased respiratory exchange rate in *Manf*-deficient mice. (A) Food intake in 6-week-old mice appears similar between genotypes. Data is expressed as an average of two light periods (day) and three dark periods (night). (B) In comparison to WT mice, KO mice display increased water intake. KO mice (C) consume less O_2 and (D) produce less CO_2 and compared to their WT littermates both during day and night. (E) The respiratory exchange ratio during night is decreased in KO mice in comparison to WT animals. (F) Locomotor activity is similar between the genotypes (n=4 males/group). Data are expressed as average +/- SEM, evaluated by one way ANOVA (***$p<0.001$).

Figures 4A-4J. Decline in beta cell mass in *Manf*-deficient mice is caused by reduced beta cell proliferation and enhanced islet beta cell death. Insulin immunohistochemistry on pancreas sections from WT (A, C, E) and KO (B, D, E) animals at P1 (A, B), P14 (C, D) and P56 (E, F). Clear reduction in islet size and loss of islet architecture can be detected in KO pancreas from 2-week-old (D) and 8-week-old (F) animals. Scale bar, 50 μm. (G) Reduction in beta cell mass in MANF KO mice at P1, P14 and at P56 (n=5/genotype). (H) Beta cell proliferation assessed by Ki67 and insulin double-staining. Relative number of Ki67-positive beta cells is equal between genotypes at E18.5. The number of proliferating beta cells in KO mice is reduced compared to WT mice in P1 and P14, respectively (E18.5

n=3/genotype, P1 *n*=5/genotype, P14 *n*=5/genotype, 2000-7500 insulin-positive cells counted). (I) Number of proliferative Ki67-positive nuclei from the pancreatic exocrine tissue was equal in WT and KO pancreas quantified from sections at P1 and P14 (*n*=5-6, 5000-10000 DAPI-stained nuclei counted digitally). (J) Islet cell death is increased in P14 5 and P56 KO compared to WT pancreas assessed by TUNEL and insulin double-staining (*n*=5/genotype, 2000-6800 insulin-positive cells counted). Data are expressed as average +/- SEM, evaluated using two-tailed Student t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Figures 5A-5G. Glucagon immunohistochemistry of pancreas sections from WT and KO 10 mice at E18.5 (A, B), P14 (C, D) and P56 (E, F). Alpha cells are dispersed in the islets of P14 (D) and P56 (F) *Manf*-ko pancreas. However, alpha cell mass (G) is not reduced in KO mice pancreata at any stage analysed (*n*=5 animals/genotype). Results are expressed as average +/- SEM, evaluated using two-tailed Student t-test. No significant differences were found between groups.

15

Figures 6A-6I. Quantitative PCR for mRNA levels of beta cell specific genes in islets from P1 (A), P14 (B) and P56 (C) pancreases. Results are expressed as average +/- SEM, evaluated using two-tailed Student t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Islets were from 4-10 pancreases/group). GLUT2 immunohistochemistry on pancreas sections from 20 WT (D, E, F) and KO (G, H, I) animals at P1 (D, G), P14 (E, H) and P56 (F, I). Scale bar, 35 μm.

Figures 7A-7E. Unfolded protein response genes are upregulated and EIF2α protein 25 phosphorylated in islets from *Manf*-deficient mice. (A and B) Quantitative real-time PCR analysis of *Atf4*, *Grp78*, *Chop*, spliced *Xbp1s*, total *Xbp1t* and *Atf6α* gene expression in wild-type and KO islets isolated from P1 (A) and P14 (B) pancreases. Relative mRNA levels were obtained after normalization to actin mRNA (*n*=4-10/genotype). (C) Western blot of lysates from P14, P28 and P56 islets probed with antibodies against phosphorylated 30 (p)EIF2α, total (t)EIF2α and house-keeping protein Gapdh (Glyceraldehyde 3-phosphate dehydrogenase) from WT and KO pancreases. (D) Intensity of Western blot bands were quantified by Image J software and levels of intensity of phosphorylated (p)EIF2α was compared to total amount of (t)EIF2α and tEIF2a to levels of Gapdh (*n*= 3/genotype). (E) Quantitative RT-PCR analysis of MANF mRNA expression from human islets after 35 cytokine treatment (48 h) (n=4/condition). Abbreviations: IL = interleukin, IFN =

interferon. Data are expressed as average +/- SEM, evaluated using two-tailed Student t-test (*p<0.05, **p<0.01, ***p<0.001).

Figures 8A-8Q. MANF rescues islet size and selectively induces beta cell proliferation in streptozotocin-induced diabetic mice. (A-L) Insulin and MANF immunohistochemistry on pancreatic sections taken 6 weeks after intrapancreatic AAV6-RFP or AAV6-MANF injection of WT mice that either were treated with buffer (A-D), or treated for 5 consecutive days with low dose of STZ (E-L). High levels of MANF can be detected in AAV6-MANF injected pancreas (J-K) suggesting successful viral transduction and expression of AAV6-transferred human MANF cDNA and protein. High levels of MANF-expression can be seen in some islet beta cells but is also present in some exocrine areas (J and K). Equal exposure time was used to take photographs of the insulin stained sections, as well as for all MANF stained sections. Scale bar A-C, E-G and I-K, 100 μm. (D, H, L) Representative immunoperoxidase (POD) stained pancreas sections for insulin antibody show reduced islet sizes in the STZ-injected diabetic AAV-RFP mouse pancreas (H) compared to buffer-injected AAV-RFP control pancreas (D) and AAV-MANF-treated STZ-injected pancreas (L). Scale bar (in D, H, and L), 1 mm. (M) Quantification of islet size in the RFP-Buffer, RFP-STZ and MANF-STZ groups indicate rescued islet size in AAV6-MANF treated group. Each symbol in the graph represents one islet and average islet size/group is shown by horizontal lines. Statistical analysis was performed using GraphPad Prism 5 and data were subjected to Cruskal-Wallis one-way analysis of variance test and differences were evaluated by Dunn´s Multiple Comparison Test (***p<0.001) (n=5-8/group). (N) Beta cell proliferation in AAV6-virus injected non-diabetic and STZ-treated mice assessed by Ki67 and insulin double–staining on pancreata taken 18-19 days after first STZ-injection. The number of Ki67-positive cells relative to the total number of insulin-positive cells was significantly higher in AAV6-MANF-treated animals compared to control STZ-and buffer-injected animals (n=6/group, 4000-5000 insulin-positive cells counted/animal). Results are expressed as average +/- SEM, evaluated using two-tailed Student t-test (**p<0.01, ***p<0.001). (O) The number of proliferative Ki67-positive nuclei from the exocrine tissue were similar in all three experimental groups (n=6/group, 90000-104000 DAPI-stained nuclei digitally counted/animal). (P) Blood glucose levels in AAV6-injected mice 18-19 days after first STZ-or buffer-injection. (Q) Serum insulin levels in AAV6-injected mice 18-19 days after first STZ-or buffer-injection.

Figure 9. MANF recombinant protein increases beta cell proliferation in culture.

Incorporation of Click EdU in beta cells after *in vitro* culturing of mouse islets for 5 days with placental lactogen (PL) or MANF or both (n=3 wells/point). **, $p > 0.01$.

Figures 10A and 10B. (A) Quantification of islet size in the RFP-Buffer, RFP-STZ and CDNF-STZ groups indicate rescued islet size in AAV6-CDNF treated group. Each symbol in the graph represents one islet and average islet size/group is shown by horizontal lines. Statistical analysis was performed using GraphPad Prism 5 and data were subjected to Cruskal-Wallis one-way analysis of variance test and differences were evaluated by Dunn´s Multiple Comparison Test (**$p<0.01$, ***$p<0.001$) (n=6/group). (B) Beta cell proliferation in AAV6-virus injected non-diabetic and STZ-treated mice assessed by Ki67 and insulin double–staining on pancreases taken 18-19 days after first STZ-injection. The number of Ki67-positive cells relative to the total number of insulin-positive cells was significantly higher in AAV6-MANF- and AAV6-CDNF-treated animals compared to control STZ-and buffer-injected animals (n=6/group, 4000-6000 insulin-positive cells counted/animal). Results are expressed as average +/- SEM, evaluated using two-tailed Student t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Figures 11A-11I. Assessment of diabetic phenotype in MANF conditional knockout mice (A) Schematic illustration of the $Manf^{+/+}$wild-type, $Manf^{-/-}$, $Manf^{flox/flox(fl/fl)}$ and Cre::$Manf^{fl/fl}$ targeted locus. β-galactosidase reporter cassette (β-gal), strong splicing acceptor site (En2SA), exon (E), Frt (F)-sites, LoxP (L)-sites, bAct::Neo; human β-actin promoter driven neomycin resistance gene. Arrowheads indicate priming sites used in genotyping by PCR and in RT-PCR. **(B)** RT-PCR analysis for *Manf* mRNA expression in tissues from P56 $Manf^{fl/fl}$ and $PGK^{Cre/+}$::$Manf^{fl/fl}$ mice. $PGK^{Cre/+}$::$Manf^{fl/fl}$ mice lack *Manf* exon 3 generating a shorter non-sense *Manf* mRNA. Primers for *Gapdh* were used to normalize mRNA levels in each sample. **(C-D)** *Ad libitum* fed blood glucose and serum insulin levels at P56-P70. Similarly to $Manf^{-/-}$ mice at the same age, $PGK^{Cre/+}$:: $Manf^{fl/fl}$ mice display hyperclycemia (D) and hypoinsulinemia in contrast to $Manf^{fl/fl}$ animals which were phenotypically indistinguishable from wild-type mice, n = 4-5 per group. **(E-F)** $Pdx1^{Cre/+}$:: $Manf^{fl/fl}$ mice develop hyperglycemia (E) and hypoinsulinemia (F) similarly to $Manf^{-/-}$ mice by P56, n = 9-11 per group. (**G-H**) Blood glucose (G) and serum insulin levels (H) are not changed in random fed 1.25-1.5 year old $Nestin^{Cre/+}$:: $Manf^{fl/fl}$ mice compared to controls, n = 4-5 per group. Mean ± s.e.m., *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus corresponding control. **(I)** Insulin and glucagon immunohistochemistry reveals

decreased intensity of insulin immunoreactivity and loss of islet architecture in *Pdx1*$^{Cre/+}$:: *Manf*$^{fl/fl}$ mice at P56, scale bar, 100 μM.

Figures 12A-12F. MANF is important for growth of body size. (A-B) Body size is
5 decreased in P56 *Manf*$^{-/-}$ mice, WT 9.25 ± 0.1 cm vs. *Manf*$^{-/-}$ 7.8 ± 0.01 cm, *n* = 3-4 mice per group. **(C)** Alike *Manf*$^{-/-}$ mice, *PGK*$^{Cre/+}$:: *Manf*$^{fl/fl}$ mice gain significantly less weight compared to *Manf*$^{fl/fl}$ mice, *n* = 4-5 per group. **(D)** Similarly to *Manf*$^{-/-}$ mice at the same age, *PGK*$^{Cre/+}$:: *Manf*$^{fl/fl}$ mice display gain significantly less weight compared to *Manf*$^{fl/fl}$ animals, *n* = 4-5 per group. **(E-F)** The body weight of pancreas-specific *Pdx1*$^{Cre/+}$:: *Manf*
10 $^{fl/fl}$ (E) or *Nestin*$^{Cre/+}$:: *Manf*$^{fl/fl}$ (F) mice was not statistically different compared to *Manf*$^{fl/fl}$ mice, *n* = 4-5 per group.

DETAILED DESCRIPTION OF THE INVENTION

15

The present invention is related to neurotrophic factor protein MANF and a genetic sequence encoding the same. MANF nucleic acids, and MANF polypeptides expressed by said nucleic acids, are useful in the design of a range of gene therapy vectors useful in the treatment of type 1 and/or type 2 diabetes.

20

The MANF polypeptide expressed by the gene vector as described in the present invention may be naturally glycosylated or may comprise an altered glycosylation pattern depending on the cells in which it is synthesised. The molecule may be a full length, naturally occurring form or may be a truncated or otherwise derivatised form. Particularly important
25 MANF polypeptides are the full-length human MANF with a signal peptide having the total length of 179 amino acids and the mature human MANF without the signal peptide having the total length of 158 amino acids (see Table 1). The MANF nucleic acid encoding mature MANF polypeptide without a signal peptide may be combined with a nucleic acid encoding another signal peptide to provide a full-length MANF polypeptide with a
30 different signal peptide than the native MANF signal peptide.

The present invention is also related to a neurotrophic factor protein CDNF and a genetic sequence encoding the same. CDNF nucleic acids, and CDNF polypeptides expressed by said nucleic acids, are useful in the design of a range of gene therapy vectors useful in the
35 treatment of type 1 and or type 2 diabetes. CDNF polypeptides are the full-length human

CDNF with a signal peptide having the total length of 187 amino acids and the mature human CDNF without the signal peptide having the total length of 161 amino acids (see Table 1).

5 Functional fragments of the MANF and CDNF polypeptides are also available for a skilled person as the production of fragments of whole polypeptides is known in the art and the proliferative effect for MANF and CDNF fragments in cultured isolated beta islets from adult mice and/or in pancreatic beta cells after streptozotocin-induced beta cell death in a mouse can be routinely measured as defined in Experimental Section below for whole 10 polypeptides.

The MANF or CDNF gene therapy vectors disclosed herein will be useful in the development of a range of therapeutic and/or diagnostic applications alone or in combination with other molecules such as GDNF. The present invention extends, 15 therefore, to pharmaceutical compositions comprising the MANF or CDNF gene therapy vector or parts, fragments, derivatives, homologues or analogues thereof together with one or more pharmaceutically acceptable carriers and/or diluents. Furthermore, the present invention extends to vectors comprising the nucleic acid sequence encoding any of the sequences set forth in SEQ ID NO:1-4 or having at least about 15%, more preferably about 20 40%, even more preferably around 60-79% or even still more preferably around 80-95% similarity thereto and host cells comprising the same. In an embodiment of the invention, a host cell comprising a MANF or CDNF vector secretes a MANF or CDNF polypeptide expressed from said vector and is used in the intrapancreatic treatment of pancreatic beta cells to induce proliferation of beta cells. Suitable host cells are, e.g., human stem cells.
25

Unless defined otherwise, all technical and scientific terms have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. The definitions below are presented for clarity.

30 As used herein, a "polynucleotide" or “nucleic acid” refers to a molecule having a nucleic acid sequence encoding SEQ ID NO:1 or a fragment or variant thereof, a nucleic acid sequence encoding SEQ ID NO:3 or the complement thereof. For example, the polynucleotide can contain the nucleotide sequence of the full-length cDNA sequence, including the 5' and 3' untranslated sequences, the coding region, as well as fragments, 35 epitopes, domains, and variants of the nucleic acid sequence. Moreover, as used herein, a

"polypeptide" refers to a molecule having an amino acid sequence encoded by a polynucleotide of the invention as broadly defined. As used herein, the term "fragment" as applied to a nucleic acid, may ordinarily be at least about 10 nucleotides in length, typically, at least about 20 nucleotides, more typically, from about 20 to about 50 nucleotides, preferably at least about 50 to about 100 nucleotides, even more preferably at least about 100 nucleotides to about 300 nucleotides, yet even more preferably at least about 300 to about 400, and most preferably, the nucleic acid fragment will be greater than about 500 nucleotides in length.

As used herein, the term "fragment" as applied to a polypeptide, may ordinarily be at least about seven contiguous amino acids, typically, at least about fifteen contiguous amino acids, more typically, at least about thirty contiguous amino acids, typically at least about forty contiguous amino acids, preferably at least about fifty amino acids, even more preferably at least about sixty amino acids and most preferably, the peptide fragment will be greater than about seventy contiguous amino acids in length.

"Nucleic acid molecule", includes DNA molecules (e.g. cDNA or genomic DNA), RNA molecules (e.g., mRNA), analogs of the DNA or RNA generated using nucleotide analogs, and derivatives, fragments and homologs. The nucleic acid molecule may be single-stranded or double-stranded, but preferably comprises double-stranded DNA.

"Isolated nucleic acid molecule" is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an isolated nucleic acid is free of sequences that naturally flank the nucleic acid (i.e. sequences located at the 5'- and 3'-termini of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, isolated MANF DNA molecules can contain less than about 5 kb, 4 kb, 3kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell/tissue from which the nucleic acid is derived (e.g., brain, heart, liver, spleen, etc.). Moreover, an isolated nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material or culture medium when produced by recombinant techniques, or of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the invention, e.g., a MANF nucleic acid molecule, or a complement of this aforementioned nucleotide sequence, can be isolated using standard

molecular biology techniques and the provided sequence information. Using all or a portion of a MANF nucleic acid sequence of interest as a hybridization probe, MANF molecules can be isolated using standard hybridization and cloning techniques (Ausubel et al, In Current protocols in Molecular Biology, John Wiley and Sons, publishers, 1989).

5

“Analogs” are nucleic acid sequences or amino acid sequences that have a structure similar to, but not identical to, the native compound but differ from it in respect to certain components or side chains. Analogs may be synthetic or from a different evolutionary origin and may have a similar or opposite metabolic activity compared to wild type.

10

Derivatives and analogs may be full length or other than full length, if the derivative or analog contains a modified nucleic acid or amino acid, as described below. Derivatives or analogs of the nucleic acids or proteins of the invention include, but are not limited to, molecules comprising regions that are substantially homologous to the nucleic acids or
15 proteins of the invention, in various embodiments, by at least about 70%, 80%, or 95% identity (with a preferred identity of 80-95%) over a nucleic acid or amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art, or whose encoding nucleic acid is capable of hybridizing to the complement of a sequence encoding the aforementioned
20 proteins under stringent, moderately stringent, or low stringent conditions (Ausubel et al., supra). MANF or CDNF polypeptides may also be, e.g., phosphorylated, glycosylated, acylated, or acetylated. A MANF-CDNF hybrid is an example of preferred derivative disclosed in this specification.

25 “Encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if
30 transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of agene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

35

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

5

“Operably-linked nucleic acid” is operably-linked when it is placed into a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operably-linked to a coding sequence if it affects the transcription of the sequence, or a ribosome-binding site is operably-linked to a coding sequence if positioned to facilitate
10 translation. Generally, "operably-linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

15

"Variant" refers to a polynucleotide or polypeptide differing from the polynucleotide or polypeptide of the present invention, but retaining essential properties thereof. Generally, variants are overall closely similar, and, in many regions, identical to the polynucleotide or polypeptide of the present invention.

20

PCR amplification techniques can be used to amplify MANF using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers. Such nucleic acids can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to MANF sequences can be
25 prepared by standard synthetic techniques, e.g., an automated DNA synthesizer.

