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(54) **Title**: DOWNREGULATION OF MIR-7 FOR PROMOTION OF BETA CELL DIFFERENTIATION AND INSULIN PRODUCTION

(57) **Abstract**: A method of ex-vivo increasing insulin content in beta cells or stem cells is disclosed. The method comprising contacting the beta cells or stem cells with an agent for downregulating an activity or expression of miR-7, thereby increasing the insulin content in the beta cells or stem cells.

DOWNREGULATION OF miR-7 FOR PROMOTION OF BETA CELL DIFFERENTIATION AND INSULIN PRODUCTION

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to downregulation of microRNA-7 and, more particularly, but not exclusively, to the use of same for promoting insulin production from pancreatic beta cells.

The development of the endocrine pancreas is governed by a network of transcription factors that specify the different endocrine cell types, including insulin-producing beta cells, glucagon-producing alpha cells, delta cells (somatostatin producing cells), PP cells (pancreatic peptide producing cells) and epsilon cells (ghrelin producing cells). The transcription factor Neurogenin3 (Ngn3) initiates the endocrine differentiation program and then a complex network of transcription factors is activated to differentially specify the endocrine lineages.

Pax6 is one such transcription factor acting downstream of Ngn3. Pax6 is pivotal in the differentiation of pancreatic beta-cells and alpha-cells, as islet morphogenesis has been shown to be disrupted when Pax6 expression is attenuated. In both humans and mice, two Pax6 alleles are required in order to maintain glucose homeostasis and loss of one allele results in glucose intolerance. The development of multiple other organs is sensitive to Pax6 haplo-insufficiency, including the iris and the lens. Normal embryonic development cannot tolerate high levels of Pax6. Thus, for example, Pax6 over-expression in mice causes eye abnormalities and induces apoptosis in the brain and the endocrine pancreas. Thus, it appears that Pax6 expression is tightly controlled to ensure appropriate levels of expression.

Genome-encoded miRNAs bind to specific sites on the 3' untranslated region (3'UTR) of their target mRNAs, to impart posttranscriptional silencing. This regulatory layer acts in concert with transcription factors to refine gene expression and confer robustness to developmental transitions. Total inactivation of miRNA maturation causes pancreas agenesis [Lynn et al. (2007) Diabetes 56(12): 2938-45], indicating that miRNAs are essential for early pancreas development. Melkman-Zehavi et al. disclosed that miRNAs control insulin content in pancreatic beta cells by downregulation of

transcriptional repressors, thus allowing reactivation of insulin transcription [Melkman-Zehavi et al. (2011) EMBO Journal 1-11]. Furthermore, specific miRNAs were shown to control insulin synthesis and exocytosis in differentiated cells. For example, loss of function of miR-375 in mice disrupts islet morphogenesis and endocrine cell differentiation [Poy et al., (2009) Proc Natl Acad Sci U S A (106) 5813-5818] while specific knockdown of miR-24, miR-26, miR-182 or miR-148 in beta cells downregulates insulin promoter activity and insulin mRNA levels [Melkman-Zehavi et al. (2011), supra].

miR-7 is another miRNA that is highly and specifically expressed in the endocrine pancreas in mice and humans [Bravo-Egana et al. (2008) Biochem Biophys Res Commun (366) 922-926; Correa-Medina et al. (2009) Gene Expr Patterns (9) 193-199]. miR-7 is an evolutionarily conserved miRNA, encoded by three different genomic loci in humans and mice (mouse: mmu-mir-7a-1 at Chr13, mmu-mir-7a-2 at Chr7 and mmu-mir-7b at Chr17). The duplication of the miR-7 gene in vertebrates hampers genetic loss-of-function analysis.

PCT Application No. WO 2009/067644 (to Pastori Ricardo et al.) discloses that mir-7 is a marker of differentiated endocrine cells and plays a role in β-cell biogenesis. WO 2009/067644 further discloses that inhibition of mir-7 activity in the fetal pancreas results in inhibition of formation of insulin in the fetal pancreas.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of ex-vivo increasing insulin content in beta cells or stem cells, the method comprising contacting the beta cells or stem cells with an agent for downregulating an activity or expression of miR-7, thereby increasing the insulin content in the beta cells or stem cells.

According to an aspect of some embodiments of the present invention there is provided an isolated population of cells generated according to the methods of the present invention.

According to an aspect of some embodiments of the present invention there is provided an isolated population of cells comprising an exogenous agent for downregulating an activity or expression of miR-7, wherein the cells secrete insulin.

According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising the isolated population of cells and a pharmaceutically acceptable carrier or diluent.

According to an aspect of some embodiments of the present invention there is provided a use of the isolated population of cells for the manufacture of a medicament identified for treating a medical condition associated with an insulin deficiency.