By the term "vector” as used herein, is meant any plasmid or virus encoding an exogenous nucleic acid. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into virions or cells, such as, for
30 example, polylysine compounds and the like. The vector may be a viral vector which is suitable as a delivery vehicle for delivery of the nucleic acid encoding the desired protein, or mutant thereof, to acell, or the vector may be a non-viral vector which is suitable for the same purpose. Examples of viral and non-viral vectors for delivery of DNA to cells and tissues are well known in the art and are described, for example, in Ma et al. (1997, Proc.
35 Natl. Acad. Sci. U.S.A. 94:12744-12746). Examples of viral vectors include, but are not

limited to, a recombinant vaccinia virus, a recombinant adenovirus, a recombinant retrovirus, a recombinant adeno-associated virus, a recombinant avian pox virus, and the like (Cranage et al., 1986, EMBO J. 5.3057-3063; International Patent Application No. W094/17810, published August 18, 1994; International Patent Application No.
5 W094/23744, published October 27, 1994). Examples of non-viral vectors include, but are not limited to, liposomes, polyamine derivatives of DNA, and the like.

“Homologs” are nucleic acid sequences or amino acid sequences of a particular gene that are derived from different species.
10

A "homologous nucleic acid sequence" or "homologous amino acid sequence," or variations thereof, refer to sequences characterized by a homology at the nucleotide level or amino acid level. Homologous nucleotide sequences encode those sequences coding for isoforms of MANF. Isoforms can be expressed in different tissues of the same organism as
15 a result of, for example, alternative splicing of RNA. Alternatively, different genes can encode isoforms. In the invention, homologous nucleotide sequences include nucleotide sequences encoding for a MANF of species other than humans, including, but not limited to vertebrates, and thus can include, e.g., frog, mouse, rat, rabbit, dog, cat cow, horse, and other organisms. Homologous nucleotide sequences also include, but are not limited to,
20 naturally occurring allelic variations and mutations of the nucleotide sequences set forth herein. A homologous nucleotide sequence does not, however, include the exact nucleotide sequence encoding a human MANF. Homologous nucleic acid sequences include those nucleic acid sequences that encode conservative amino acid substitutions in a MANF sequence of interest, as well as a polypeptide possessing MANF biological activity.
25

"Percent (%) nucleic acid sequence identity" with respect to a MANF is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in that particular MANF, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity.
30

"Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to transform a suitable host cell.

"Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic nonnaturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Synthetic polypeptides can be synthesized, for
5 example, using an automated polypeptide synthesizer.

"Physiologically acceptable" carriers, excipients, or stabilizers are ones which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples
10 of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other
15 carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

"Treatment" refers to both therapeutic treatment and prophylactic or preventative
20 measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

In addition to naturally occurring allelic variants of MANF, changes can be introduced by mutation into MANF sequences that incur alterations in the amino acid sequences of the
25 encoded MANF polypeptide. Nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in the sequence of a MANF polypeptide. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequences of MANF without altering its biological activity, whereas an "essential" amino acid residue is required for such biological activity. For example, amino
30 acid residues that are conserved among the MANF molecules of the invention are predicted to be particularly non-amenable to alteration. Amino acids for which conservative substitutions can be made are well known in the art.

Moreover, MANF from other species that have a nucleotide sequence that differs from the
35 human sequence of MANF are contemplated. Nucleic acid molecules corresponding to

natural allelic variants and homologues of MANF cDNAs of the invention can be isolated based on their homology to MANF using cDNA-derived probes to hybridize to homologous MANF sequences under stringent conditions.

5 "MANF variant polynucleotide" or "MANF variant nucleic acid sequence" means a nucleic acid molecule which encodes an active MANF that (1) has at least about 80% nucleic acid sequence identity with a nucleotide acid sequence encoding a full-length native MANF, (2) a full-length native MANF lacking the signal peptide, or (3) any other fragment of a full-length MANF. Ordinarily, a MANF variant polynucleotide will have at least about 80% 10 nucleic acid sequence identity, more preferably at least about 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% nucleic acid sequence identity and yet more preferably at least about 99% nucleic acid sequence identity with the nucleic acid sequence encoding a full-length native MANF. A MANF variant polynucleotide may encode full-length native MANF lacking the signal peptide 15 with or without the signal sequence, or any other fragment of a full-length MANF. Variants do not encompass the native nucleotide sequence.

Ordinarily, MANF variant polynucleotides are at least about 30 nucleotides in length, often at least about 60, 90, 120, 150, 180, 210, 240, 270, 300, 400 nucleotides in length, more 20 often at least about 500 nucleotides in length, or more.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such 25 sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding MANF.

30 Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

Transformation means introducing DNA into an organism so that the DNA is replicable, 35 either as an extrachromosomal element or by chromosomal integrant. Depending on the

host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd edition (New York: Cold Spring Harbor Laboratory Press, 2001), or electroporation is generally used for prokaryotes or other cells
5 that contain substantial cell-wall barriers.

General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216 issued Aug. 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and
10 Hsiao et al., Proc. Natl. Acad. Sci. USA, 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, etc., may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352
15 (1988).

TREATMENT

In type 1 diabetes insulin-producing pancreatic beta cells die. We have now discovered that
20 neurotrophic factors MANF and CDNF have potential to promote survival of dying beta cells. The present invention thus provides viral vector (e.g. AAV and lentivirus vector) expressing recombinant human MANF or CDNF.

By delivering MANF, e.g. by a virus vector expressing MANF proteins, to the pancreatic
25 duct it is possible to effectively protect and repair insulin-producing cells in pancreas in streptozotocin-induced diabetic mice suffering from type 1 diabetes as described in the Experimental Section below. Further, in Yi et al., Cell 153, 1–12, 2013, it is disclosed that replenishing insulin-producing pancreatic β cell mass will benefit both type 1 and type 2 diabetics. Therefore, targeted treatment of pancreatic β cells with a gene therapy vector
30 expressing or a host cell secreting a neurotrophic factor MANF or CDNF is thus beneficial for a patient suffering from type 1 or type 2 diabetes.

Any suitable vector may be used to introduce a transgene of interest into an animal. Exemplary vectors that have been described in the literature include replication-deficient retroviral vectors, including but not limited to lentivirus vectors [Kim et al., J. Virol.,

72(1): 811-816 (1998); Kingsman & Johnson, Scrip Magazine, October, 1998, pp. 43-46.]; adenoviral (*see*, for example, U.S. Patent No. 5,824,544; U.S. Patent No. 5,707,618; U.S. Patent No. 5,792,453; U.S. Patent No. 5,693,509; U.S. Patent No. 5,670,488; U.S. Patent No. 5,585,362; Quantin et al., Proc. Natl. Acad. Sci. USA, 89: 2581-2584 (1992);
5 Stratford-Perricadet et al., J. Clin. Invest., 90: 626-630 (1992); and Rosenfeld et al., Cell, 68: 143-155 (1992)), retroviral (*see*, for example, U.S. Patent No. 5,888,502; U.S. Patent No. 5,830,725; U.S. Patent No. 5,770,414; U.S. Patent No. 5,686,278; U.S. Patent No. 4,861,719), adeno-associated viral (*see*, for example, U.S. Patent No. 5,474,935; U.S. Patent No. 5,139,941; U.S. Patent No. 5,622,856; U.S. Patent No. 5,658,776; U.S. Patent
10 No. 5,773,289; U.S. Patent No. 5,789,390; U.S. Patent No. 5,834,441; U.S. Patent No. 5,863,541; U.S. Patent No. 5,851,521; U.S. Patent No. 5,252,479; Gnatenko et al., J. Investig. Med., 45: 87-98 (1997), an adenoviral-adenoassociated viral hybrid (*see*, for example, U.S. Patent No. 5,856,152) or a vaccinia viral or a herpesviral (*see*, for example, U.S. Patent No. 5,879,934; U.S. Patent No. 5,849,571; U.S. Patent No. 5,830,727; U.S.
15 Patent No. 5,661,033; U.S. Patent No. 5,328,688); Lipofectin-mediated gene transfer (BRL); liposomal vectors [See, e.g., U.S. Patent No. 5,631,237 (Liposomes comprising Sendai virus proteins)] ; and combinations thereof. All of the foregoing documents are incorporated herein by reference in the entirety. Replication-deficient adenoviral vectors, adeno-associated viral vectors and lentiviruses constitute preferred embodiments.

20

In embodiments employing a viral vector, preferred polynucleotides include a suitable promoter and polyadenylation sequence to promote expression in the target tissue of interest. For many applications of the present invention, suitable promoters/enhancers for mammalian cell expression include, e.g., cytomegalovirus promoter/enhancer [Lehner et
25 al., J. Clin. Microbiol., 29:2494-2502 (1991); Boshart et al., Cell, 41:521-530 (1985)]; Rous sarcoma virus promoter [Davis et al., Hum. Gene Ther., 4:151 (1993)]; simian virus 40 promoter, long terminal repeat (LTR) of retroviruses, keratin 14 promoter, and α myosin heavy chain promoter.

30 Gene therapy is generally disclosed in U.S. Pat. No. 5,399,346. Gene therapy means genetic modification of cells by the introduction of exogenous DNA or RNA into these 222cells for the purpose of expressing or replicating one or more peptides, polypeptides, proteins, oligonucleotides, or polynucleotides in vivo for the treatment or prevention of disease or deficiency in humans or animals.

In gene therapy applications, genes are introduced into cells in order to achieve in vivo synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy" includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes in vivo. It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane. (Zamecnik et al., Proc. Natl. Acad. Sci. USA, 83:41434146 (1986)). The oligonucleotides can be modified to enhance their uptake, e.g., by substituting their negatively charged phosphodiester groups by uncharged groups.

There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, ex vivo, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes (Nicolau and Sene, *Biochim. Biophys. Acta*, 721:185-190 (1982); Fraley, *et al.*, *Proc. Natl. Acad. Sci. USA*, 76:3348-3352 (1979); Felgner, *Sci. Am.*, 276(6):102-6 (1997); Felgner, *Hum. Gene Ther.*, 7(15):1791-3, (1996)), electroporation (Tur-Kaspa, *et al.*, *Mol. Cell Biol.*, 6:716-718, (1986); Potter, *et al.*, *Proc. Nat. Acad. Sci. USA*, 81:7161-7165, (1984)), direct microinjection (Harland and Weintraub, *J. Cell Biol.*, 101:1094-1099 (1985)), cell fusion, DEAE-dextran (Gopal, *Mol. Cell Biol.*, 5:1188-1190 (1985), the calcium phosphate precipitation method (Graham and Van Der Eb, *Virology*, 52:456-467 (1973); Chen and Okayama, *Mol. Cell Biol.*, 7:2745-2752, (1987); Rippe, *et al.*, *Mol. Cell Biol.*, 10:689-695 (1990), cell sonication (Fechheimer, *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:8463-8467 (1987)), gene bombardment using high velocity microprojectiles (Yang, *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:9568-9572 (1990). The currently preferred in vivo gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection (Dzau et al., Trends in Biotechnology, 11:205-210 (1993)). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with

endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for
5 example, by Wu et al., J. Biol. Chem., 262:4429-4432 (1987); and Wagner et al., Proc. Natl. Acad. Sci. USA, 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson et al., Science, 256:808-813 (1992).

In a particular embodiment of the invention, the expression construct (or indeed the
10 peptides discussed above) may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation
15 of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, "In Liver Diseases, Targeted Diagnosis And Therapy Using Specific Receptors And Ligands," Wu, G., Wu, C., ed., New York: Marcel Dekker, pp. 87-104 (1991)). The addition of DNA to cationic liposomes causes a topological transition from liposomes to optically birefringent liquid-crystalline condensed globules (Radler, *et*
20 *al.*, *Science*, 275(5301):810-4, (1997)). These DNA-lipid complexes are potential non-viral vectors for use in gene therapy and delivery.

Also contemplated in the present invention are various commercial approaches involving "lipofection" technology. In certain embodiments of the invention, the liposome may be
25 complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda, *et al.*, *Science*, 243:375-378 (1989)). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato, *et al.*, *J. Biol. Chem.*, 266:3361-3364 (1991)). In yet further
30 embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention.

Other vector delivery systems that can be employed to deliver a nucleic acid encoding a therapeutic gene into cells include receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific.

In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above that physically or chemically permeabilize the cell membrane. This is applicable particularly for transfer *in vitro*, however, it may be applied for *in vivo* use as well. Dubensky, *et al.*, *Proc. Nat. Acad. Sci. USA*, 81:7529-7533 (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif, *Proc. Nat. Acad. Sci. USA*, 83:9551-9555 (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein, *et al.*, *Nature*, 327:70-73 (1987)). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang, *et al.*, *Proc. Natl. Acad. Sci USA*, 87:9568-9572 (1990)). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Those of skill in the art are aware of how to apply gene delivery to *in vivo* and *ex vivo* situations. For viral vectors, one generally will prepare a viral vector stock. Depending on the type of virus and the titer attainable, one will deliver 1 x 10^4, 1 x 10^5, 1 x 10^6, 1 x 10^7, 1 x 10^8, 1 x 10^9, 1 x 10^{10}, 1 x 10^{11} or 1 x 10^{12} infectious particles to the patient. Similar figures may be extrapolated for liposomal or other non-viral formulations by comparing

relative uptake efficiencies. Formulation as a pharmaceutically acceptable composition is discussed below.

Various routes are contemplated for various cell types. For practically any cell, tissue or
5 organ type, systemic delivery is contemplated. In other embodiments, a variety of direct, local and regional approaches may be taken. For example, the cell, tissue or organ may be directly injected with the expression vector or protein.

In a different embodiment, *ex vivo* gene therapy is contemplated. In an *ex vivo*
10 embodiment, cells from the patient are removed and maintained outside the body for at least some period of time. During this period, a therapy is delivered, after which the cells are reintroduced into the patient.

The present invention thus provides a method of treating a subject suffering from type 1 or
15 type 2 diabetes, wherein the method comprises the step of administering by intrapancreatic delivery to the subject a gene vector expressing effective amount of a MANF polypeptide comprising the sequence of SEQ ID NO:1. Preferably, said gene vector expresses MANF polypeptide comprising the sequence according to SEQ ID NO:2.

20 The above embodiments can be performed by using CDNF molecules instead of MANF molecules, since the inventors were able to provide results showing that CDNF also induces proliferation of pancreatic beta cells after streptozotocin-induced beta cell death in mouse (see the Experimental Section below).

25 Accordingly, the present invention also provides a method of treating a subject suffering from type 1 or type 2 diabetes, wherein the method comprises the step of administering by intrapancreatic delivery to the subject a gene vector expressing effective amount of a CDNF polypeptide comprising the sequence of SEQ ID NO:3. Preferably, said gene vector expresses CDNF polypeptide comprising the sequence according to SEQ ID NO:4.

30

Viral vectors expressing MANF or CDNF

Although other vectors may be used, preferred vectors for use in the methods of the present invention are viral vectors. The vector selected should meet the following criteria: 1) the vector must be able to infect targeted cells and thus viral vectors having an appropriate host range must be selected; 2) the transferred gene should be capable of persisting and being expressed in a cell for an extended period of time (without causing cell death) for stable maintenance and expression in the cell; and 3) the vector should do little, if any, damage to target cells.

Because adult pancreatic cells are non-dividing, the recombinant expression vector chosen must be able to transfect and be expressed in non-dividing cells. At present, vectors known to have this capability include DNA viruses such as adenoviruses, adeno-associated virus (*AAV*), and certain RNA viruses such as HIV-based lentiviruses, feline immunodeficiency virus (FIV) and equine immunodeficiency virus (EIV. Other vectors with this capability include herpes simplex virus (HSV). However, some of these viruses (e.g., *AAV* and HSV) can produce toxicity and/or immunogenicity. An HIV-based lentiviral vector system has been developed which, like other retroviruses, can insert a transgene into the nucleus of host cells (enhancing the stability of expression) but, unlike other retroviruses, can make the insertion into the nucleus of non-dividing cells. Lentiviral vectors have been shown to stably transfect brain cells after direct injection, and stably express a foreign transgene without detectable pathogenesis from viral proteins (see, Naldini, et al., Science, 272:263-267 (1996), the disclosure of which is incorporated by reference). Following the teachings of the researchers who first constructed the HIV-1 retroviral vector, those of ordinary skill in the art will be able to construct lentiviral vectors suitable for use in the methods of the invention (for more general reference concerning retrovirus construction, see, e.g., Kriegler, Gene Transfer and Expression, A Laboratory Manual, W. Freeman Co. (NY 1990) and Murray, E J, ed., Methods in Molecular Biology, Vol. 7, Humana Press (NJ 1991)).

The use of recombinant AAV vectors is efficient; their infection is relatively long-lived and is generally non-toxic, unless a toxic transgene is recombined therein. AAV is a helper-dependent parvovirus consisting of a single strand 4.7 kb DNA genome surrounded by a simple, non-enveloped icosahedral protein coat. About 85% of the adult human population is seropositive for AAV. Nonetheless, no pathology has been associated with AAV infection. AAV is dependent on Adenovirus or herpesvirus as a helper virus to establish productive infection by AAV. In the absence of helper virus, the AAV genome

also amplifies in response to toxic challenge (UV irradiation, hydroxyurea exposure). If there is no toxic challenge or helper virus, wild-type AAV integrates into human chromosome 19 site-specifically. This is driven by the AAV Rep proteins that mediate the formation of an AAV-chromosome complex at the chromosomal integration site. Most of the viral genome (96%) may be removed, leaving only the two 145 base pair (bp) inverted terminal repeats (ITRs) for packaging and integration of the viral genome. Techniques for efficient propagation of recombinant AAV, rAAV, have been developed in the art: the use of mini-adenoviral genome plasmids, plasmids encoding AAV packaging functions and adenovirus helper functions in single plasmids. Moreover, methods of rAAV for isolation of highly purified rAAV are a relatively straightforward and rapid undertaking, as is titration of rAAV stocks. To trace rAAV-mediated transgene expression the green fluorescent protein (GFP), a well-characterized 238 amino acid fluorescent protein, is frequently used in a bicistronic arrangement in rAAV. Selective and specific expression of rAAV mediated gene transfer through different promoters has also been identified. We use a commercial available AAV Helper-free system (Invitrogen) to construct our recombinant AAVs. MANF is cloned into vectors/plasmids of the AAV system using conventional recombinant DNA techniques.

Construction of vectors for recombinant expression of MANF or CDNF for use in the invention may be accomplished using conventional techniques which do not require detailed explanation to one of ordinary skill in the art. Specifics for construction of AAV vector is set forth in here. For further review, those of ordinary skill may wish to consult Maniatis et al., in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, (NY 1982).

Briefly, construction of recombinant expression vectors employs standard ligation techniques. For analysis to confirm correct sequences in vectors constructed, the ligation mixtures may be used to transform a host cell and successful transformants selected by antibiotic resistance where appropriate. Vectors from the transformants are prepared, analyzed by restriction and/or sequenced by, for example, the method of Messing, et al., (Nucleic Acids Res., 9:309, 1981), the method of Maxam, et al., (Methods in Enzymology, 65:499, 1980), or other suitable methods which will be known to those skilled in the art. Size separation of cleaved fragments is performed using conventional gel electrophoresis as described, for example, by Maniatis, et al., (Molecular Cloning, pp. 133-134, 1982).

Expression of a gene (MANF/CDNF) is controlled at the transcription, translation or post-translation levels. Transcription initiation is an early and critical event in gene expression. This depends on the promoter and enhancer sequences and is influenced by specific cellular factors that interact with these sequences. The transcriptional unit of many prokaryotic genes consists of the promoter and in some cases enhancer or regulator elements (Banerji et al., Cell 27:299 (1981); Corden et al., Science 209:1406 (1980); and Breathnach and Chambon, Ann. Rev. Biochem. 50:349 (1981)). For retroviruses, control elements involved in the replication of the retroviral genome reside in the long terminal repeat (LTR) (Weiss et al., eds., The molecular biology of tumor viruses: RNA tumor viruses, Cold Spring Harbor Laboratory, (NY 1982)). Moloney murine leukemia virus (MLV) and Rous sarcoma virus (RSV) LTRs contain promoter and enhancer sequences (Jolly et al., Nucleic Acids Res. 11:1855 (1983); Capecchi et al., In: Enhancer and eukaryotic gene expression, Gulzman and Shenk, eds., pp. 101-102, Cold Spring Harbor Laboratories (NY 1991). Other potent promoters include those derived from cytomegalovirus (CMV) and other wild-type viral promoters.

An alternative approach to change the expression of MANF is to change the pre-region (signal sequence) of the protein to an equivalent signal sequence from a protein known to be expressed in high amounts, such as the immunoglobulin heavy chain. Alternatively, the signal sequence of MANF can be changed to that of a protein known to be expressed in the appropriate target tissue or organ. Likewise, deletion or insertion variants of MANF might be more efficiently expressed and more stable than the native MANF protein.

Methods of making and using rAAV and delivery of rAAV to various cells in vivo are found in U.S. Pat. Nos. 5,720,720; 6,027,931; 6,071,889; WO 99/61066; all of which are hereby incorporated by reference for this purpose. Different serotypes of AAV are available, and they show tissue tropism. Thus, the use of the accurate serotype depends on which tissue is to be transduced.

With regard to methods for the successful, localized, long-term and non-toxic transgene expression in the pancreas beta cells using adeno-associated virus (AAV) and selected promoters, reference is made to Jimenez et al., 2011, Diabetologia 51 (5), 1075-86, “In vivo genetic engineering of murine pancreatic beta cells mediated by single-stranded adeno-associated viral vectors of serotypes 6,8 and 9”. and Wang et al., 2006, Diabetes 55

(4), 875-884 , "Widespread and stable pancreatic gene transfer by adeno-associated virus vectors via different routes.

A further important parameter is the dosage of MANF to be delivered into the target tissue. The unit dosage refers generally to the MANF/ml of MANF composition. For viral vectors, MANF concentration may be defined by the number of viral particles/ml of the composition. Optimally, for delivery of MANF using a viral expression vector, each unit dosage of MANF will compromise 2.5 to 25 ul of MANF compostion, wherein the composition includes viral expression vector in pharmaceutically acceptable fluid and provides from 10^{10} to 10^{15} MANF expressing viral particles per ml of MANF composition. Such high titers are particularly useful for AAV. For lentivirus, the titer is normally lower, from 10^{8} to 10^{10} transducing units per ml (TU/ml).

PHARMACEUTICAL AND THERAPEUTICAL COMPOSITIONS AND FORMULATIONS

The MANF nucleic acid molecules, defined herein as gene therapy vectors, and derivatives, fragments, analogs and homologs thereof, can be incorporated into pharmaceutical compositions.

Such compositions of MANF are prepared for storage by mixing MANF nucleic acid molecule having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., (1980)), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients, or stabilizers are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

The MANF nucleic acid molecule may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, 5 albumin microspheres, microemulsions, nano-particles, and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

The MANF nucleic acid molecules can be inserted into vectors and used as gene therapy 10 vectors. The route of MANF nucleic acid molecule administration is in accord with known methods, e.g., those routes set forth above for specific indications, as well as the general routes of injection or infusion by pancreatic ductal, intravenous, intraperitoneal, intramuscular, intraocular, intraarterial, or intralesional means, or sustained release systems as noted below. MANF nucleic acid molecule is administered continuously by infusion or 15 by bolus injection. Generally, where the disorder permits, one should formulate and dose the MANF nucleic acid molecule for site-specific delivery. Administration can be continuous or periodic. Administration can be accomplished by a constant- or programmable-flow implantable pump or by periodic injections. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration 20 (Nabel and Nabel, US Patent No. 5, 328, 470, 1994), or by stereotactic injection (Chen et al., Proc. Natl. Acad. Sci. USA 91:3054-3057 (1994)). The pharmaceutical preparation of a gene therapy vector can include an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded.

25 Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system.

Sustained-release MANF compositions also include liposomally entrapped MANF nucleic 30 acid molecule. Liposomes containing MANF nucleic acid molecule are prepared by methods known per se: Epstein et al., Proc. Natl. Acad. Sci. USA, 82:3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA, 77:40304034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar 35 type in which the lipid content is greater than about 30 mol % cholesterol, the selected

proportion being adjusted for the optimal MANF nucleic acid molecule therapy.

Accordingly, also included as a gene therapy method is a method for preventing or treating type 1 or type 2 diabetes comprising implanting cells that secrete recombinant MANF into 5 the body of patients in need thereof. The patient's own cells, transformed to produce MANF ex vivo, could be implanted directly into the patient, optionally without encapsulation. 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 under experimentation.

10

An effective amount of MANF nucleic acid molecule to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titre the dosage and modify the route of administration as required to obtain the optimal therapeutic 15 effect. Typically, the clinician will administer the MANF gene vector until a dosage is reached that achieves the desired effect. As an alternative general proposition, the MANF nucleic acid molecule is formulated and delivered to the target site or tissue at a dosage capable of establishing in the tissue a MANF level that is efficacious but not unduly toxic.

20 The efficacy of virally-delivered or non-virally delivered MANF polynucleotides can be tested in any of a number of animal models of the type 1 or type 2 diabetes, known in the art.

Accordingly, the present invention is directed to a use of a MANF nucleotide comprising a 25 nucleotide sequence encoding a MANF polypeptide comprising the sequence of SEQ ID NO:1 for the manufacture of a gene therapy vector for the intrapancreatic treatment of pancreatic beta cells, and preferably for the treatment of type 1 or type 2 diabetes.

The present invention is also directed to a use of a CDNF nucleotide comprising a 30 nucleotide sequence encoding a CDNF polypeptide comprising the sequence of SEQ ID NO:3 for the manufacture of a gene therapy vector for the intrapancreatic treatment of pancreatic beta cells, and preferably for the treatment of type 1 or type 2 diabetes.

Further, the present invention provides a gene therapy vector expressing effective amount 35 of a MANF polypeptide comprising the sequence of SEQ ID NO:1 or 2 for use in the

intrapancreatic treatment of pancreatic beta cells, and preferably for use in the treatment or prevention of type 1 or type 2 diabetes. The present invention also provides a gene therapy vector expressing effective amount of a CDNF polypeptide comprising the sequence of SEQ ID NO:3 or 4 for use in the intrapancreatic treatment of pancreatic beta cells, and
5 preferably for use in the treatment or prevention of type 1 or type 2 diabetes. The present invention is also directed to pharmaceutical compositions comprising a gene therapy vector expressing effective amount of a MANF or CDNF polypeptide as defined herein for use in the intrapancreatic treatment of pancreatic beta cells, and preferably for use in the treatment or prevention of type 1 or type 2 diabetes.

10

In another embodiment of the invention, the present invention provides an isolated host cell transfected with the gene therapy vector as defined above, wherein said host cell secrets a MANF or CDNF polypeptide, for use in the intrapancreatic treatment of pancreatic beta cells.

15

In preferred embodiments of the invention, the intrapancreatic treatment results in regeneration of pancreatic beta cells, i.e. the above mentioned vectors and host cells are for use in the regeneration of pancreatic beta cells.

20 TRANSGENIC ANIMALS

The present invention is directed to a genetically-modified non-human animal, preferably a mouse or rat. The non-human animal of the invention comprises a disrupted allele for the gene that naturally encodes and expresses a functional MANF gene, wherein the disrupted
25 allele is a non-functional MANF gene and the non-human animal displays progressive postnatal reduction of pancreatic beta cell mass due to the disrupted non-functional MANF gene. Preferably, the non-human animal is homozygous for disruption of the MANF gene. Further, the non-human animal comprising said disrupted non-functional MANF gene preferably displays type 1 diabetes.

30

A method of selecting a compound preventing pancreatic beta cell apoptosis and dysfunction using the non-human animal comprising a disrupted allele for the gene that naturally encodes and expresses a functional MANF gene as defined in the present invention is also contemplated. The present animal model is useful for screening and
35 evaluating possible therapeutic applications of drugs which can compensate the loss of

MANF activity in beta cells. The present invention is thus directed to a method of screening a compound for preventing pancreatic beta cell apoptosis and dysfunction. This method comprises the following steps: (a) administering a compound to be tested to a non-human animal comprising a disrupted allele for the gene that naturally encodes and
5 expresses a functional MANF gene and displaying progressive postnatal reduction of pancreatic beta cell mass; (b) observing the effect of said compound on said animal; and (c) relating the observed effect of said compound on said animal to the beta cell preservation and/or regeneration activity of said compound, wherein a compound showing said preservation and/or regeneration activity is a candidate compound for the treatment of
10 type 1 diabetes.

Accordingly, the present invention is also directed to a use of the non-human animal as defined above as a model of postnatal pancreatic beta cell death and, consequently, as a model of type 1 diabetes.
15

Nucleic acids which encode MANF, preferably from non-human species, such as murine or rat protein, can be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse) is an animal having cells that contain a
20 transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic, stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, the human and/or mouse cDNA encoding MANF, or an appropriate sequence thereof, can be used to clone genomic DNA encoding MANF in accordance with established techniques and the
25 genomic sequences used to generate transgenic animals that contain cells which express DNA encoding MANF. Methods for generating transgenic animals, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Pat. Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for MANF transgene incorporation with tissue-specific enhancers, which could result in desired effect
30 of treatment. Transgenic animals that include a copy of a transgene encoding MANF introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding MANF. Such animals can be used as tester animals for reagents thought to confer protection from, for example, diseases related to MANF. In accordance with this facet of the invention, an animal is treated with the
35 reagent and a reduced incidence of the disease, compared to untreated animals bearing the

transgene, would indicate a potential therapeutic intervention for the disease.

It is now well-established that transgenes are expressed more efficiently if they contain introns at the 5' end, and if these are the naturally occurring introns (Brinster et al. Proc. 5 Natl. Acad. Sci. USA 85:836-840 (1988); Yokode et al., Science 250:1273-1275 (1990)).

Transgenic offspring are identified by demonstrating incorporation of the microinjected transgene into their genomes, preferably by preparing DNA from short sections of tail and analyzing by Southern blotting for presence of the transgene ("Tail Blots"). A preferred 10 probe is a segment of a transgene fusion construct that is uniquely present in the transgene and not in the mouse genome. Alternatively, substitution of a natural sequence of codons in the transgene with a different sequence that still encodes the same peptide yields a unique region identifiable in DNA and RNA analysis. Transgenic "founder" mice identified in this fashion are bred with normal mice to yield heterozygotes, which are backcrossed to create 15 a line of transgenic mice. Tail blots of each mouse from each generation are examined until the strain is established and homozygous. Each successfully created founder mouse and its strain vary from other strains in the location and copy number of transgenes inserted into the mouse genome, and hence have widely varying levels of transgene expression. Selected animals from each established line are sacrificed at 2 months of age and the expression of 20 the transgene is analyzed by Northern blotting of RNA from pancreas, liver, muscle, fat, kidney, brain, lung, heart, spleen, gonad, adrenal and intestine.

PRODUCTION OF “KNOCK OUT” ANIMALS

25 Alternatively, the non-human homologs of MANF can be used to construct a MANF "knock out" animal, i.e., having a defective or altered gene encoding MANF, as a result of homologous recombination between the endogenous MANF gene and an altered genomic MANF DNA introduced into an embryonic cell of the animal. For example, murine MANF cDNA can be used to clone genomic MANF DNA in accordance with established 30 techniques. A portion of the genomic MANF DNA can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas and Capecchi, Cell 51:503 (1987) for a description of homologous recombination vectors). The vector is introduced into an 35 embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA

has homologously recombined with the endogenous DNA are selected (see e.g., Li et al., Cell 69:915 (1992)). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see e.g., Bradley, in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E. J. Robertson, ed. (IRL, Oxford, 1987),
5 pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harbouring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for
10 their ability to display development of severe diabetes due to progressive postnatal reduction of beta cell mass caused by decreased beta cell proliferation and increased beta cell apoptosis.

Accordingly, the present invention also provides a method of producing a genetically-
15 modified non-human animal, preferably a mouse or rat, comprising a disrupted allele for the gene that naturally encodes and expresses a functional MANF gene, the method comprising: mating non-human animals heterozygous for disruption of the MANF gene to produce a non-human animal homozygous for said MANF disrupted allele and identifying progeny displaying progressive postnatal reduction of pancreatic beta cell mass and in
20 which both alleles of the endogenous MANF gene are disrupted.

EQUIVALENTS

Although particular embodiments have been disclosed herein in detail, this has been done
25 by way of example for purposes of illustration only, and is not intended to be limiting with respect to the scope of the appended claims that follow. In particular, it is contemplated by the inventors that various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims. The choice of nucleic acid starting material, clone of interest, or library type is
30 believed to be a matter of routine for a person of ordinary skill in the art with knowledge of the embodiments described herein. Other aspects, advantages, and modifications considered to be within the scope of the following claims.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

5 Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting.

10

EXPERIMENTAL SECTION

EXAMPLE 1

15 **Materials and methods**

***Manf*-targeted embryonic stem cells**

The targeted mouse embryonic stem (ES) cell clone MANF_D06 (EPD0162_3_D06,
20 C57Bl/6N-Manf $^{tm1a(KOMP)Wtsi}$) used for this research project was generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). Genetically modified ES-cells were amplified for freezing and aggregation on gelatinized (0.1%) plates in Knockout™ DMEM (Gibco, Life Technologies, NY, USA), 15% fetal bovine serum (HyClone, ThermoScientific,
25 Cramlington, UK), 2 mM GlutaMAX™ (Gibco), 1 mM non-essential amino acids (Gibco), LIF 1000 U/ml (Gibco), 1 µm 2-Mercaptoethanol (Gibco) and penicillin 100U/ml, streptomycin 10 mg/ml (Sigma-Aldrich, USA), in a 37°C humidified 5% CO_2 incubator. Medium was changed daily until 80% confluent (approx. 1.5-2 x 10^7 cells). ES cells were aggregated with morula-stage pre-implantation embryos (ICR strain) at the GM mouse unit
30 of the Laboratory Animal Centre, University Helsinki. ES cells were grown in RESGRO™ culture medium (*Merck* KGaA, Darmstadt, Germany) before morula aggregation. From 120 aggregates produced and transferred to pseudopregnant ICR females, we obtained 9 chimeric males of which 2 gave germ-line transmission and heterozygote progeny by crossings to WT ICR mice.

35

Animals

All experimental procedures involving mice were approved by the Finnish Animal Ethics committee of the State Provincial Office of Southern Finland. Mice were maintained in
5 pathogen-free facility with a 12 hr light/dark cycle and unlimited access to food (Harlan, Teklad Global, 16% protein rodent diet, 2916) and water. In all studies comparing wild-type (WT) and MANF homozygous knockout mice (KO), we used sex-matched siblings derived from crossings of MANF heterozygous (HZ) animals in hybrid C57Bl/6 x ICR mixed background. The day of vaginal plug was designated as E0.5. Age-matched NMRI
10 male mice for the *in vivo* MLD-STZ experiment were obtained from Harlan Teklan, UK. 129/Sv and C57Bl6 mice from Harlan Teklan, UK were used for the islet proliferation assay. PGK-Cre mice were originally described in [45]. CaqFlp mice were a gift from Prof. Frank Grosvelt (Erasmus MC, Rotterdam, The Netherlands).

15 **Genomic DNA isolation and genotyping**

For genotyping animals, DNA was isolated from ear-marks using standard Proteinase K digestion 0.25 mg/ml in lysis buffer (100 mM Tris-HCl, pH8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) o/n in 55°C shaker or Fast Tissue-to-PCR kit (Fermentas Life Sciences,
20 Thermo Scientific) according to manufacturer´s instructions. Genotyping was carried out using the following primers: MANF KO primers f2 5´-TGG AGTGAG CACAAC TCA GG-3´ (SEQ ID NO:5), r2 5´-GGC TTC GAC ACC TCA TTG AT-3´ (SEQ ID NO:6) and r4 5´-CCA CAA CGG GTT CTT CTG TT-3´ (SEQ ID NO:7) (WT f2/r2 PCR product 547 bp, KO f2/r4 product 352 bp). Primers used to genotype β-gal reporter excised mice
25 ($MANF^{\Delta Gal/+}$) were f2, r2 and r5 5´-CTC AGG TCC TCC ACA AGA GC-3´ (SEQ ID NO:8) (WT PCR product 350 bp, Δβ-gal reporter excised product 547 bp). Primers used to identify $MANF^{fl/fl}$ mice derived from crossings of PGK-Cre mice and $MANF^{\Delta Gal/\Delta Gal}$ mice were f2 and r6 5´-TGC CAT GGT GAT GCT GTA AC-3´ (SEQ ID NO:9). Expected PCR products are as follows: 531 bp for the allele lacking exon 3, 1300 bp containing exon 3
30 and Frt-and LoxP-sites and 1051 bp for the WT product. Primers for genotyping Caq-Flp transgenic mice were F 5´-CCC ATT CGA TGC GGG GTA TCG-3´ (SEQ ID NO:10) and R 5´-GCA TCT GGG AGA TCA CTG AG-3´ (SEQ ID NO:11) and for PGK-Cre transgenic mice were F 5´-AAT CTC CCA CCG TCA GTA CG-3´ (SEQ ID NO:12), R 5´-CGT TTT CTG AGC ATA CCT GGA-3´ (SEQ ID NO:13).

35

Islet isolation

Pancreases from mice were treated with collagenase P digestion (Collagenase P, Roche Diagnostics GmbH Mannheim, Germany) followed by hand-picking of islets under a stereomicroscope [46]. Human pancreases (Otonkoski lab.)? Human islets were obtained through the European Consortium for Islet Transplantation (ECIT).

For RNA isolation all islets from one mouse pancreas (or from 2-3 if pancreases contained too few or small islets) were dissolved in 200-300 µl TriReagent® (Molecular Research Center, Inc., Ohio, USA) and RNA was isolated according to manufacturer´s instructions. Islets for Western blot analysis were cultured overnight in RPMI-1640 (Life Technologies) medium with 10% FCS and dissolved in 1x Laemmli buffer.

To verify successful removal of exocrine pancreas tissue, isolated islets were stained for 30-60 minutes in 10% dithizone diluted in HBSS (Gibco, Invitrogen, NY, USA) containing dimethyl sulphoxide (DMSO) Hybri-Max® (Sigma®, Life Science, Sigma-Aldrich, St. Louis, MO, USA).