According to an aspect of some embodiments of the present invention there is provided a method of treating a medical condition associated with an insulin deficiency in a subject in need thereof, the method comprising administering to the subject the isolated population of cells, thereby treating the medical condition associated with the insulin deficiency.

According to an aspect of some embodiments of the present invention there is provided a method of treating a medical condition associated with an insulin deficiency in a subject in need thereof, the method comprising administering to the subject an agent for downregulating an activity or expression of miR-7, thereby treating the medical condition associated with the insulin deficiency.

According to an aspect of some embodiments of the present invention there is provided a method of increasing insulin content in beta cells or stem cells, the method comprising expressing in the beta cells or stem cells a target gene of miR-7 selected from the group consisting of epidermal growth factor receptor (EGFR), insulin-degrading enzyme (IDE), Kruppel-like factor 4 (KLF4), GLI family zinc finger 3 (GLI3), insulin receptor substrate 1 (IRS1), Sp1 transcription factor (SP1), O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) (OGT), insulin-like growth factor 1 receptor (IGF1R) and one cut homeobox 2 (ONECUT2), thereby increasing the insulin content in the beta cells or stem cells.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising a nucleic acid sequence encoding a target gene of miR-7 selected from the group consisting of epidermal growth factor receptor (EGFR), insulin-degrading enzyme (IDE), Kruppel-like factor 4 (KLF4), GLI family zinc finger 3 (GLI3), insulin receptor substrate 1 (IRS1), Sp1 transcription factor (SP1), O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-

acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) (OGT), insulin-like growth factor 1 receptor (IGF1R) and one cut homeobox 2 (ONECUT2) wherein the target gene of miR-7 is under a transcriptional regulation of a cis-acting regulatory element.

According to an aspect of some embodiments of the present invention there is provided an isolated population of beta cells or stem cells exogenously expressing a target gene of miR-7 selected from the group consisting of epidermal growth factor receptor (EGFR), insulin-degrading enzyme (IDE), Kruppel-like factor 4 (KLF4), GLI family zinc finger 3 (GLI3), insulin receptor substrate 1 (IRS1), Sp1 transcription factor (SP1), O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) (OGT), insulin-like growth factor 1 receptor (IGF1R) and one cut homeobox 2 (ONECUT2).

According to some embodiments of the invention, the isolated population of cells is for treating a medical condition associated with an insulin deficiency.

According to some embodiments of the invention, the stem cells comprise embryonic stem cells.

According to some embodiments of the invention, the stem cells comprise human pluripotent stem cells.

According to some embodiments of the invention, the stem cells comprise mesenchymal stem cells.

According to some embodiments of the invention, the beta cells comprise precursor beta cells.

According to some embodiments of the invention, the precursor beta cells comprise de-differentiated beta cells.

According to some embodiments of the invention, the dedifferentiated beta cells comprise induced pluripotent stem cells generated from beta cells.

According to some embodiments of the invention, the precursor beta cells comprise transdifferentiated liver cells.

According to some embodiments of the invention, the beta cells comprise mature beta cells.

According to some embodiments of the invention, the agent is a polynucleotide agent.

According to some embodiments of the invention, the agent is an antagomir.

According to some embodiments of the invention, the medical condition associated with an insulin deficiency comprises diabetes mellitus.

According to some embodiments of the invention, the subject is a human subject.

According to some embodiments of the invention, the method being effected ex-vivo.

According to some embodiments of the invention, the method being effected in-vivo.

According to some embodiments of the invention, the cis-acting regulatory element is a beta cell or stem cell specific promoter.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-Y depict miR-7 expression in endocrine cells of the pancreas. Figures 1A-T and 1V-Y illustrate miR-7 fluorescent in situ hybridization combined with protein immunofluorescence analysis of E13.5-E15.5 pancreas sections. Figure 1A illustrates miR-7 *in situ* hybridization on E15.5 pancreas sections, insets (Figure 1B) is higher magnifications of the field marked by the dashed square. Figure 1C illustrates fluorescent miR-7 *in situ* hybridization; Figure 1D illustrates U6 *in situ* hybridization

(positive control); Figure 1E illustrates scrambled probe *in situ* hybridization (negative control); Figures 1F-H illustrate fluorescent miRNA *in situ* hybridization combined with immunostaining. miR-7 (red) co-localization with insulin (Ins; green, Figure 1G) or glucagon (Gcg; white, Figure 1H). Blue, nuclei. Insets (Figures 1V-W) are higher magnifications of the field marked by the dashed square. Figures 1I-N illustrate that miR-7 (red) is not expressed in acinar cells marked by Cpa1 at E15.5 (green, Figures 1J-K) or in duct cells marked by HNF1b at E14.5 (green, Figure 1M). Figure 1N is a higher magnification of the field marked by the dashed square in 1M. Figures 1O-T illustrate expression of miR-7 in many Ngn3-positive cells at E13.5 (white, Figures 1P-Q) as well as at E14.5 (Figures 1S-T). Higher magnification insets with arrowheads indicating representative cells that co-express miR-7 and Ngn3 (Figures 1X-Y). Scale bars: 50 μm. Figure 1U is a graph illustrating that miR-7 expression is dependent on Ngn3. qPCR analysis of miR-7, miR-375, miR-17, let-7b in E14.5 Ngn3 knockout (KO) pancreatic buds, relative to Ngn3 heterozygous controls (WT). Data were normalized to sno234. qPCR data of Pax6 and insulin expression in the same samples, normalized to Hprt and Gapdh, and then presented relative to control. Error bars represent ±SEM $^{**}P<0.05$.