RNA isolation, reverse transcription and quantitative PCR

RNA from tissues and islets was isolated by TriReagent® (Molecular Research Center) or by using NucleoSpin RNA® II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to manufacturer´s instructions. Reverse transcription reactions were performed with RevertAid™ Premium Reverse Transcriptase (Fermentas UAB, Thermo Fisher Scientific Inc., Vilnius, Lithuania), and dNTP mix, 10 mM each (Fermentas UAB), oligo(dT) 15 Primer, 500 µg/ml (Promega Corporation, Madison, Wisconsin, USA) for 40 minutes in 55°C according to the manufacturer´s protocol. All reactions were performed in duplicates. Quantitative PCR was performed using LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and Roche LightCycler® 480 Real-Time PCR System with the following program: 95 °C (5 min.), 45 cycles of 95 °C (10 s.), 60 °C (10 s.), 72 °C (10 s.). The expression levels were normalized to the levels of β-actin in the same samples. Standard curve assisted relative quantification method was used. Duplicates with CP difference over 1 were excluded from the statistical analysis. Primers used in quantitative PCR for *Grp78*, *Xbp1s*, *Xbp1t*, *Glut2* and *MafA* were synthetized using previously published sequences [47]. Primer sequences for *Atf4* were F 5´-ATG GCC GGC TAT GGA TGA T-3´ (SEQ ID NO:14), R 5´-CGA AGT CAA ACT CTT TCA GAT CCA TT-3´ (SEQ ID NO:15), for *Chop* F 5´-CCA ACA GAG GTC ACA CGC

AC-3´ (SEQ ID NO:16), R 5´-TGA CTG GAA TCT GGA GAG CGA-3´ (SEQ ID NO:17), for *Atf6α* F 5´- GGA CGA GGT GGT GTC AGA G-3´ (SEQ ID NO:18), R 5´- *GAC AGC TCT TCG CTT TGG AC* (SEQ ID NO:19), *for Ins1/2* F 5´-GCG TGG CTT CTT CTA CAC AC-3´ (SEQ ID NO:20), R 5´-GTT CTC CAG CTG GTA GAG GG-3´ (SEQ ID NO:21) and for *Pdx1* were F 5´-GAA ATC CAC CAA AGC TCA CG-3´ (SEQ ID NO:22), R 5´-CGG GTT CCG CTG TGT AAG-3´ (SEQ ID NO:23).

The following primers were used in RT-PCR (see Fig. 1A): primers to amplify full length *Manf* cDNA (540 bp), f1 5´- ACC ATG TGG GCT ACG CGC GGG CT-3´ (SEQ ID NO:24), r1 5´-CTA CAG ATC AGT CCG TGC GCT GGC TG-3´ (SEQ ID NO:25), primers for *β-galactosidase* (510 bp) f3 5´-ACC AGC GAA ATG GAT TTT TG-3´ (SEQ ID NO:26), r3 5´- AGT AAG GCG GTC GGG ATA GT-3´ (SEQ ID NO:27), primers for *Gapdh* (425 bp) 5´- GTGGAAGGGCTCATGACCACAG-3´ (SEQ ID NO:28), 5´-GGAGTTGCTGTTGAAGTCGCAGG-3´ (SEQ ID NO:29).

Comprehensive laboratory animal monitoring system

For metabolic measurements, 6-week-old male WT and MANF-KO mice were used. Mice were then individually placed in CLAMS (Columbus Instruments, Columbus, USA) cages for 60 hrs (2 days and 3 nights) for automated, non-invasive and simultaneous monitoring of feeding and drinking, energy expenditure (O_2 consumption and CO_2 production using indirect calorimetry) and locomotor activity. The lights were on between 6:00 and 18:00. The experiment started at 10:00 and continued for 72 h. The period until beginning of the first dark cycle was allowed for acclimatization. The data from the following 60 hours (3 dark and 2 light periods) were used for analysis and expressed as mean values per hour during light and dark phase, respectively. The samples were recorded every 30 minutes. Body weights were determined just before and after testing.

Food consumption was continuously_monitored from a food container on an electronic weigh scale. The consumption was monitored and stored each time a metabolic measure was recorded. Individual water columns containing pressure transducers supplied drinking water to each cage. Drinking reduced pressure in the column and was converted to volume (ml). Each cage is an indirect open circuit calorimeter that provides measures of oxygen consumption and carbon dioxide production. The system compared oxygen and carbon dioxide gas concentrations by volume at the inlet and outlet ports of the cage chamber through which ambient air flows at a constant rate (0.60 l/min). The difference in concentration between the two ports and the flow information was used to calculate

oxygen consumption (ml/kg/h), carbon dioxide production (ml/kg/h) and the Respiratory Exchange Ratio (RER). Heat production (kcal/h) was also estimated with standard formulas using oxygen consumption and the RER.

An array of infrared beams (2.5 cm inter-beam distance) surrounded each
5 cage. Ambulatory activity was defined as a movement producing sequential horizontal beam breaks of different beams. Activity was measured continuously and recorded with intervals of one hour as number of beam breaks.

Western analysis

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Tissues were dissected and snap frozen in liquid N_2. Thereafter, tissues were homogenized in lysis buffer (300 mM NaCl, 100 mM TrisHCl, 4 mM EDTA, 2% BSA, 0.2% Triton, 2 mM PMSF, 1 mM Sodium Orthovanadate, Protease cocktail inhibitor (Complete, Mini EDTA, Boehringer Mannheim, Germany) using a teflon homogenizer. Tissue lysates were
15 incubated on ice for 15-30 minutes and centrifuged at 13 000 rpm for 30 min. and for MANF protein detection 1 M HCl was added to the supernatant (pH <2), and the samples were incubated 30 minutes on ice. The pH of the samples was neutralized to pH 7.6 using 1 M NaOH. Protein content was determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA). Pancreatic islets were homogenized directly in 1x Laemmli buffer. Proteins
20 were separated by SDS-PAGE and transferred to nitrocellulose membrane. Immunoblot analysis using primary antibodies pEF2α (1:1000, rabbit, Cell Signaling Technology, Danvers, MA, USA), tEIF2α (1:1000, rabbit, Cell Signaling Technology), Gapdh (1:2000, mouse, Millipore Corporation, Billerica, MA, USA), MANF (rabbit, Icosagen, Tartu, EST), ATF6 (1:1000, mouse, Life Span, BioSciences, Seattle, WA, USA) were used to
25 determine protein of interest. Immunoreactivity was detected through chemiluminescence by ECL Blotting Substrate (Pierce, Thermo Scientific, Rockford, USA) or Super Signal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, USA). Pixel intensities of bands from scanned X-ray films were calculated using Image J (Media Cybernetics, Bethesda, MD, USA) software and intensities compared as peIF2α ratio
30 compared to teIF2α and teIF2a to Gapdh.

Immunohistochemistry and quantification of beta- and alpha-cell masses

For immunohistochemistry, mice were anaesthetized with (i.p. chloral hydrate 3.5%
35 solution in 0.9% NaCl, 0.8 ml/adult mouse) and perfused transcardially with 4%

paraformaldehyde (wt/vol, PFA) in phosphate-buffered saline (PBS, pH 7.5) or tissues were postfixed in 4% PFA for at least o/n. For cryosections, tissue samples were cryoprotected overnight in 30% sucrose at +4°C followed by embedding in Tissue Tek® OCT™ compound (Sakura Finetek Europe). Paraffin-embedded 5 μm sections were
5 deparaffinized and subjected to antigen retrieval by treating sections in 10 mM sodium citrate buffer (pH 6.0) or 0.05% citraconic anhydride buffer (pH 7.4) for 10 minutes in microwave oven (800W). For immunoperoxidase staining endogenous peroxidase was quenched by 0.5% H_2O_2 in TBS, 0.1% Tween for 30 minutes prior to blocking. Immunohistochemistry was performed using the following antibodies: insulin (guinea-pig
10 1:200, Abcam, Cambridge, UK), pro-insulin (mouse 1:50, Beta Cell Biology Consortium, Målöv, DK) glucagon (1:150 rabbit, Abcam, Cambridge, UK), Ki67 (1:250 rabbit, Thermo Fisher Scientific, CA, USA), mRFP1 (1:500 rabbit, Invitrogen, Molecular Probes, OR, USA), MANF (1:500 rabbit, Icosagen, Tartu, EST), somatostatin (1:200 rabbit, DAKO, CA, USA), pancreatic polypeptide (1:600 rabbit, Dako, CA, USA), GLUT2 (1:1000 goat,
15 Santa Cruz Biotechnology, Inc., CA, USA). For fluorescence microscopy appropriate secondary antibodies conjugated with Alexa Fluor® 488 or 568 (1:400, Molecular Probes, Life Technologies, CA, USA) were used to visualize the labels. Slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). For detection of signal in light microscopy biotinylated secondary antibody and
20 peroxidase-conjugated streptavidin Vectastain ABC-detection system (Vector Laboratories, Inc., CA, USA) were used. Sections were developed with Vector diaminobenzidine peroxidase substrate kit (Vector Laboratories, Inc., CA, USA) and mounted in DePeX mounting medium Gurr® (BDH Prolabo, VWR, USA). Fluorescence or light microscopy images were captured and analyzed with light microscope Olympus
25 BX61 equipped with a high resolution color digital camera and Cell Life Science Microscopy software (Olympus Soft Imaging Solution, GmbH, Germany) or Zeiss AxioImager M2 482 epifluorescence microscope equipped with 40x/Plan-Apochromat/0.95 Corr M27 and 63x/Plan-Apochromat/1.40 Oil/M27 and 483 AxioCam HRm camera. Images were acquired with the AxioVision4 software.

30 For proliferation analysis, pregnant mice were injected intraperitoneally with BrdU labeling reagent (0.1mg/g body weight, i.p.), Amersham Cell Proliferation Reagent Bromo-deoxyuridine) and sacrificed after 4 hrs. Tissues were collected from embryos, immersed in 4% PFA and processed into paraffin. For antigen retrieval, paraffin sections were boiled for 10 minutes in 10 mM citrate buffer (pH6) and proliferating S-phase cells
35 were detected by BrdU antibody staining (Thermo Fisher Scientific., Inc.). To study beta

cell proliferation, Ki67- and insulin-stained double positive cells were counted from 2-5 randomly chosen pancreas sections. Analysis of exocrine acinar cell proliferation was carried out by counting Ki67- and DAPI-stained double positive cells in exocrine pancreas.

For cell death analysis, In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, Germany) TUNEL System was used followed after last wash by insulin antibody staining and fluorescent Alexa Fluor® 568-conjugated secondary antibody staining.

For beta- and alpha- cell mass analysis pancreases were dissected, weighed and fixed in 4% paraformaldehyde (in PBS) following routine dehydration and paraffin embedding procedures. Pancreases were serially sectioned five sections, one per 200 µm were stained with insulin or glucagon antibodies as described above, counterstained with haematoxylin and morphometrically analyzed in blind for genotype by Image Pro Plus-program (Media Cybernetics, Bethesda, MD, USA) from digitalized photographs taken under light microscope Olympus BX61 equipped with a high resolution color digital camera and Cell Life Science Microscopy software (Olympus Soft Imaging Solution, GmbH, Germany). The beta/alpha cell mass was quantified by multiplying the total pancreatic weight with the relative insulin/glucagon-positive area per total pancreatic tissue area [46,48].

Analysis of blood samples

Blood samples were collected from the tail vein and assayed for glucose (Accucheck Aviva Glucometer, Roche Diagnostics, Mannheim, Germany, glucose range 0.6-33.3 mmol/l) and insulin levels (ultrasensitive mouse insulin ELISA (Crystal Chem. Inc., Downers Grove, IL, USA) (from ad libitum fed mice). For glucose challenge test P14 mice were fasted for 1 h and P56 mice 5-6 h before animals were injected i.p. with 2 g/kg body weight of glucose (in 0.9% NaCl). Blood glucose levels were measured from tail vein 30 minutes after glucose injection and animals were sacrificed for terminal blood collection from heart. Serum was separated from whole blood by centrifugation. Sera were snap-frozen and stored in -70°C until use.

***In vivo* gene therapy study design**

AAV construction, packaging and purification

The construction of the AAV packaging plasmids have been previously described for dsAAV-MANF [5] and for dsAAV-RFP [49]. For the construction of dsAAV-CDNF, the cDNA of human CDNF was excised from pCR3.1 CDNF [2] using KpnI and XbaI restriction endonucleases and used to replaced eGFP in pdsAAV-eGFP [50] using the same restriction enzymes. The resulting plasmid, pdsAAV-CDNF was sequenced and tested for protein production by transfecting HEK293 cells and Western blot analysis.

To generate AAV vectors, viral stocks were prepared using the triple-transfection method with modifications [51]. Briefly, twenty 15 cm dishes containing HEK293 cells at 85–95% confluency were transfected by the $CaCl_2$ method with pHelper (Stratagene, La Jolla, CA), pdsAAV-RFP, pdsAAV-MANF, pdsAAV-CDNF and pAAV6 [52], a plasmid containing the rep/cap genes for serotype 6. Plasmids used for packaging AAV were generously provided by Dr. Xiao Xiao (UNC, Chapel Hill, NC). Approximately 48 hours post-transfection, cells were harvested, lysed by freeze/thaw, and purified by centrifugation on a CsCl gradient. Final samples were dialyzed in PBS, aliquoted and stored at −80°C until use. Viral transduction and expression was verified using rat primary cortical neurons.

In vivo administration of AAV6-vectors

Retrograde pancreatic duct injections were carried out as described previously [42] with some modifications. Mice were anaesthetized with a mixture of ketamine (73 mg/kg) and xylazine (12 mg/kg) and the abdomen was carefully shaved and the skin disinfected. Surgery was performed in sterile conditions and under a dissection microscope. A surgical ventral midline incision (2-3 cm) was made and the abdomen was entered through another ventral midline through the linea alba. The bile duct next to the liver was closed with a microclamp and a needle (30G) was pushed through the duodenum, and advanced retrogradely through the spinchter of Oddi into the common bile duct. The needle was slowly removed and replaced with a 30 G Hamilton needle (Cat. No. 7762-03) attached to a Hamilton syringe (100 µl, Cat. No. 710RN) containing $1x10^{12}$ AAV6 viral genome particles diluted in saline and briefly sonicated (10 s). The Hamilton needle was secured in place by hand, and 100 µl of the fluid containing the virus and Evans blue dye was slowly injected into the pancreatic duct over approximately 1 min. Scattered blue spots in the pancreas marked successful infusion [53]. Throughout the infusion, gut and other internal organs were kept moist using sterile saline. At 1 minute post-injection the cannula was gently and slowly removed from the duct and the microclamp was removed from the bile

duct. The peritoneum and skin was sutured using 6-0 Dafilon® suture (B. Braun Melsungen AG, Germany). Carprofen 5 mg/kg was given for postoperative analgesia s.c. immediately after wound closure and the mouse was returned to its cage placed on a heat pad (+37°C) until recovered. Operated mice were monitored daily. Three weeks after AAV administration, all AAV6-MANF and six AAV6-RFP animals were injected on 5 consecutive days with a low-dose of streptozotocin (40 mg/kg/day, i.p.), freshly dissolved in 0.1 M citrate buffer (pH4.5). Six control mice containing AAV6-RFP were injected with 0.1 M citrate buffer. Body weight and blood glucose levels from tail vein were measured at the initiation of experiment and ~every third day until sacrifice at day 18-19 after the initial STZ-injection. Tissue-samples and terminal blood were collected 18-19 days after the first STZ-injection.

Beta cell proliferation assay

Islets from female, virgin 8 weeks old mice were isolated as described above. Islets were recovered o/n in growth medium and the next day equal numbers of islets/well (70/well) were treated for 5 days with placental lactogen (PL 500 ng/ml) or recombinant human MANF (100 n/ml) or a mixture of both. Half of the medium was changed daily to fresh medium with growth factors. Edu, a nucleoside analog alternatively to BrdU (Click-iT®Edu proliferation kit, Invitrogen,) was be added 48 hrs prior to islet harvesting. Islets were broken with trypsin and centrifuged onto glass slides in cytocentrifuge. Cells were fixed after cytospins and proliferating cells stained with Click-iT AlexaFluor azide color reagent, followed by insulin staining (guinea-pig 1:200, Abcam, Cambridge, UK) o/n at +4°C to detect beta cells. Cells were washed and stained with secondary antibodies conjugated with Alexa Fluor® 488 (1:400, Molecular Probes, Life Technologies, CA, USA). Slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). Twelve images (10 x magnification) were acquired with Fluorescence Zeiss AxioImager M2 482 epifluorescence microscope equipped with 40x/Plan-Apochromat/0.95 Corr M27 and 63x/Plan-Apochromat/1.40 Oil/M27 and 483 AxioCam HRm camera using AxioVision4 software and analysed by Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA) to quantify number of DAPI-positive nuclei. The relative numbers of proliferating beta cells were quantified and compared to wells of three to five repeats/treatment.

Statistical Analysis

Significance of differences between groups was measured by Student´s unpaired two-tailed *t* test. Microsoft Excel software was used for calculation of *P* values. All data are presented as mean ± SEM. Differences between more than two groups were calculated by one-way ANOVA followed by appropriate post-hoc test, using SPSS Inc. PASW Statistics 18 program. For statistical analysis of islet size distribution we used GraphPad Prism 5 and data were subjected to Cruskal-Wallis one-way analysis of variance test and differences were evaluated by Dunn´s Multiple Comparison Test. $P < 0.05$ or * was considered statistically significant.

Results

Generation of the *Manf*-deficient mice

We have developed *Manf*-deficient mice from an embryonic stem cell clone MANF_D06 (EPD0162_3_D06, C57Bl/6N-Manf $^{tm1a(KOMP)Wtsi}$), containing a *beta*-galactosidase reporter cassette with a strong splice acceptor site inserted in the intron between exon 2 and exon 3 of the *Manf* gene, creating a constitutive null mutation in the *Manf* gene through efficient splicing to the reporter cassette (Fig. 1A). *Manf* chimeric mice were generated through morula aggregation using targeted *Manf* ES cells, and routine transgenic methods. Two of the chimeric *Manf* male mice generated heterozygote offspring and the homozygote offspring from both lines harboured similar phenotypes. Therefore we randomly chose one of the lines for systematic phenotypic analysis.

Splicing of the Manf exon 2 to the strong splicing acceptor site preceding the reporter beta-galactosidase cassette generates truncated *Manf* mRNA transcript and a Manf null allele. To ensure that alternative splicing resulting in wild-type (WT) transcripts does not occur in MANF KO tissues, we performed RT-PCR for mRNA isolated from tissues of KO and WT littermate mice using primers amplifying full-length *Manf* mRNA transcript (Fig. 1 C). In most WT tissues, *Manf* mRNA could be detected, whereas no transcript of correct size could be seen in tissues of KO mice (Fig. 1 C and data not shown). In testis and brain, a PCR product of larger size was detected and sequenced but found to be a transcript aberrantly spliced generating a non-sense mRNA (data not shown). In tissues from KO mice β-galactosidase mRNA was amplified accordingly under the *Manf* promoter (Fig. 1C). In agreement with RT-PCR results, high levels of Manf protein could be detected by Manf antibody in Western blot analysis in glandular tissues such as pancreas,

salivary gland and testis from WT mice. No Manf protein was present in tissue lysates from KO mice confirming the specificity of MANF antibody (Fig. 1D). These results confirm an overall successful targeted disruption of the *Manf* gene.

5 **Loss of Manf results in growth retardation, decreased postnatal survival, and diabetes mellitus**

MANF KO mice could already be distinguished from their WT and heterozygote (HZ) littermates at birth based on their smaller body size. The weights of KO E18.5 embryos
10 and new born mice were already markedly reduced compared to the weights of WT and HZ littermates (Fig. 2A, inset). The growth retardation of MANF KO mice became more evident with age, was gender independent and heterozygous mice did not differ from their WT littermates (data not shown). At postnatal day 56 (P56) the difference in body weight between WT and KO mice was 43% (26.5 ± 1.03 g vs. 15 ± 0.68 g). Consistent with the
15 smaller body weight of adult MANF KO mice, most tissues in KO mice were also smaller (35-45 %). Of 1358 offspring generated from MANF HZ inter-crosses genotyped after weaning, 213 (16 %) were MANF KO, 797 (58 %) were MANF HZ, and 348 (26 %) were WT indicating a 36 % lethality in the homozygous animals. This lethality of homozygote mice, mainly the smallest pups, was observed during the first to third postnatal days and
20 was due to maternal cannibalism (data not shown). Compared to their litter-mates, MANF KO survivors were equally active until 8-12 weeks of age after which the health of the mutant mice quickly deteriorated and they had to be sacrificed.

Routine monitoring of cages housing MANF KO mice noted increased water intake and wet bedding indicating high urinary output, classical symptoms for diabetes [32].
25 Therefore, we measured ad libitum fed blood glucose and insulin levels from KO mice and their littermates at different ages. Blood glucose levels were within normal range for KO compared to WT mice at P1 (3.2 ± 0.25 mmol/l vs. 3.7 ± 0.12 mmol/l) and at P14 (6.5 ± 0.41 mmol/l vs. 6.25 ± 0.38 mmol/l) (Fig. 2B). However, at P56 the KO mice showed a severe hyperglycemia (30.6 ± 0.62 mmol/l vs. 9.2 ±0.31 mmol/l) (Fig. 2B). At P14, serum
30 insulin levels were comparable between genotypes (432.2 ± 83 pg/ml vs. 487.8 ± 118 pg/ml) (Fig. 2C). However, significantly reduced levels of insulin could be detected from sera of P28 KO mice compared to WT littermates (194.8 ± 70 pg/ml vs. 1091 ±210 pg/ml). At P56, insulin levels were barely detectable (40.8 ± 5.36 pg/ml vs. 1013.5 ± 86 pg/ml) consistent with the high blood glucose levels at the same time-point.

To determine glucose clearance we challenged fasted P14 and P56 mice with glucose (2g/kg, i.p.) and measured the blood glucose levels and insulin levels 30 minutes after glucose injection. In contrast to WT control mice, P14 KO mice showed a severely compromised glucose tolerance compared to WT control mice after glucose administration (19.4 ± 1.1 mmol/l vs. 8.5 ± 1.79 mmol/l, Fig. 2D). As expected, the blood glucose levels in all glucose challenged P56 KO mice reached maximum detection limit of glucometer whereas WT mice cleared additional glucose well (33.3 ± 0 vs. 12.1 ± 1.04 mmol/l Fig. 2 D). Despite of displaying decreased glucose clearance, P14 mice had similar insulin values after glucose administration (536 pg/ml ± 156 vs. 708 pg/ml ± 241). However, barely detectable levels of insulin could be detected in sera from glucose challenged P56 KO mice compared to WT mice (54 pg/ml ± 41 vs. 2642 pg/ml ± 678). These results show that MANF KO mice gradually became overtly diabetic with age, showing compromised glucose tolerance already at P14.

We next monitored drinking and feeding behaviour and energy expenditure to assess the effects of loss of MANF to energy balance using CLAMS monitoring system in P42 MANF KO and WT mice with body weights of 14.7 ± 0.4 g vs. 24.9 ± 0.5 g. The average food intake did not significantly differ between KO mice and their WT controls during day (1.4 ± 0.18 g vs. 0.84 ± 0.24 g, p= 0.12) or night (2.8 ± 0.13 g vs. 3.43 ± 0.35 g, p= 0.179), (Fig. 3 A). In line with diabetic phenotype, MANF KO mice consumed significantly more water compared to their WT littermates both during day (5.47 ± 1.49 ml vs. 0.92 ± 0.2 ml) and night (11.6 ± 2.4 ml vs. 5.1 ± 0.7 ml) (Fig. 3 B). In contrast to WT littermates, MANF KO mice displayed decreased oxygen consumption and CO_2 production suggesting that decreased metabolic rate in MANF KO mice is a direct result of low body mass (Fig.3 C and D). However, in comparison to WT animals, the respiratory exchange ratio (RER) was increased during night in MANF KO mice indicating predominant utilization of fat as energy substrate (0.87 ± 0.009 vs. 0.97 ± 0.013). In both day and night, locomotor activity did not differ significantly between groups (Fig. 3 F).

As an independent assessment of diabetic phenotype, we removed the β-galactosidase reporter cassette by crossing of MANF heterozygote mice to deleter Flp (CaqFlp) transgenic mice generating $MANF^{\Delta Gal/+}$ mice, where the β-galactosidase cassette between Frt-sites was removed (Fig. 1 A). Homozygous $MANF^{\Delta Gal/\Delta Gal}$ mice were viable and fertile and expressed *Manf* mRNA transcript (data not shown). To recapitulate the MANF KO phenotype, *Manf* exon 3 was conditionally removed by crossing the $MANF^{\Delta Gal/\Delta Gal}$ mice with deleter Cre mice (PgkCre). Homozygous removal of exon 3 ($MANF^{fl/fl}$) in all cells resulted in truncated *Manf* mRNA, where exon 2 was spliced to

exon 4 leading to frame-shift and a premature stop codon. The PGK-Cre:MANF$^{fl/fl}$ mice developed a similar severe diabetic phenotype in the same time-frame as the global MANF KO mice (data not shown), confirming the specific beta cell phenotype.

5 **Progressive reduction in MANF KO pancreatic beta cell mass results from low beta cell proliferation and enhanced apoptosis**

The beta cells, the most abundant cell type in the islet, secrete insulin in response to high blood glucose levels, which stimulates the uptake of glucose by cells of the body and 10 stimulates the conversion of glucose to glycogen. Glucagon, secreted by the islet alpha cells, is released in response to low blood sugar levels, to help to convert glycogen into glucose in the liver and therefore increase blood glucose levels. Progressive hyperglycemia and hypoinsulinemia in MANF KO mice prompted us to study the pancreas in more detail at different developmental stages by histology. Staining of pancreatic sections with insulin 15 antibody revealed smaller islets in MANF KO pancreas at P1 but no visible difference in islet architecture compared to WT (Fig. 4A, B). In P14 MANF KO pancreas, we found smaller islets, loss of beta cell architecture and weak insulin staining of many beta cells compared to WT (Fig. 4C, D). In P56 MANF KO pancreas most of the islet insulin-positive beta cells were lost (Fig. 4E, F), which correlates well with the low serum insulin 20 levels in the MANF KO mice at this age. Islet mass per pancreas was calculated by multiplying relative insulin positive area with wet mass of pancreas. Relative insulin-positive area compared to pancreas area was quantified for sections of E16.5 pancreas, as the pancreas was too small at that age for accurate pancreas weight. We found no difference in the insulin-positive area between genotypes at E16.5 (data not shown). 25 Furthermore, the beta cell mass was equal in E18.5 pancreases from MANF KO and WT mice (0.159 ± 0.006 mg vs. 0.173 ± 0.006 mg) confirming that beta cell neogenesis from endocrine progenitor cells was not compromised (Fig. 4G). However, one day after birth, the beta cell mass was reduced by 50% in MANF KO compared to WT pancreas (0.142 ± 0.006 mg vs. 0.293 ± 0.013 mg, $p< 0.001$) suggesting defects in the early postnatal 30 expansion of beta cells (Fig. 4G). At P14 the beta cell mass for KO pancreas was reduced by 60% compared to WT (0.65 ± 0.01 mg vs. 1.61 ± 0.08 mg, $p< 0.0001$) and for P56 adult MANF KO pancreas the beta cell mass was reduced by 85% compared to that of WT pancreas (0.33 ± 0.01 mg vs. 2.25 ± 0.16 mg, $p<0.001$). Consequently, post-natal age-dependent progressive reduction in beta cell mass was the reason for reduced serum insulin 35 levels and diabetes in MANF KO mice.

Glucagon immunohistochemistry was performed on MANF KO and WT pancreas sections in order to study whether *Manf*-deficiency also affected the glucagon-producing alpha cells in the islets (Fig. 5 A-F). Instead of localizing in the periphery of the islets as in WT pancreas, the glucagon-producing alpha cells were found to be dispersed in the core of the islets at P14 and P56 in the MANF KO pancreas (Fig. 5 D and F). However, we found no difference in the alpha cell mass quantified from pancreases of animals of both genotypes at different stages (Fig. 5G).

The islet beta cells are generated through neogenesis of endocrine progenitor cells but massive beta cell proliferation with waves of apoptosis leads to expansion of beta cell mass between E16 and first weeks of life but slows down considerably in adult animals [33].

To study the mechanism which contributes to progressive beta cell reduction, we analysed proliferation and cell death in MANF KO and WT mice at various developmental stages. There was no difference between genotypes in the number of BrdU-labelled insulin-positive beta cells quantified from pancreases taken from E16.5 and E18.5 embryos, derived from heterozygote pregnant mice injected with BrdU (data not shown). Furthermore, there was no difference in the relative number of proliferating beta cells in the MANF KO and WT pancreases at E18.5 quantified from Ki67 and insulin-stained sections (Fig. 4 H,). However, at P1, the peak beta cell proliferative phase, when 17% of all beta cells were replicating in WT pancreas, the relative number of proliferating beta cells in MANF KO pancreases was only 7.7% (Fig. 4 H). At P14, the relative beta cell proliferation rate had reduced to 7% (± 0.21%) in WT pancreas whereas it was significantly lower in the MANF KO pancreas (4.2 ± 0.22%) (Fig. 4H). To study whether the decline in proliferation was specific only for beta cells in the pancreas, we quantified also relative number of Ki67-positive acinar nuclei of the exocrine part of the pancreas. There were similar relative numbers of Ki67-positive DAPI-stained acinar nuclei in pancreas of both genotypes indicating that *Manf*-removal does not affect acinar cell proliferation rate (Fig. 4 I).

To study whether apoptosis contributed to the beta cell loss, we analysed beta cell death by TUNEL staining followed by insulin immunohistochemistry and counted the number of the beta cells double positive for TUNEL and insulin staining in the islets (). No significant differences in the relative number of dying beta cells was found between MANF KO and WT pancreases at P1 (0.7 ± 0.05% vs. 0.62 ± 0.18%, *n.s.*) but in P14 and in P56 MANF KO islets the cell death was significantly increased (1.52 ± 0.18% vs. 0.59 ± 0.04%, $p<0.01$ and 3.24 ± 0.22% vs. 0.51 ± 0.06%, $p<0.001$ respectively) (Fig. 4 J).

Results based on the quantification of proliferating and dying beta cells demonstrated that the observed difference in beta cell mass between MANF KO and WT pancreas from P1 and older animals was mainly due to a significantly reduced beta cell proliferation rate which at P14 was accompanied by higher rate of beta cell death.

5

Manf is expressed in the pancreatic beta cells and exocrine acinar cells and is needed to maintain beta cell specific phenotype

Manf mRNA and protein has been found to be widely expressed in mouse embryonic and
10 adult tissues [7]. Expression has been detected in mouse pancreatic acinar cells at E17 and in adult pancreatic islet cells by immunohistochemistry [7,10]. We studied in more detail the expression of Manf in mouse pancreas by double immunohistochemistry using MANF KO pancreas as specificity control for the Manf antibody. High Manf expression was co-localized with insulin positive beta cells in islets. Manf was not found in the glucagon-
15 producing alpha cells, in the somatostatin-producing delta cells or in the pancreatic polypeptide producing cells (data not shown). However, Manf-positive immunostaining at different intensity was found in the exocrine acinar and duct cells. Importantly, none to very weak background staining of Manf could be detected on sections from KO pancreas. Similarly to expression in mouse, we detected MANF expression in the islets and exocrine
20 tissue of human embryonic and adult pancreas tissue. The MANF antibody staining pattern for human was in line with the MANF expression data found for human pancreas (http://www.proteinatlas.org/ENSG00000145050/normal).

Next we set out to elucidate other changes in beta cell phenotype of MANF KO mice. GLUT2, a low-affinity glucose transporter, is present on the plasma membrane
25 of pancreatic beta cells, hepatocytes and intestine and it has been proposed to control glucose-stimulated insulin release [34]. Its expression is reduced in beta cells of different animal models of diabetes and total disruption of Glut2 leads to reduced insulin biosynthesis and release from beta cells and premature death [35]. We examined *Glut2* mRNA expression in WT and MANF KO islets by quantitative PCR and found
30 significantly reduced expression of *Glut2* mRNA already in islets of P1 MANF KO pancreases (Fig. 6 A) and declining in islets from P14 and P56 KO mice (Fig. 6 B and C). In comparison to WT mice, also immunohistochemical staining indicated reduced expression of GLUT2 protein at P14 and P56 in MANF KO beta cells accompanied with a reduced cell membrane localization (Fig. 6H and I).

In mouse pancreas pre-proinsulin is encoded by two different genes, *Ins1* and *Ins2* giving rise to identical peptides [36]. In line with reduced beta cell mass in P1 MANF KO pancreas, a reduction in *Ins1/Ins2* mRNA was found by quantitative PCR (6A). Interestingly, there was a progressive decline (from P1 to P56 KO islets) in *Ins1* and *Ins2* mRNA expression, supporting the observation that KO beta cell mass declines progressively with age (Fig. 6 A-C).

Transcription factor pancreatic and duodenal homeobox factor-1 (Pdx1) plays a crucial role in pancreas development, beta cell differentiation and in maintaining beta cell function [37]. Furthermore, Pdx1 is important for the transactivation of the insulin and glucose transporter 2 (*Glut2*) genes [38]. Therefore we studied whether the expression of *Pdx1* mRNA was differently regulated in islets of MANF KO pancreas at different developmental stages. Reduced Pdx1 mRNA expression was observed in islets isolated from P1, P14 and P56 MANF KO pancreases, however in P14 the reduction did not reach statistical significance (Fig. 6 C). Maf transcription factors are important for the final stage of the beta cell maturation, and may regulate replication/survival and function of beta cells after birth [39]. While MafB is produced by immature beta cells, postnatal maturation is accompanied with switch to MafA expression. Therefore, we studied whether the expression of MafA is differently regulated in P1, P14 and P56 MANF KO islets. We found significant reduction in MafA mRNA expression already in P1 KO islets (Fig. 6 A) probably contributing to the reduced proliferation of beta cells.

In conclusion, we found reduced expression of beta cell specific marker genes *Glut2*, *Ins1/2*, *Pdx1* and *MafA* in neonatal MANF KO islets, indicating the progressive loss of beta cell phenotype, reduced proliferation and enhanced beta cell death.

Loss of MANF results in activated endoplasmic reticulum stress and unfolded protein response pathways

Gradual decline in the beta cell number is a typical hallmark of diabetes. As ER stress is one proposed mechanism underlying the beta cell decline in diabetes and Manf is shown to play a role in ER stress and possibly in UPR *in vitro* we set out to study whether UPR genes and proteins are differently regulated in MANF KO islets. For this purpose, expression of common ER stress- and UPR-genes was studied by quantitative real-time PCR using islets isolated from MANF KO and WT pancreases at different developmental stages. Slightly higher, but not statistically significant, levels of *Atf4*, *Grp78*, *Chop*, spliced *Xbp1* and *Atf6α* mRNA expression was observed in KO P1 islets compared to WT (Fig. 7 A). A significant elevation of mRNA levels for *Atf4*, *Grp78*, *Chop* and *Atf6α* was found in

the islets isolated from P14 KO pancreas compared to mRNA levels in WT islets (Fig. 7 B). In addition, the mRNA levels for spliced *Xbp1* were elevated in P14 MANF KO islets but did not reach statistical significance ($p = 0.11$, Fig. 7 B). Taken together, out results demonstrate that Grp78, the general marker for ER stress and several genes in PERK and ATF6 pathways are upregulated in the MANF KO islets.

To study levels of activated eIF2α, Western blot was carried out from lysates of islets from pancreases of individual animals blotted against phosphorylated (p)eIF2α, total (t)eIF2α and Gapdh antibodies (Fig. 7 C). Quantification of pEIF2α/tEIF2α ratio revealed a significantly higher level of phosphorylated eIF2α in P14 and P56 KO islets compared to WT islets (Fig. 7 D). In P28 KO islets the levels of pEIF2α was higher compared to WT islets but did not reach statistical significance. Thus, higher levels of activated EIF2α in KO islets studied at three different time-points suggested that the PERK pathway was chronically activated in islets of MANF KO.

For clinical translation, it is essential to validate the mouse studies in human islets. Interleukin (IL)-1α and interferon (IFN)-α, are well-established mediators that trigger ER stress and apoptotic death of human beta cells in type 1 diabetes [27]. It has recently been shown that IL-17 markedly potentiates the beta cell targeted toxicity of these cytokines [40]. Our preliminary results demonstrate that cytokine exposure appears to upregulate MANF expression in the human islets (Fig. 7 E), suggesting that MANF may modify the responses against these cytokines and can be protective against cytokine-induced ER stress.

MANF induces proliferation of pancreatic beta cells after streptozotocin-induced beta cell death in mouse

As Manf-removal resulted in reduced beta cell proliferation and survival we next examined whether gene therapy using over-expression of MANF could result in enhanced beta cell proliferation and survival *in vivo*. To study the beta cell specific proliferative effect of MANF *in vivo*, we used streptozotocin-induced beta cell depletion, followed by intrapancreatic delivery of MANF-expressing adeno-associated virus serotype 6 (AAV6). Streptozotocin (STZ) is an antimicrobial toxic glucose analogue that preferentially accumulates in pancreatic beta cells via the GLUT2 glucose transporter which modifies macromolecules and fragments DNA and eventually destroys the beta cells [41]. Double-stranded (ds) AAV6 which has been used previously for pancreatic gene delivery was used for gene-therapy [42,43]. Ds AAV6 expressing MANF or Mouse Red Fluorescent Protein 1 (mRFP1) was injected into the pancreases of 6-week-old NMRI mice through the common

bile duct using $1x10^{11}$vg/mouse in 100 μl saline. Three weeks after AAV6-administration, animals were injected for 5 consecutive days using low-dose streptozotocin administration (5x40 mg/kg, i.p.) to induce beta cell deficiency [44]. As additional control groups, six mice treated with AAV6-RFP were given sodium citrate buffer instead of STZ. Blood glucose levels were monitored and weights were measured every third day after the first STZ-injection. We observed no significant weight reduction in STZ-treated mice through the whole experiment. As expected hyperglycemia (>15 mmol/l) could be detected in STZ-treated animals 8 days after the first injection. To study transduction efficiency of AAV6, the expression of mRFP1 and MANF in the pancreases of injected mice was carried out. Immunohistological analysis from AAV6-RFP injected pancreases revealed patchy expression of mRFP1 in exocrine tissue accompanied with expression in a small population of islet beta cells (Fig. 8 B, C, F and G). Analysis of transduction efficiency in exocrine- and endocrine pancreas was carried out by quantifying the RFP-positive pancreatic exocrine area and the relative number of RFP-antibody stained insulin positive cells in AAV6-RFP-injected buffer-treated animals. Quantification revealed successful gene-delivery to 4.2 ± 1.5% of the exocrine acinar cells, and 4.3 ± 1.5% of the islet beta cells. Importantly, RFP expression was not observed in other tissues examined (liver, kidney, spleen and brain), indicating pancreas specific gene delivery (data not shown).