FIGs. 2A-K depict Pax6 as the target of miR-7. Figure 2A is a schematic illustration of a gene ontology analysis of miR-7 predicted targets. Gene ontology analysis of miR-7 depicted 'Regulation of transcription" as significantly enriched ($P<2.35E^{-7}$). Within this list, Pax6 was the only characterized factor known to control pancreas development. Figure 2B shows the predicted base pairing of mature miR-7 sequence (SEQ ID NO: 28) at the 3'UTRs of *Pax6* (SEQ ID NO: 27). The seed sequence is marked with red; Figure 2C is a histogram demonstrating the relative luciferase activity of a reporter that harbors the Pax6 3'UTR (742 bp). The expression of the luciferase reporter is repressed by miR-7 overexpression (miR-7 OE). Introduction of 'anti-miR' oligos (miR-7 KD) partially abrogates the repression. A reporter that harbors Pax6 3'UTR with deleted miR-7 seed sequence is completely insensitive to miR-7 OE ('mut UTR'). All data is normalized to the activity of firefly luciferase co-expressed from the dual reporter and to a negative control miRNA vector (Ctrl). n=3 independent experiments in triplicates, each; Figures 2D-E are representative western blots of PAX6 protein levels in MIN6 cells that were treated with miR-7 KD or miR-7 OE; Figures 2F-G are bar graph quantification of band densitometry of four

independent experiments in duplicates (of Figures 2D-E), each (ANOVA test, **P<0.05); Figures 2H-J are photographs depicting co-localization of miR-7 *in situ* hybridization (red), and Pax6 immunofluorescence (green) on E15.5 pancreas sections. Scale bar represent 50 μm; Figure 2K is a qPCR analysis of miR-7 and Pax6 expression during pancreas development (E12.5-E15.5). A negative correlation between miR-7 expression (red, normalized to sno234 and then relative to E12.5) and Pax6 expression (black, normalized to Gapdh and then relative to E12.5). n=4, each pool of 3 pancreata from at least 2 litters. Error bars represent ±SEM (**P<0.05).

FIGs. 3A-T depict miR-7 knockdown and over expression in pancreatic explants. Figure 3A is a scheme showing the experimental set up. Cholesterol-conjugated oligos introduced into the medium of E12.5 dorsal pancreatic buds that were grown in hanging drops for 48 hrs; Figures 3B-I illustrate miR-7 knockdown analysis (miR-7-KD, blue), relative to knockdown of control miRNA, miR-122 (Ctrl-KD, gray): Figure 3B is a graph illustrating upregulated Pax6 mRNA expression upon miR-7 knockdown; Figure 3C is a graph illustrating increased insulin and glucagon mRNA expression upon miR-7 knockdown; Figure 3D is a graph illustrating that insulin protein levels are upregulated upon miR-7 KD, as measured by ELISA assay. Error bars represent ± s.e.m. **P<0.05; Figures 3E-J are photographs representing immunostaining of insulin (green), glucagon (red) and ghrelin (white), taken for quantification. Scale bars represent 50 μm; Figure 3K is a qPCR analysis of transcription factors expression. Figure 3L is a graph illustrating the quantification of endocrine cell types (SS, somatostatin; Ghr, ghrelin; Gcg, glucagon; Ins, insulin). The percentage of cells in each individual population was calculated from the total number of counted cells in serial sections of the whole pancreas anlagen. At least three explants per treatment, average of three to eight independent experimental repeats. qPCR data are normalized to Hprt and Gapdh mRNA, and then to Ctrl KD treatment; Figures 3M-R illustrate analysis of miR-7 overexpression (miR-7-OE oligos, pink), relative to overexpression of control miRNA oligo, miR-67 (Ctrl-OE, gray): Figure 3M is a graph illustrating repressed Pax6 mRNA expression in miR-7-OE explants; Figure 3N is a graph illustrating decreased insulin mRNA expression in miR-7-OE explants, whereas glucagon levels remain similar; Figure 3O reveals a significant reduction of beta cell mass in a morphometry of miR-7-OE explants; Figures 3P-Q are photographs representing immunostaining of insulin (green) and Pax6 (red), taken for

quantification; Figure 3R is a graph of a qPCR analysis of transcription factors expression. n=pools of 2-3 explants per treatment in the same litter, 3-6 independent experiments. All qPCR data normalized to Hprt and Gapdh mRNA and then to treatment with control oligos. Error bars represent ±SEM (** $P<0.05$); and Figures 3S-T are photographs illustrating Pax6 immunostaining in control and miR-7 OE.

FIGs. 4A-H depict explant differentiation in culture. Figures 4A-C are graphs illustrating qPCR analysis of Ngn3 mRNA, miR-7 and insulin mRNA expression in explants grown in culture. Of note, these results reveal the dynamics of endocrine gene expression under *ex-vivo* differentiation conditions. Data normalized to Gapdh or sno234 and to the expression at E12.5 (time-point 0). n=4 per time-point in two repeats; Figures 4D-E are photographs illustrating immunoflorescent detection of insulin (red) and glucagon (green) at 24 hrs or 70 hrs in culture. Of note, these results exemplify the propagation of endocrine differentiation *ex-vivo*; Figures 4F-H are photographs illustrating the efficiently taken up Cy3-bound-antagomir by pancreatic explants. Upper confocal optical section captures the periphery of the explants (Figure 4F) and a central optical section reveals penetration of the Cy3-labled antagomir deep into the explants (Figure 4G). Cy3-labled antagomir depicted in the cells in higher magnification. (Figure 4H; nuclei, blue).

FIG. 4I is a graph depicting a Luciferase assay. HEK-293T cells were transfected with a luciferase reporter that harbors multiple miR-7 binding sites on its 3'UTR. The histogram demonstrates the ratio of firefly to renilla luciferase activity in negative control (Ctrl), cells overexpressing miR-7 (miR-7 OE) or cells overexpressing miR-7 and co-transfected with anti-miR-7 (miR-7 KD), normalized to control (3 experiments in triplicates).

FIGs. 5A-L depict reduced expression of endocrine genes by *in-vivo* overexpression of miR-7. Figure 5A is a schematic representation of the targeting construct for conditional miR-7-IRES-GFP misexpression; Figure 5B shows a southern blot analyses of genomic DNA from wild-type (lanes 2,3) and targeted ES clone (lanes 1,4), using the probe denoted in red (see Figure 5A); Figure 5C is a graph showing that miR-7 up-regulation is Cre dependent. qPCR analysis of miR-7 and miR-199 levels in primary embryonic fibroblasts harvested from Rosa-miR-7 transgenic mice and infected with an adenovirus that expresses either Cre-GFP ('Ad-Cre') or GFP alone ('Ad-GFP').

Data normalized to sno234 (three independent MEF lines, each in triplicates); Figures 5D-E are photographs showing that GFP is specifically expressed in E13.5 Pdx1-Cre;Rosa-miR7 pancreatic epithelium but not in littermates that do not harbor the Cre recombinase ('Ctrl'). Asterisks depict erythrocyte autofluorescence; Figures 5F-J show that miR-7 mis-expression specifically repressed endocrine genes: Figure 5F is a graph showing qPCR analysis of pancreatic genes in E15.5 Pdx1-Cre;Rosa-miR-7 samples, as indicated. Data normalized to Hprt and Gapdh mRNA and to the expression levels in littermate controls (n=6 each genotype, three litters). Error bars represent ±SEM (** $P<0.05$); Figures 5G-J are photographs showing immunostaining for insulin (green) and glucagon (red) in E15.5 sections of Pdx1-Cre;Rosa-miR-7 pancreas and control littermates [insets (Figures 5I-J) are higher magnifications of the field marked by the dashed squares]. Scale bar represents 50 μm; Figures 5K-L are graphs illustrating Pax6-miR-7 interaction upstream of insulin promoter activation in MIN6 cells. Of note, insulin promoter activity was downregulated by miR-7 overexpression, relative to control ('miR-7 OE', 'Ctrl OE', respectively) and was suppressed by siRNA against Pax6 siPax6). Combining miR-7 OE with siPax6 enhanced the suppressions of the insulin promoter (Figure 5K). Insulin promoter activity was upregulated by miR-7 knockdown, relative to negative control scrambled oligo ('miR-7 KD', Ctrl KD', respectively). Concomitant introduction of miR-7 KD with siPax6 restored insulin promoter activity (Figure 5L). All firefly luciferase data was normalized to the activity of Renilla luciferase co-transfected and presented relative to the control experiment. N=3 independent experiments. Error bars represent ±SEM **$P<0.05$.