Next we studied whether virus-delivered MANF was over-expressed in the pancreases of AAV6-MANF-injected mice. Stronger MANF positive antibody staining was observed in AAV6-MANF vs. AAV6-RFP-injected pancreases, confirming a successful delivery and over-expression of MANF (Fig. 8 I-K).

STZ-induced beta cell loss could be detected in the islets from mice treated with STZ (Fig. 8 E, I) compared to the buffer injected (Fig. 8 A). To study the effect of gene therapy on pancreatic morphology, islet profiles were quantified from insulin stained pancreatic sections on STZ-injected and AAV6-MANF and AAV6-RFP treated mice (Fig. 8 M). Overall islet size in the AAV6-MANF-treated (Fig. 8 L) pancreatic sections stained with insulin antibody seemed larger compared to the STZ-injected, AAV6-RFP-treated mice (Fig. 8 H). Importantly, injection of AAV6-MANF resulted in increased islet size compared to administration of AAV6-RFP, indicating successful gene therapy using MANF (Fig. 8 M).

Quantification of Ki67 positive insulin-expressing beta cells revealed that the relative number of proliferating beta cells was significantly higher in the AAV6-MANF-treated STZ-injected group compared both to the AAV6-RFP buffer-injected and the AAV6-RFP STZ-injected (Fig. 8 N), demonstrating that over-expressed MANF could enhance beta cell

proliferation *in vivo*. Importantly, we found no significant difference in the number of proliferating exocrine acinar cells between groups, suggesting that proliferation promoting effect of MANF is specific for endocrine pancreas (Fig. 8 O). In line with beta cell deficiency, increased blood plasma glucose and decreased plasma insulin levels were observed in STZ treated animals (Fig. 8 P and Q). However, the blood glucose and insulin levels were comparable between AAV6-RFP and AAV6-MANF STZ-injected groups. Taken together, the current data indicates that gene therapy using MANF is able to promote normal islet morphology and enhance beta cell proliferation, but likely due to low transduction efficiency is not sufficient to rescue drug-induced hyperglycemia and insulin deficiency.

MANF affects beta cell proliferation *in vitro*

Indirect evidence from the global MANF KO mice and from the mice overexpressing MANF through AAV6 gene delivery in the pancreas *in vivo* suggests that MANF is important for the postnatal beta cell growth and survival. Therefore, we assessed whether MANF had any direct effect on mouse beta cell proliferation *in vitro*. Results from four independent experiments show a proliferative effect for recombinant human MANF measured by EdU incorporation in the DNA of beta cells in cultured isolated islets from adult mice (Fig. 9). Importantly, MANF together with placental lactogen (PL) showed an additive effect in the proliferation efficiency suggesting an independent signalling pathway for MANF. Hence, extracellular MANF seems to have a direct proliferative effect on mouse beta cells suggesting a yet unidentified receptor on the beta cells mediating intracellular mitogenic cascades.

CDNF also induces proliferation of pancreatic beta cells after streptozotocin-induced beta cell death in mouse

In contrast to MANF-removal in mice, CDNF-deficient mice are viable and fertile and do not develop pancreatic beta cell deficiency and diabetes. However, CDNF mRNA is expressed in pancreatic islets of Langerhan´s. Therefore, we examined whether gene therapy using over-expression of CDNF could result in enhanced beta cell proliferation and survival *in vivo*. In the same *in vivo* experiment as with MANF-AAV6 we used streptozotocin-induced beta cell depletion, followed by intrapancreatic delivery of CDNF-expressing adeno-associated virus serotype 6 (AAV6). Ds AAV6 expressing CDNF was injected into the pancreases of 6-week-old NMRI mice through the common bile duct

using $1x10^{11}$vg/mouse in 100 μl saline. Three weeks after AAV6-administration, animals were injected for 5 consecutive days using low-dose streptozotocin administration (5x40 mg/kg, i.p.) to induce beta cell deficiency. Blood glucose levels were monitored and weights were measured every third day after the first STZ-injection. We observed no significant weight reduction in STZ-treated mice through the whole experiment. As expected hyperglycemia (>15 mmol/l) could be detected in STZ-treated animals 8 days after the first injection. Virus-delivered over-expressed CDNF was detected from AAV6-CDNF-injected pancreas by in-house-built CDNF ELISA[75]. Homogenized pancreatic tissue from STZ-treated AAV6-CDNF injected mice contained ~37 ± 1.5 ng/ml whereas pancreatic tissue from AAV6-RFP injected mice contained 0.08 ± 0.02 ng/ml CDNF protein. Pancreas from control mice not treated with STZ contained 0.24 ± 0.9 ng/ml CDNF protein measured by CDNF ELISA.

To study the effect of gene therapy on pancreatic morphology, islet profiles were quantified from insulin stained pancreatic sections on STZ-injected and AAV6-CDNF and AAV6-RFP treated mice. Overall islet size in the AAV6-CDNF-treated pancreatic sections stained with insulin antibody seemed larger compared to the STZ-injected, AAV6-RFP-treated mice. Importantly, injection of AAV6-CDNF resulted in increased islet size compared to administration of AAV6-RFP, indicating successful gene therapy using CDNF (Fig. 10 A).

Quantification of Ki67 positive insulin-expressing beta cells revealed that the relative number of proliferating beta cells was significantly higher in the AAV6-CDNF-treated STZ-injected group compared both to the AAV6-RFP buffer-injected and the AAV6-RFP STZ-injected (Fig. 10 B), demonstrating that over-expressed CDNF could enhance beta cell proliferation *in vivo*. Importantly, we found no significant difference in the number of proliferating exocrine acinar cells between groups, suggesting that proliferation promoting effect of MANF is specific for endocrine pancreas. In line with beta cell deficiency, increased blood plasma glucose and decreased plasma insulin levels were observed in STZ treated animals. However, the blood glucose and insulin levels were comparable between AAV6-RFP and AAV6-CDNF STZ-injected groups. Taken together, the current data indicates that gene therapy using MANF is able to promote normal islet morphology and enhance beta cell proliferation, but likely due to low transduction efficiency is not sufficient to rescue drug-induced hyperglycemia and insulin deficiency.

Taken together, the current data indicates that gene therapy using CDNF is able to promote normal islet morphology and enhance beta cell proliferation.

EXAMPLE 2

5 **Assessment of diabetic phenotype in independent *Manf*$^{flox/flox}$ knockout mouse lines**

To confirm that the diabetic phenotype in the global *Manf*$^{-/-}$ mice was due to MANF-removal from the pancreatic beta cells and not secondary due to lack of MANF in other tissues, we first removed the β-galactosidase reporter cassette by crossing of *Manf*$^{+/-}$ mice
10 to *CAG-Flp* transgenic mice generating *Manf*$^{flox/flox(fl/fl)}$ mice, where the β-galactosidase cassette between *Frt*-sites was removed (Figure 11 A). Homozygous *Manf*$^{fl/fl}$ mice were viable and fertile and expressed *Manf* mRNA transcript (data not shown). To recapitulate the *Manf*$^{-/-}$ phenotype, *Manf* exon 3 was conditionally removed by crossing the *Manf*$^{fl/fl}$ mice with ubiquitously *Cre* expressing deleter *Cre* mice (*PGK-Cre*). Homozygous removal
15 of exon 3 (*PGK*$^{Cre/+}$::*Manf*$^{fl/fl}$) in all cells resulted in truncated *Manf* mRNA (Figure 11 B) where exon 2 was spliced to exon 4 leading to frame-shift and a premature stop codon. The *PGK*$^{Cre/+}$::*Manf*$^{fl/fl}$ mice developed a similar severe diabetic phenotype, in the same time-frame as the global *Manf*$^{-/-}$ mice, confirming the specific β-cell phenotype (Figure 11 C-D). *PGK-Cre* (Cre expressed under the phosphoglycerate kinase-1 promoter) mice were
20 originally described in (Lallemand et al., 1998). To confirm that the diabetic phenotype was caused by MANF-removal from the pancreas we crossed the *Manf*$^{fl/fl}$ mice with transgenic mice expressing *Cre* under the mouse *Pdx1* (pancreatic and duodenal homeobox 1) promoter, which has been shown to direct *Cre* expression throughout the early pancreatic epithelium and in adult is restricted mainly to endocrine pancreas (Hingorani et
25 al., 2003). Bi-allelic removal of *Manf* by *Pdx1-Cre* resulted in the development of diabetes, albeit less severe as for the conventional *Manf*$^{-/-}$ mice (Figure 11 E-F), which reflected the fact that MANF was removed from most but not all β-cells in *Pdx1*$^{Cre/+}$:: *Manf*$^{fl/fl}$ pancreas (data not shown). Nestin is a marker for neuronal stem cells but is also expressed in multipotent stem cells of the pancreatic primordium that generate both exocrine and
30 endocrine cells (Keedes et al., 2007). However, cell linage analysis in *Nestin-Cre* mice show Cre-mediated recombination in pancreatic exocrine cell lineage but not in the islet endocrine progenitor cells (Delacour et al., 2004). Specific removal of MANF by crossings to *Nestin*$^{Cre/+}$ mice did not result in diabetes assayed from 1.25-1.5 year old *Nestin*$^{Cre/+}$:: *Manf*$^{fl/fl}$ mice (Figure 11 G-H), highlighting the indispensable role of pancreatic MANF in
35 regulation of beta cell mass.

Global loss of MANF in mice results in growth retardation

5 We found that global MANF-deficient mice display progressive gender independent growth retardation. Furthermore, ubiquitous removal of MANF in $PGK^{Cre/+}$::$Manf^{fl/fl}$ resulted in the same growth defect. Conversely, removal of MANF exclusively from the pancreas in $Pdx1^{Cre/+}$:: $Manf^{fl/fl}$ mice or from the brain in 1.25-1.5 year old $Nestin^{Cre/+}$:: $Manf^{fl/fl}$ mice does not result in a growth defect (Figure 12), suggesting that MANF is an
10 important regulator of body size growth.

Table 1. MANF and CDNF amino acid sequences.

Mature MANF amino acid sequence:

LRPGDCEVCI SYLGRFYQDL KDRDVTFSPA TIENELIKFC REARGKENRL
CYYIGATDDA ATKIINEVSK PLAHHIPVEK ICEKLKKKDS QICELKYDKQ
IDLSTVDLKK LRVKELKKIL DDWGETCKGC AEKSDYIRKI NELMPKYAPK
AASARTDL (SEQ ID NO:1)

MANF amino acid sequence with a signal sequence:

MWATQGLAVA LALSVLPGSR ALRPGDCEVC ISYLGRFYQD LKDRDVTFSP
ATIENELIKF CREARGKENR LCYYIGATDD AATKIINEVS KPLAHHIPVE
KICEKLKKKD SQICELKYDK QIDLSTVDLK KLRVKELKKI LDDWGETCKG
CAEKSDYIRK INELMPKYAP KAASARTDL (SEQ ID NO:2)

Mature CDNF amino acid sequence:

QEAGGRPGAD CEVCKEFLNR FYKSLIDRGV NFSLDTIEKE LISFCLDTKG
KENRLCYYLG ATKDAATKIL SEVTRPMSVH MPAMKICEKL KKLDSQICEL
KYEKTLDLAS VDLRKMRVAE LKQILHSWGE ECRACAEKTD YVNLIQELAP
KYAATHPKTE L (SEQ ID NO:3)

CDNF amino acid sequence with a signal sequence:

MWCASPVAVV AFCAGLLVSH PVLTQGQEAG GRPGADCEVC KEFLNRFYKS
LIDRGVNFSL DTIEKELISF CLDTKGKENR LCYYLGATKD AATKILSEVT
RPMSVHMPAM KICEKLKKLD SQICELKYEK TLDLASVDLR KMRVAELKQI
LHSWGEECRA CAEKTDYVNL IQELAPKYAA THPKTEL (SEQ ID NO:4)

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CLAIMS

1. A genetically-modified non-human animal comprising a disrupted allele for the gene that naturally encodes and expresses a functional MANF gene, wherein the disrupted allele is a non-functional MANF gene and said animal displays progressive postnatal reduction of pancreatic beta cell mass due to the disrupted non-functional MANF gene.

2. The non-human animal according to claim 1, wherein the animal is homozygous for disruption of the MANF gene.

3. The non-human animal according to claim 1 or 2, wherein said animal displays type 1 diabetes.

4. The non-human animal according to any one of claims 1-3, wherein said animal is a mouse or rat.

5. The non-human animal according to any one of claims 1-4, wherein the non-functionality of the MANF gene is caused by a non-conditional mutation to the MANF gene.

6. The non-human animal according to any one of claims 1-4, wherein the non-functionality of the MANF gene is caused by a conditional mutation to the MANF gene.

7. A method of screening a compound for preventing pancreatic beta cell apoptosis and dysfunction, the method comprising: (a) administering said compound to a non-human animal according to any one of claims 1-6; (b) observing the effect of said compound on said animal; and (c) relating the observed effect of said compound on said animal to the beta cell preservation and/or regeneration activity of said compound, wherein a compound showing said preservation and/or regeneration activity is a candidate compound for the treatment of type 1 diabetes.

8. Use of the non-human animal according to claim any one of claims 1-6 as a model of postnatal pancreatic beta cell death.

9. The use according to claim 8, wherein said non-human animal is used as a model of type 1 diabetes.

10. A method of screening a compound for regulation of body weight, the method
5 comprising: (a) administering said compound to a non-human animal according to claim 5; (b) observing the effect of said compound on said animal; and (c) relating the observed effect of said compound on the body weight of said animal, wherein a compound showing body weight regulating activity is a candidate compound for the treatment of body weight related conditions such as obesity.
10

11. Use of the non-human animal according to claim 5 as a model of body weight regulation in mammals.

12. A method of producing a genetically-modified non-human animal according to any one
15 of claims 1-6, the method comprising: mating non-human animals heterozygous for disruption of the MANF gene to produce a non-human animal homozygous for said MANF disrupted allele and identifying progeny displaying progressive postnatal reduction of pancreatic beta cell mass and in which both alleles of the endogenous MANF gene are disrupted.
20

13. The method according to claim 12, wherein said non-human animal is a mouse or rat.

14. A gene therapy vector expressing effective amount of a CDNF polypeptide comprising the sequence of SEQ ID NO:3 or a functional fragment thereof for use in the
25 intrapancreatic treatment of pancreatic beta cells.

15. The gene therapy vector according to claim 14, wherein said CDNF polypeptide comprises SEQ ID NO:4.

30 16. The gene therapy vector according to claim 14 and 15, wherein said vector expresses effective amount of a CDNF-MANF hybrid polypeptide.

17. The gene therapy vector according to any one of claims 14-16, wherein said treatment results in regeneration of pancreatic beta cells.

35

18. The gene therapy vector according to any one of claims 14-17 for use in the treatment of type 1 or type 2 diabetes.

19. An isolated host cell transfected with the gene therapy vector as defined in any one of
5 claims 14-16, wherein said host cell secrets a CDNF polypeptide, for use in the intrapancreatic treatment of pancreatic beta cells.

20. The host cell according to claim 19, wherein said treatment results in regeneration of pancreatic beta cells.
10

21. The host cell according to claim 19 or 20 for use in the treatment of type 1 or type 2 diabetes.

22. A gene therapy vector expressing effective amount of a MANF polypeptide comprising
15 the sequence of SEQ ID NO:1 or a functional fragment thereof for use in the intrapancreatic treatment of pancreatic beta cells.

23. The gene therapy vector according to claim 22, wherein said MANF polypeptide comprises the sequence of SEQ ID NO:2.
20

24. The gene therapy vector according to claim 22 or 23, wherein said treatment results in regeneration of pancreatic beta cells.

25. The gene therapy vector according to any one of claims 22-24 for use in the treatment
25 of type 1 or type 2 diabetes.

26. An isolated host cell transfected with the gene therapy vector as defined in claim 22 or 23, wherein said host cell secrets a MANF polypeptide, for use in the intrapancreatic treatment of pancreatic beta cells.
30

27. The host cell according to claim 26, wherein said treatment results in regeneration of pancreatic beta cells.

28. The host cell according to claim 26 or 27 for use in the treatment of type 1 or type 2
35 diabetes.

29. A pharmaceutical composition comprising a gene therapy vector or a host cell according to any one of claims 14-28 for use in the intrapancreatic treatment of pancreatic
5 beta cells.

30. The pharmaceutical composition according to claim 29 for use in the treatment or prevention of type 1 or type 2 diabetes.

10 31. Method of treating a subject suffering from type 1 or type 2 diabetes, the method comprising: administering by intrapancreatic delivery to the subject a gene vector as defined in any one of claims 14-18 or a host cell as defined in claims 19-21.

32. Method of treating a subject suffering from type 1 or type 2 diabetes, the method
15 comprising: administering by intrapancreatic delivery to the subject a gene vector as defined in any one of claims 22-25 or a host cell as defined in claims 26-28.