FIGs. 6A-J depict Pax6 haplo-insufficiency resembling miR-7 overexpression. Figure 6A is a schematic illustration of a heterozygous (mono-allelic) mice model for expression of Pax6 in-vivo; Figure 6B is a graph showing that Pax6 mRNA expression is reduced in E15.5 heterozygous pancreas ('het'), relative to wild-type littermates pancreas ('wt', two functional alleles); Figures 6C-E show reduced cellular expression of Pax6 in heterozygous pancreas, depicted by immunostaining (red). Scale bar represents 50 μm; Figure 6F is a graph of a qPCR analysis showing reduced insulin and glucagon mRNA levels in Pax6 heterozygous pancreata, relative to wt littermates; Figure 6G shows a qPCR analysis of transcription factors. All qPCR data were normalized to Hprt and Gapdh. $n\geq5$ embryos per genotype, three independent litters. Error bars represent ±SEM

(** P<0.05); Figures 6H-I are photographs of immunostaining for insulin (green) and glucagon (red) on E15.5 Pax6 heterozygous and wt littermates. Nuclei (blue); Figure 6J is a graph showing a reduction in beta cell numbers. Hormone-positive cells were counted every eighth section throughout the pancreas and the average number of positive cells per organ, from several animals was normalized to wt controls (wt n=1912 cells; heterozygous n=1751 cells counted from 16 sections each).

FIGs. 7A-C depict miR-7 expression, upstream of Pax6 and dependent on Ngn3. Figure 7A is a graph showing that miR-7 is upstream of Pax6. qPCR study of miR-7, miR-17 and let7b expression in E14.5 wild type (wt), Pax6 heterozygous (het) and knockout (KO) animals, normalized to sno234; Figure 7B is schematic illustrations of Ngn3, Pax6 and miR-7 which are wired into an 'incoherent feed-forward-loop'. This is a conserved network, described in the differentiation of neurons in the retina of Drosophila Melanogaster (Figure 7C).

FIGs. 8A-S depict that miR-7 KD controls endocrine differentiation. Figures 8A-J are photographs illustrating an analysis of endocrine-cell populations by whole mount immunostaining for Ngn3 (green), Insulin (red), Glucagon (white), Somatostatin (magenta) and Ghrelin (white) in control (Figures 8A-E) and miR-7 KD explants (Figures 8F-J); Figures 8K-O are graphs illustrating quantification of positive area by analysis of the stained area, relative to the total explant area (done with Niss elements software, additional details are described in the 'materials and experimental procedures' section, hereinbelow); Figures 8P-S are graphs illustrating the cell number quantification of Ngn3-, Insulin- (Ins) Glucagon (Gcg)- and Ghrelin (Ghr) positive cells. Positive cells were manually counted in stacked confocal images of the entire explants. 3-5 explants per treatment. Error bars represent ±SEM (** P<0.05).

FIGs. 9A-G depict that proliferation is not affected by miR-7 KD. Figures 9A-C illustrate no change in proliferating of insulin-positive cells, upon miR-7 KD relative to Ctrl. Double positive Ki67-positive/insulin-positive cells were counted from the entire explant (>3 per genotype) and their percentage from the insulin positive population is presented in Figure 9C; Figures 9D-F illustrate no change in proliferation of glucagon-positive cells, upon miR-7 KD. Figures 9D-E are photographs of double positive BrdU-positive/Glucagon-positive cell population was counted and quantified. Figure 9G

illustrates that the total numbers of BrdU-positive cells are comparable between mir-7 KD and Ctrl. Error bars represent ±SEM (** P<0.05).

FIG. 10 is a schematic model depicting miR-7-Pax6 interactions in pancreas development. The regulation of Pax6 levels (blue) by miR-7, regulates the differentiation of hormone-expressing endocrine cells. miR-7 knockdown de-represses Pax6 and results in reduced ghrelin (Ghr) expression and preference towards insulin and glucagon-positive cells (Ins and Gcg). Similarly, miR-7 overexpression or heterozygous (Het) expression of Pax6 results in reduced Pax6 and reciprocal changes in the expression of hormones.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to downregulation of microRNA-7 and, more particularly, but not exclusively, to the use of same for promoting insulin production from pancreatic beta cells.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Differentiation of endocrine pancreatic beta cells is controlled by a network of pancreatic transcription factors including Ngn3 acting upstream of Pax6. Pax6 is pivotal in the differentiation of pancreatic beta-cells and any deviation from normal Pax6 expression has severe developmental consequences (e.g. induction of cell apoptosis). Genome-encoded miRNAs bind to specific sites on the 3' untranslated region (3'UTR) of their target mRNAs and provide a posttranscriptional regulatory layer that along with transcription factors act to refine gene expression. Previous studies have shown that total inactivation of miRNA maturation causes pancreas agenesis [Lynn et al. (2007) Diabetes 56(12):2938-45], while Melkman-Zehavi et al. disclosed that specific knockdown of

miR-24, miR-26, miR-182 or miR-148 in beta cells downregulates insulin promoter activity and insulin mRNA levels [Melkman-Zehavi et al. (2011) EMBO Journal 1-11].