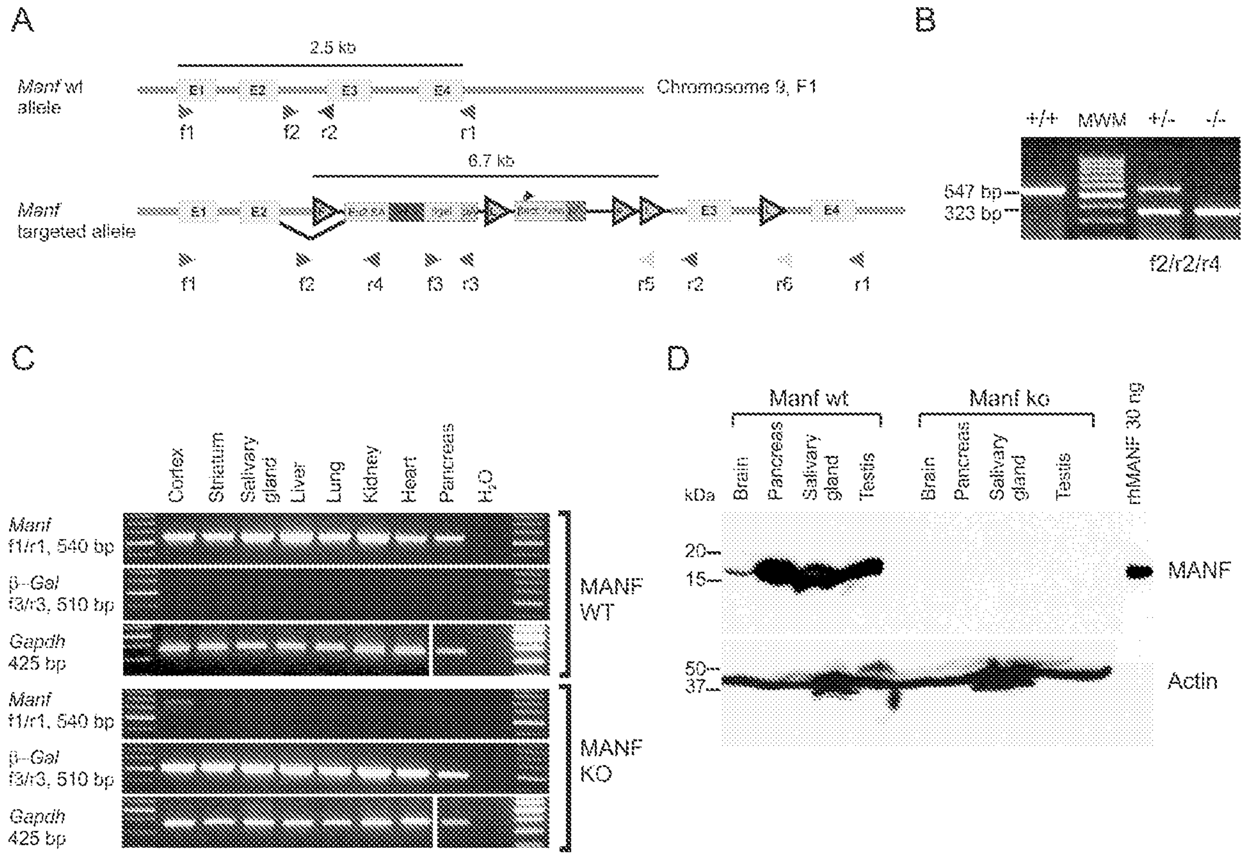
33. Use of a nucleotide sequence encoding a CDNF polypeptide comprising the sequence of SEQ ID NO:3 or a functional fragment thereof for the manufacture of a gene therapy
20 vector or a host cell comprising said vector for intrapancreatic treatment of pancreatic beta cells.

34. The use according to claim 33, wherein said vector is for the treatment of type 1 or type 2 diabetes.
25

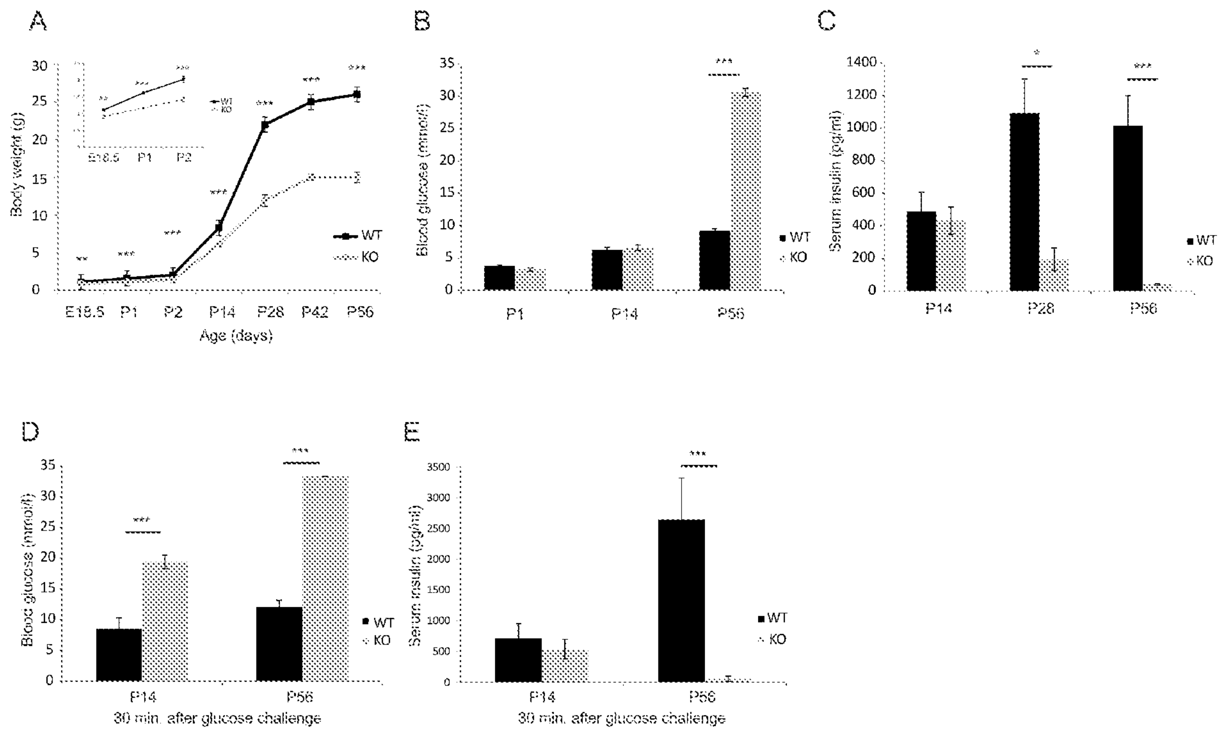
35. Use of a nucleotide sequence encoding a MANF polypeptide comprising the sequence of SEQ ID NO:1 or a functional fragment thereof for the manufacture of a gene therapy vector or a host cell comprising said vector for intrapancreatic treatment of pancreatic beta cells.
30

36. The use according to claim 35, wherein said vector is for the treatment of type 1 or type 2 diabetes.

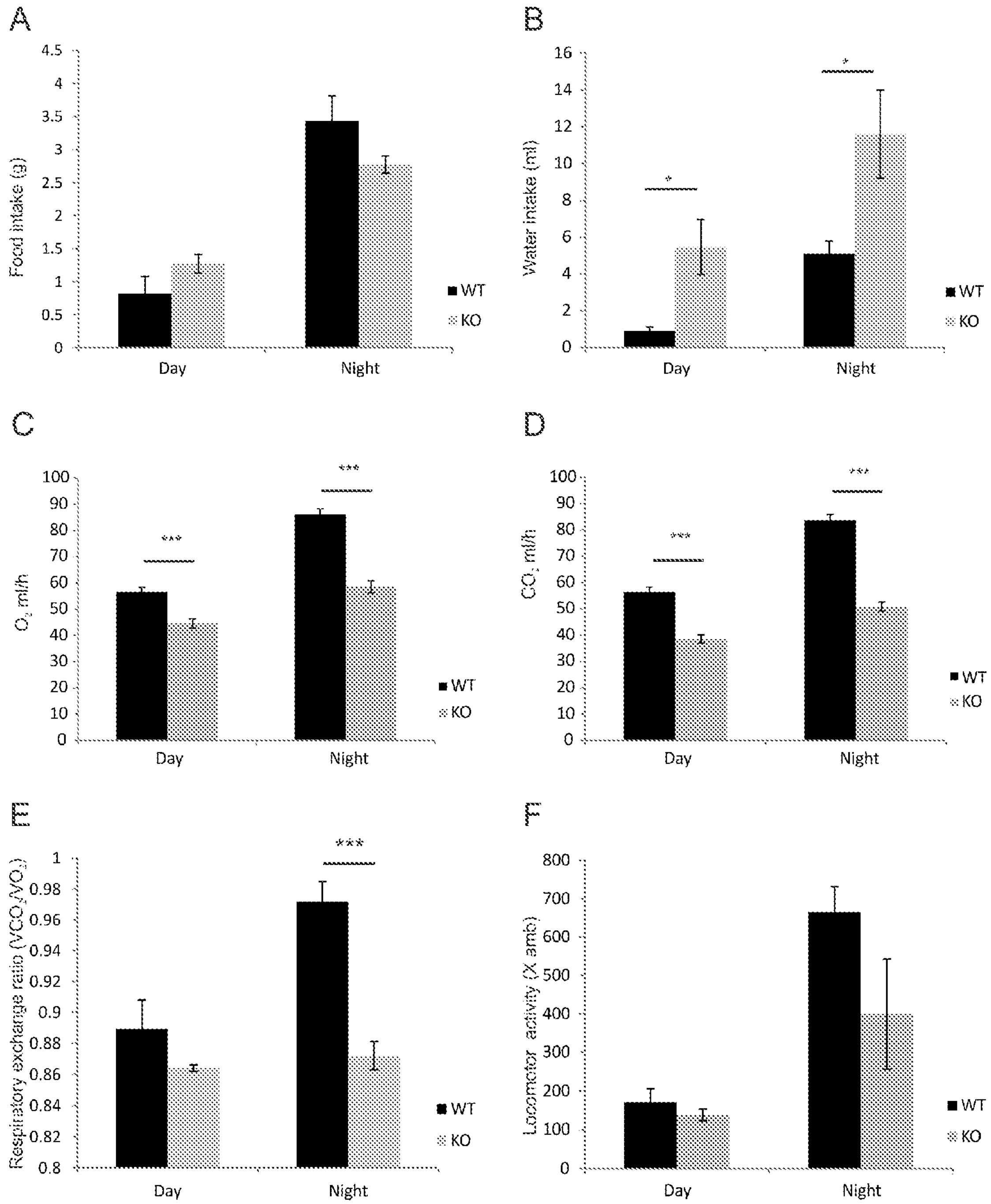
35



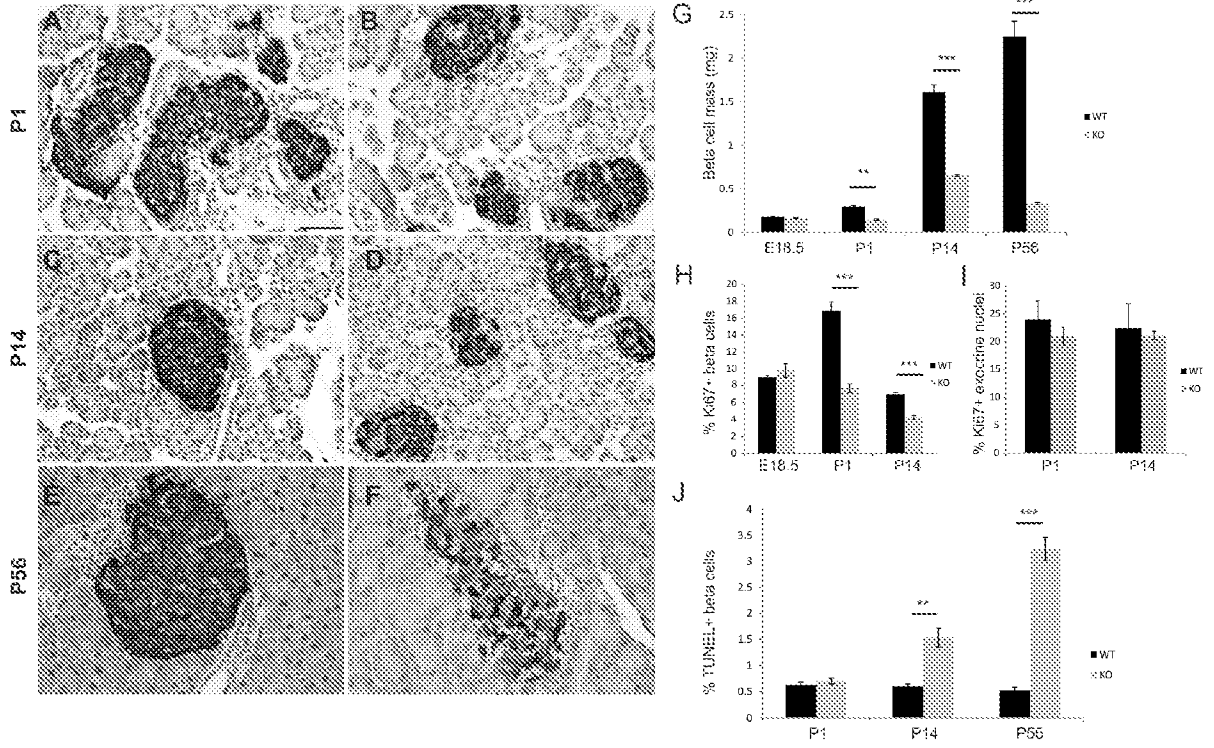
Figures 1A-1D.



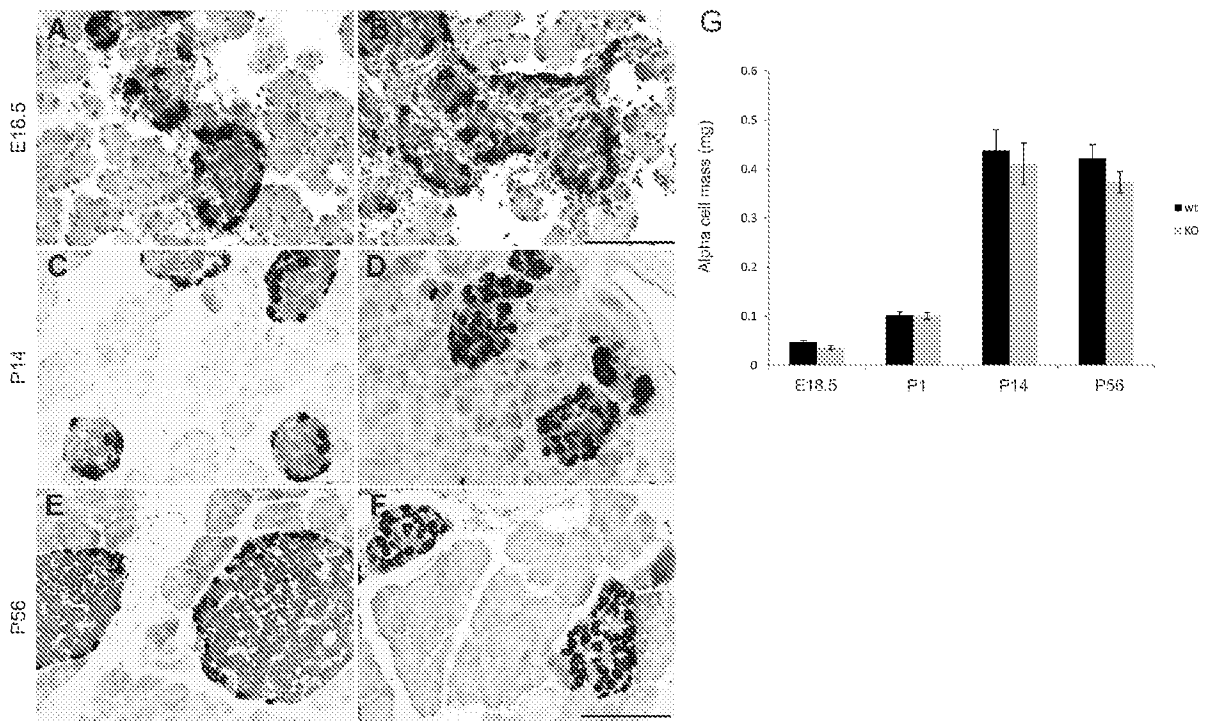
Figures 2A-2E.



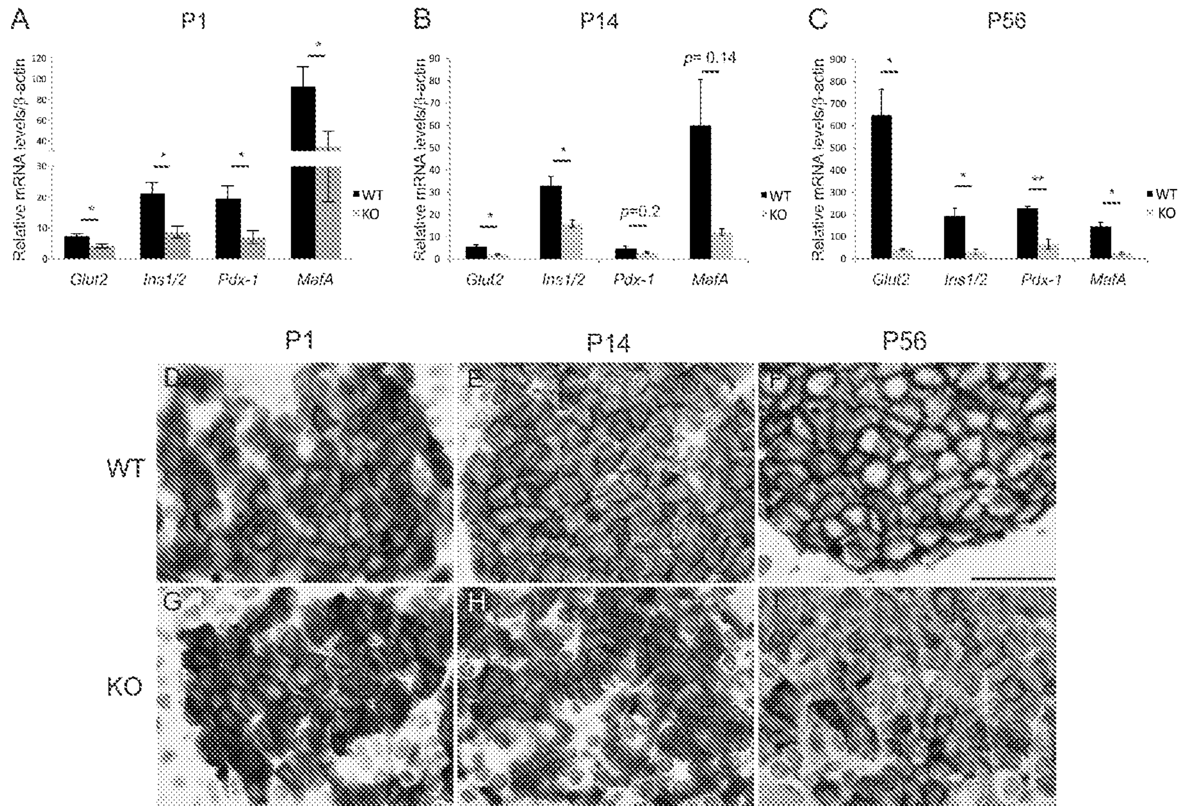
Figures 3A-3F.