While reducing the present invention to practice, the present inventors have uncovered that endocrine-specific microRNA-7 (miR-7) acts downstream of Ngn3 to directly repress Pax6 expression and to thereby modulate pancreatic beta cell differentiation and insulin production therefrom.

As is illustrated hereinbelow and in the Examples section which follows, the present inventors have shown that miR-7 knockdown resulted in Pax6 upregulation and further resulted in reduced ghrelin producing cells and preference towards insulin and glucagon-producing cells (see Example 3 of the Examples section which follows). Similarly, miR-7 overexpression in developing pancreas explants or in a new mouse transgene led to Pax6 downregulation and to preference towards ghrelin producing cells, while significantly reducing the number of insulin and glucagon-producing cells (e.g. beta and alpha cells, respectively, see Example 4 of the Examples section which follows). Taken together, these results substantiate the value of downregulation of miR-7 for promoting pancreatic beta cell regeneration and increasing insulin protein levels.

Thus, according to one aspect of the present invention there is provided a method of treating a medical condition associated with an insulin deficiency in a subject in need thereof, the method comprising administering to the subject an agent for downregulating an activity or expression of miR-7, thereby treating the medical condition associated with the insulin deficiency.

The term "treating" refers to inhibiting or arresting the development of a disease, disorder or condition and/or causing the reduction, remission, or regression of a disease, disorder or condition or keeping a disease, disorder or medical condition from occurring in a subject who may be at risk for the disease disorder or condition, but has not yet been diagnosed as having the disease disorder or condition. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a disease, disorder or condition, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a disease, disorder or condition.

As used herein, the term "subject" refers to an animal, preferably a mammal, or a human being, of any age or sex, who suffers from or is predisposed to an insulin deficiency associated disorder.

Diseases or syndromes which are associated with an insulin deficiency include, but are not limited to, type 1 and type 2 diabetes mellitus, metabolic syndrome, type 1 and type 2 diabetes mellitus subtypes, insulin deficiency syndrome, maturity onset diabetes of the young (MODY 1-11), and permanent neonatal diabetes mellitus.

According to a specific embodiment of the present invention, the insulin deficiency comprises diabetes.

According to a specific embodiment of the present invention, the insulin deficiency comprises diabetes type 1.

As used herein "diabetes" refers to a disease resulting either from an absolute deficiency of insulin (type 1 diabetes) due to a defect in the biosynthesis or production of insulin, or a relative deficiency of insulin in the presence of insulin resistance (type 2 diabetes), i.e., impaired insulin action, in an organism. The diabetic patient thus has absolute or relative insulin deficiency, and displays, among other symptoms and signs, elevated blood glucose concentration, presence of glucose in the urine and excessive discharge of urine.

In order to treat the medical condition, pancreatic beta cells may be generated ex-vivo and then used for therapy.

Thus, according to one aspect of the present invention there is provided a method of ex-vivo increasing insulin content in beta cells or stem cells, the method comprising contacting the beta cells or stem cells with an agent for downregulating an activity or expression of miR-7, thereby increasing the insulin content in the beta cells or stem cells.

As used herein the phrase "ex-vivo" refers to a process in which cells, which are removed from a living organism, are subjected to a treatment and alternatively or additionally culturing outside the organism (e.g., in a cell culture plate or test tube). According to an embodiment ex-vivo includes in vitro, especially in the case cell-lines.

As used herein, the phrase "insulin content" refers to the amount of insulin polypeptides or peptides derived therefrom (e.g. mature insulin) inside an insulin producing cell (e.g. pancreatic beta cell) or secreted therefrom.

Measurement of insulin content is well known in the art. An exemplary method is extraction of cellular insulin with 3 M acetic acid. The amount of mature insulin extracted from the pancreatic beta cell may be determined using, for example, an enzyme-linked immunosorbent assay (ELISA) kit commercially available from e.g. Mercodia, Uppsala, Sweden. Alternatively Western Blot analysis, Immunofluorescence or Immunohistochemistry may be carried out using specific antibodies available, from e.g. Cell Signaling Technology, Thermo Scientific Pierce Antibodies or GeneTex.

According to one embodiment, the beta cells comprise isolated cells. The isolated beta cells may be of homogeneous or heterogeneous nature.

The term "isolated" refers to at least partially separated from the natural environment e.g., from a body.

The phrase "beta cells" as used herein refers to pancreatic islet endocrine cells capable of producing and secreting insulin (e.g. in response to physiological signals such as elevated glucose concentrations) and expressing typical beta cell markers including, but not limited to, insulin, pdx, Hnf3β, PC1/3, Beta2, Nkx2.2, GLUT2 and PC2.

According to one embodiment, the beta cells are mature beta cells.

The phrase "mature beta cells" as used herein refers to fully differentiated and functional pancreatic islet endocrine cells. Typically such cells secrete insulin in response to glucose stimulation. Furthermore, mature beta cells typically express typical beta cell markers including, but not limited to, insulin, pdx, Hnf3β, PC1/3, Beta2, Nkx2.2, GLUT2 and PC2.

According to one embodiment, the beta cells are precursor beta cells.

The phrase "precursor beta cells" as used herein refers to cells that have the capacity to develop/differentiate into pancreatic islet endocrine cells capable of producing and secreting insulin (e.g. in response to physiological signals such as elevated glucose concentrations) and expressing typical beta cell markers including, but not limited to, insulin, pdx, Hnf3β, PC1/3, Beta2, Nkx2.2, GLUT2 and PC2.

The precursor beta cells of the present invention may comprise pancreatic progenitor cells, somatic cells (capable of transdifferentiation into insulin producing beta cells) and beta cell lines.

According to a specific embodiment, the precursor beta cells are pancreatic progenitor cells of an adult or fetal pancreas (i.e., of any gestational age). Such

pancreatic progenitor cells may be obtained from the pancreatic islets, ducts or acini of an adult or fetal pancreas.

Thus, for example, the pancreatic progenitor cells may be comprised in isolated pancreatic islets. Islet cells are typically comprised of the following: 1) beta cells that produce insulin; 2) alpha cells that produce glucagon; 3) delta cells (or D cells) that produce somatostatin; and/or F cells that produce pancreatic polypeptide. The polypeptide hormones (insulin, glucagon, somatostatin and pancreatic polypeptide) inside these cells are stored in secretary vesicles in the form of secretory granules.

Methods of isolating pancreatic progenitor cells are well known in the art. For example, pancreatic tissue may be obtained from a human subject or donor by any method known in the art (e.g. using a biopsy guided by ultrasound or CT, by laparoscopy or by laparotomy). Islets may then be isolated from pancreatic tissue using collagenase and ficoll gradients. An exemplary method is described in U.S. Patent Application No. 20080014182, incorporated herein by reference. It will be appreciated that the pancreatic progenitor cells may be further isolated from the islets (or from ducts or acini as needed) e.g. by FACS sorting or clonal analysis using identification of specific progenitor markers, such as, but not limited to, expression of nestin, Ngn-3, c-met Arx, Pax4, Pax6, insulin, glucagon, glut2, Nkx2.2, Nkx6.1, Gck, Sur1, Kir6.2 and/or NeuroD/Beta2.

According to another specific embodiment, the precursor beta cells comprise somatic cells capable of transdifferentiation into insulin producing beta cells. Somatic cells as of the present teachings comprise both fetal and adult cells including, for example, liver cells, neuroendocrine cells, intestinal cells, fibroblasts, myoblasts and monocytes.

According to a specific embodiment, the precursor beta cells comprise transdifferentiated liver cells (i.e. liver cells which underwent differentiation into pancreatic cells), as taught e.g. by Sapir T. et al., Proc. Natl. Acad. Sci. USA (2005) 102 (22) 7964-7969 and by Zalzman M. et al., Diabetes (2005) 54(9):2568-2575.

It will be appreciated that somatic cells may be isolated by any cell isolation method known in the art [e.g. for liver cell isolation see for example Alpini G. et al., Recent advances in the isolation of liver cells. Hepatology (1994) Aug;20(2):494-514]. Furthermore, the isolated somatic cells (e.g. liver cells) may undergo any molecular

manipulation (e.g. genetic modification) or culturing with specific agents [e.g. specific soluble factors (SSF) such as activin-A, nicotinamide or HGF] in order to induce transdifferentiation thereof into insulin producing beta-like cells.

As used herein, the phrase "stem cells" refers to cells which are capable of remaining in an undifferentiated state (i.e. "pluripotent stem cells") for extended periods of time in culture until induced to differentiate into other cell types having a particular, specialized function (i.e., "fully differentiated" cells).

Preferably, the phrase "stem cells" encompasses embryonic stem cells (ESCs), induced pluripotent stem cells (iPS), adult stem cells and hematopoietic stem cells. According to a specific embodiment, the stem cells are of a human origin. Alternatively, the stem cells may be any mammalian stem cells, such as for example from a human, porcine, rodent (e.g. mouse or rat) or primate (e.g. monkey) origin.

The phrase "embryonic stem cells" refers to embryonic cells which are capable of differentiating into cells of all three embryonic germ layers *(i.e.*, endoderm, ectoderm and mesoderm), or remaining in an undifferentiated state. The phrase "embryonic stem cells" may comprise cells which are obtained from the embryonic tissue formed after gestation (e.g., blastocyst) before implantation of the embryo (*i.e.*, a pre-implantation blastocyst), extended blastocyst cells (EBCs) which are obtained from a post-implantation/pre-gastrulation stage blastocyst (see WO2006/040763) and embryonic germ (EG) cells which are obtained from the genital tissue of a fetus any time during gestation, preferably before 10 weeks of gestation.