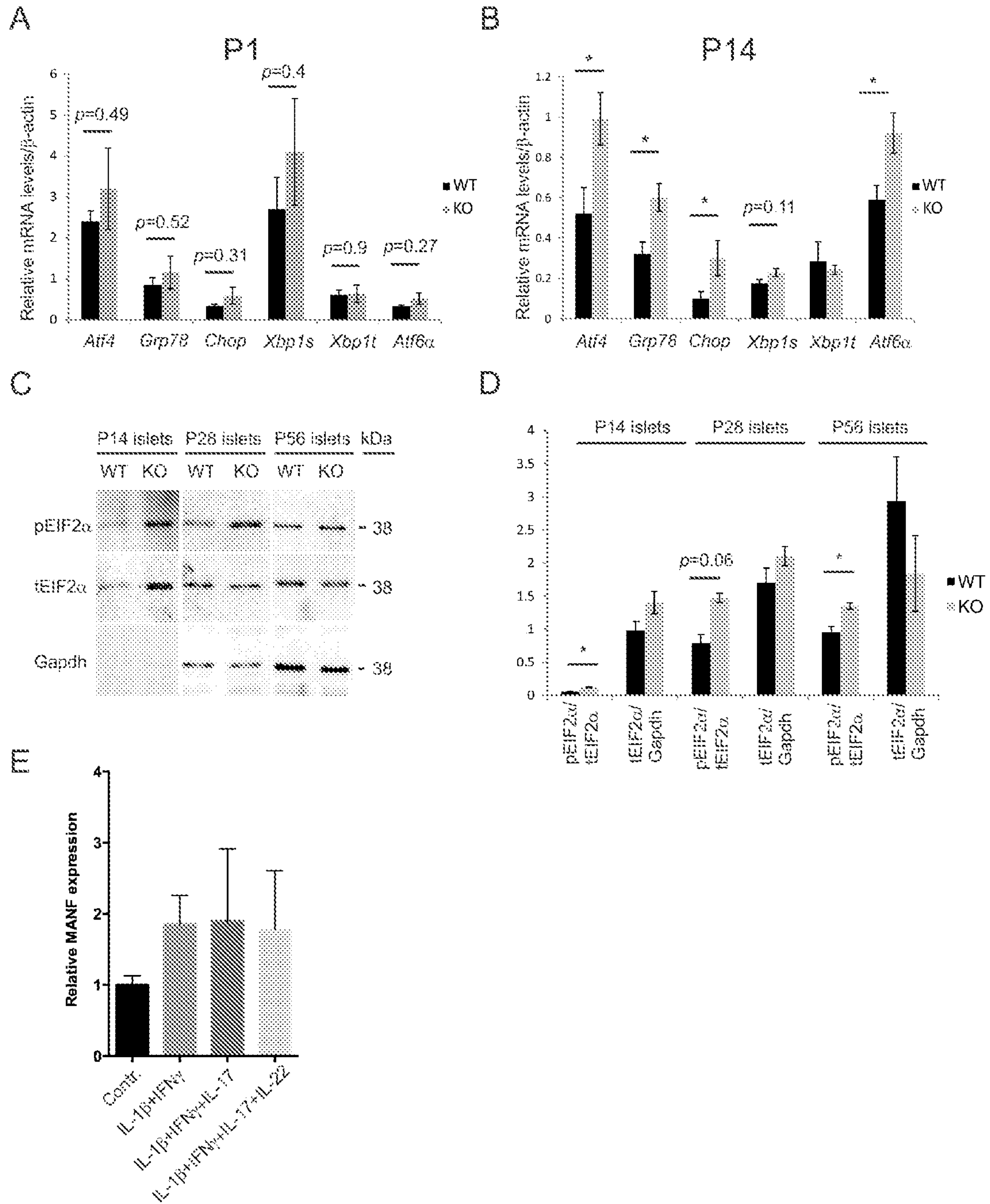
Figures 4A-4J.



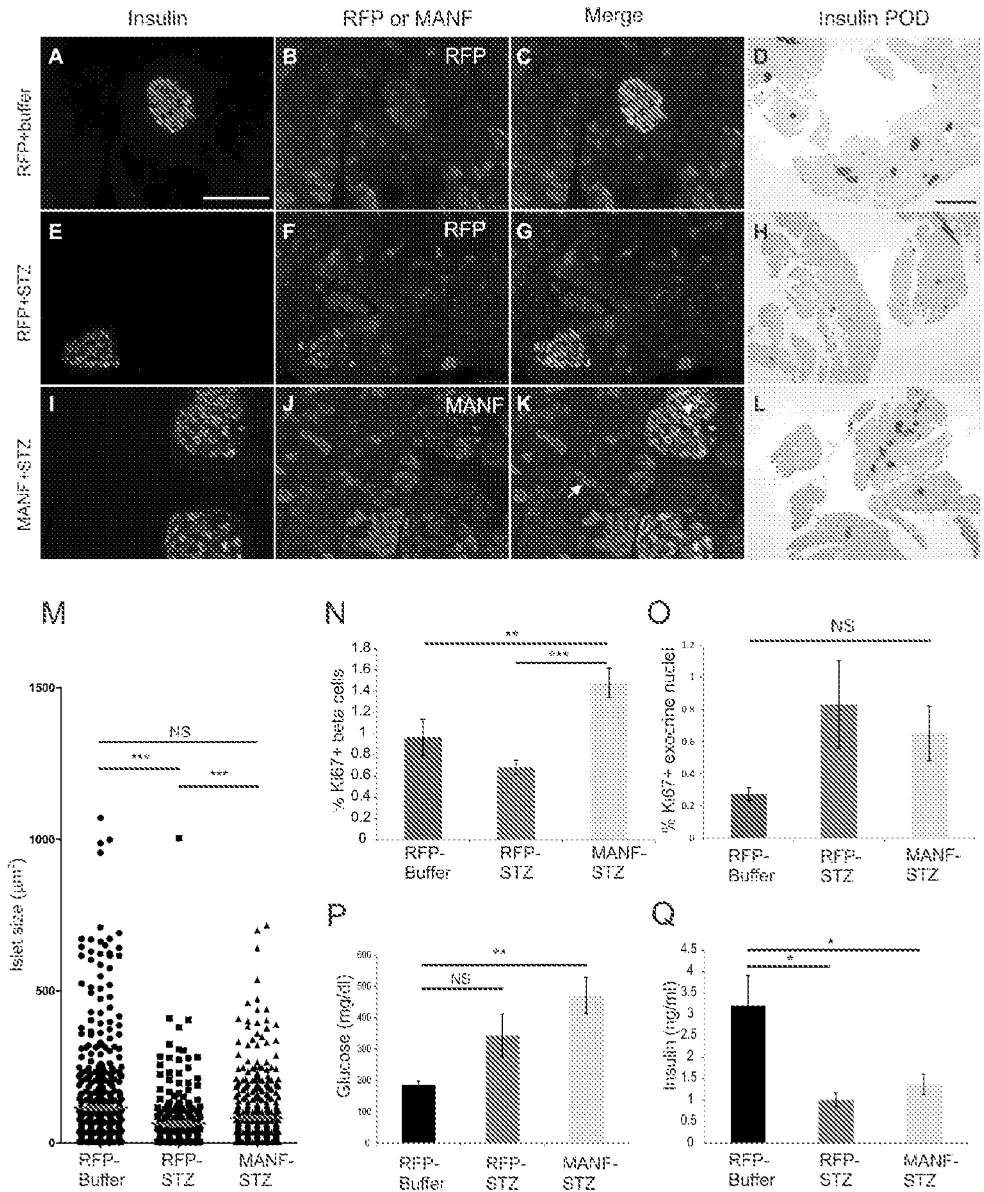
Figures 5A-5G.



Figures 6A-6I.



Figures 7A-7E.



Figures 8A-8Q.

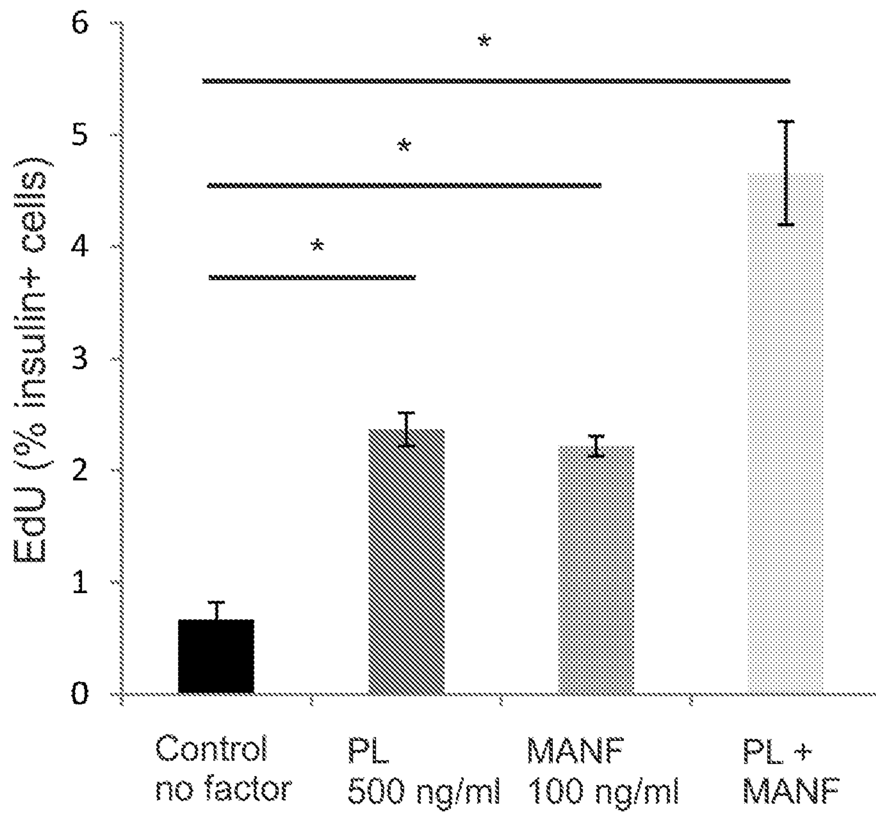
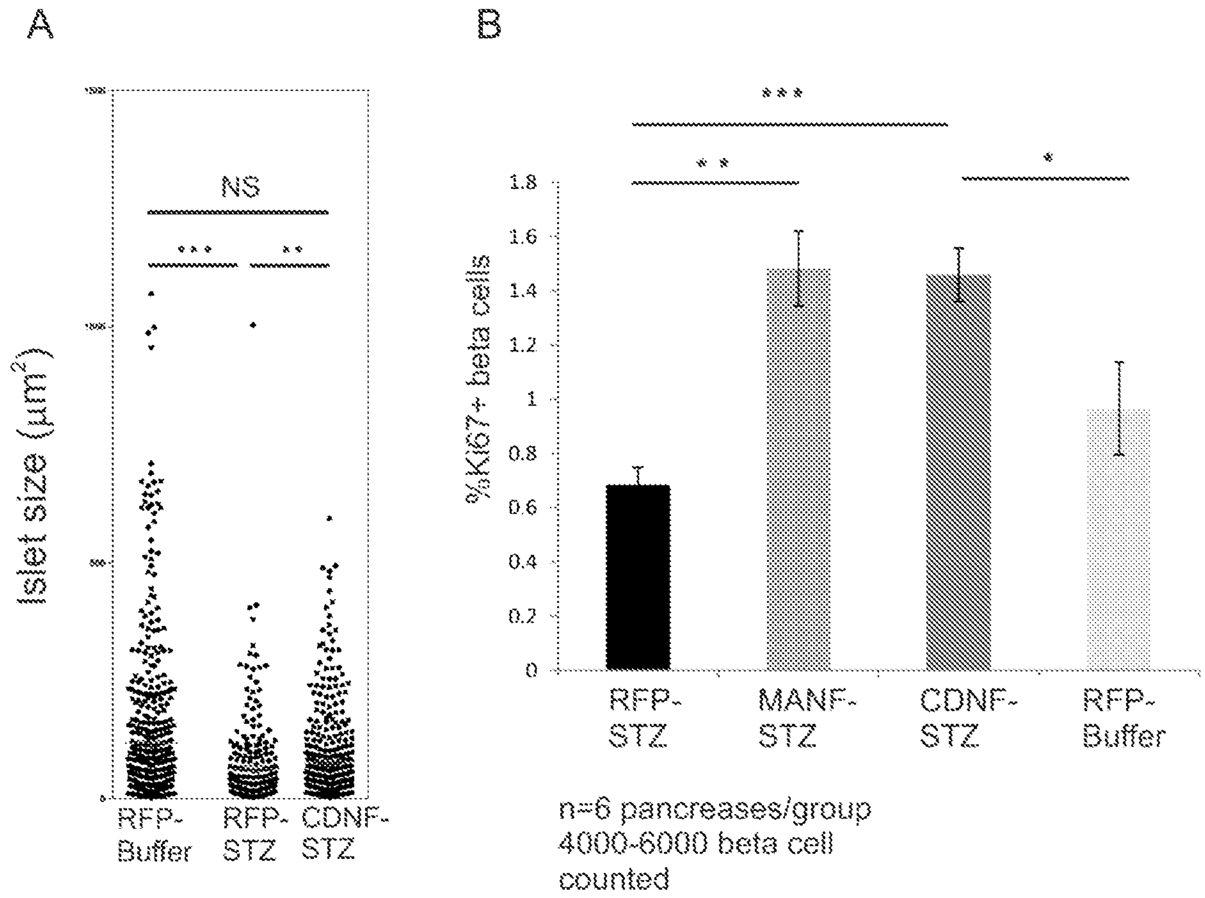
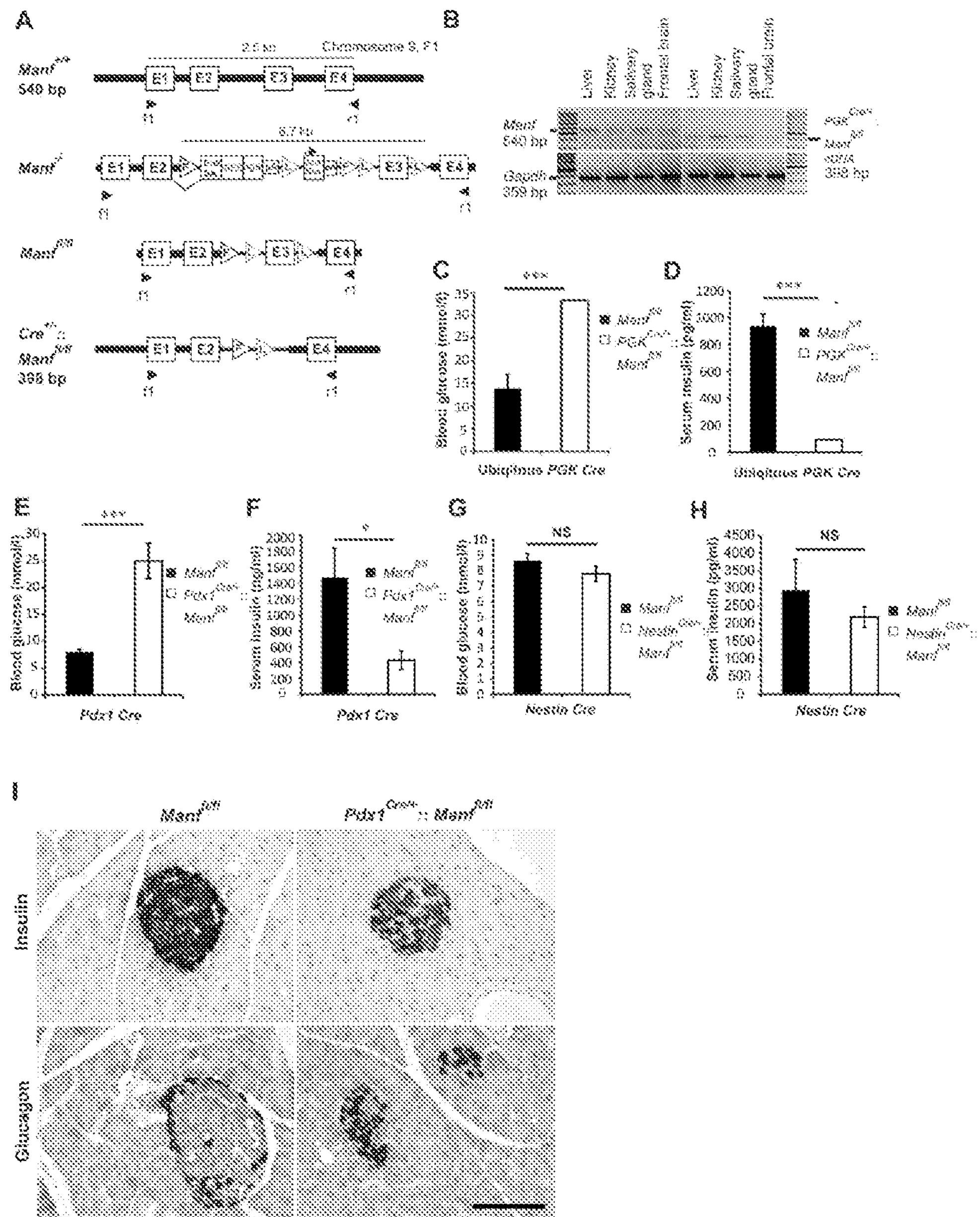


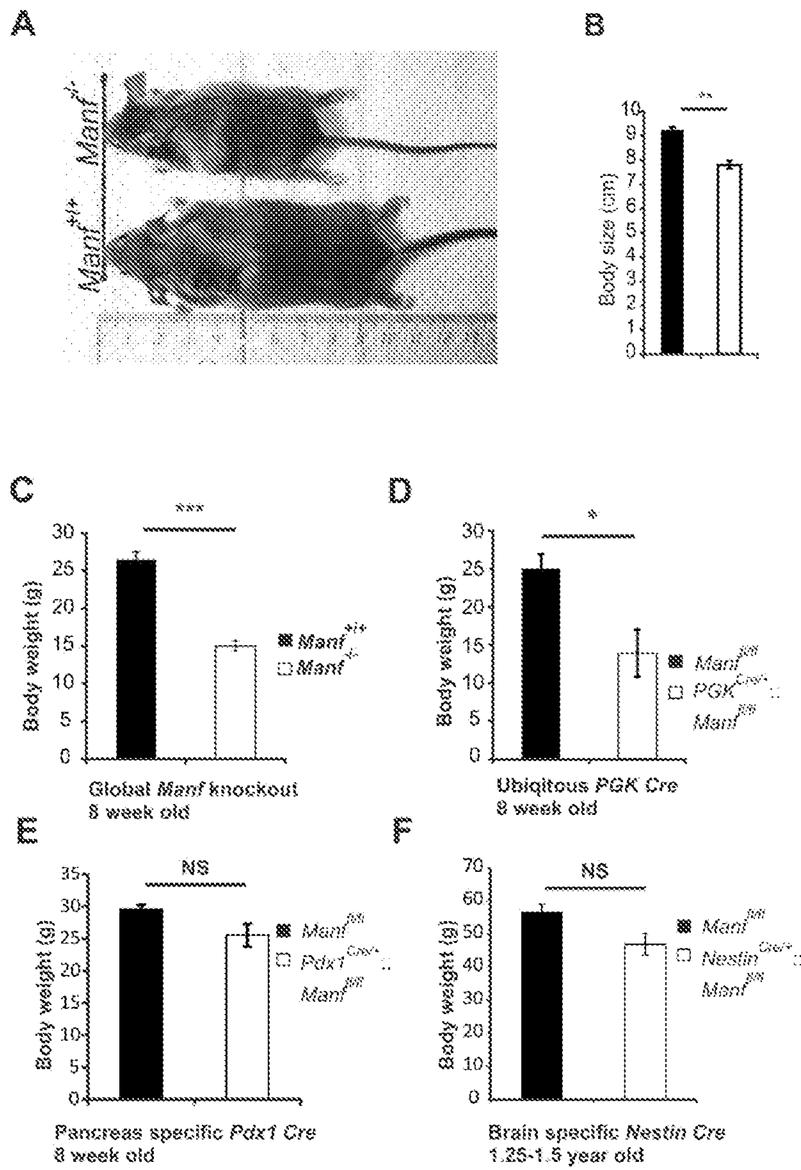
Figure 9.



Figures 10A and 10B.



Figures 11A-11I.



Figures 12A-12F.