The embryonic stem cells of the present invention can be obtained using well-known cell-culture methods. For example, human embryonic stem cells can be isolated from human blastocysts. Human blastocysts are typically obtained from human *in-vivo* preimplantation embryos or from *in vitro* fertilized (IVF) embryos. Alternatively, a single cell human embryo can be expanded to the blastocyst stage. For the isolation of human ES cells the zona pellucida is removed from the blastocyst and the inner cell mass (ICM) is isolated by immunosurgery, in which the trophectoderm cells are lysed and removed from the intact ICM by gentle pipetting. The ICM is then plated in a tissue culture flask containing the appropriate medium which enables its outgrowth. Following 9 to 15 days, the ICM derived outgrowth is dissociated into clumps either by a mechanical dissociation or by an enzymatic degradation and the cells are then re-

plated on a fresh tissue culture medium. Colonies demonstrating undifferentiated morphology are individually selected by micropipette, mechanically dissociated into clumps, and re-plated. Resulting ES cells are then routinely split every 4-7 days. For further details on methods of preparation human ES cells see Thomson et al., [U.S. Pat. No. 5,843,780; Science 282: 1145, 1998; Curr. Top. Dev. Biol. 38: 133, 1998; Proc. Natl. Acad. Sci. USA 92: 7844, 1995]; Bongso et al., [Hum Reprod 4: 706, 1989]; and Gardner et al., [Fertil. Steril. 69: 84, 1998].

It will be appreciated that commercially available stem cells can also be used with this aspect of the present invention. Human ES cells can be purchased from the NIH human embryonic stem cells registry [www(dot)escr(dot)nih(dot)gov]. Non-limiting examples of commercially available embryonic stem cell lines are BG01, BG02, BG03, BG04, CY12, CY30, CY92, CY10, TE03 and TE32.

Induced pluripotent stem cells (iPS; embryonic-like stem cells), are cells obtained by de-differentiation of adult somatic cells which are endowed with pluripotency (*i.e.*, being capable of differentiating into the three embryonic germ cell layers, *i.e.,* endoderm, ectoderm and mesoderm). According to some embodiments of the invention, such cells are obtained from a differentiated tissue (e.g., a somatic tissue such as skin) and undergo de-differentiation by genetic manipulation which re-programs the cell to acquire embryonic stem cells characteristics. According to some embodiments of the invention, the induced pluripotent stem cells are formed by inducing the expression of Oct-4, Sox2, Kfl4 and c-Myc in a somatic stem cell. Lineage specification of human iPS cells into functional glucose-responsive, insulin-producing progeny have been previously taught e.g. by Thatava T. et al., Indolactam V|[sol]|GLP-1-mediated differentiation of human iPS cells into glucose-responsive insulin-secreting progeny, Gene Therapy (2011) 18, 283–293.

Induced pluripotent stem cells (iPS) (embryonic-like stem cells) can be generated from somatic cells by genetic manipulation of somatic cells, e.g., by retroviral transduction of somatic cells such as fibroblasts, hepatocytes, gastric epithelial cells with transcription factors such as Oct-3/4, Sox2, c-Myc, and KLF4 [Yamanaka S, Cell Stem Cell. 2007, 1(1):39-49; Aoi T, et al., Generation of Pluripotent Stem Cells from Adult Mouse Liver and Stomach Cells. Science. 2008 Feb 14. (Epub ahead of print); IH Park, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency

with defined factors. Nature 2008;451:141-146; K Takahashi, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861-872]. Other embryonic-like stem cells can be generated by nuclear transfer to oocytes, fusion with embryonic stem cells or nuclear transfer into zygotes if the recipient cells are arrested in mitosis.

According to a specific embodiment, the precursor beta cells comprise de-differentiated beta cells.

The phrase "de-differentiated beta cells" refers to a partially or terminally differentiated beta cells which revert to an earlier developmental stage (e.g. non-insulin secreting cells) capable of re-differentiating into beta cells.

According to another specific embodiment, the de-differentiated beta cells comprise induced pluripotent stem cells generated from beta cells.

According to another specific embodiment, the precursor beta cells comprise de-differentiated beta cell lines.

The phrase "adult stem cells" (also called "tissue stem cells" or a stem cell from a somatic tissue) refers to any stem cell derived from a somatic tissue [of either a postnatal or prenatal animal (especially the human)] which is capable of differentiating into beta cells. The adult stem cell is generally thought to be a multipotent stem cell, capable of differentiation into multiple cell types. Adult stem cells can be derived from any adult, neonatal or fetal tissue such as adipose tissue, skin, kidney, liver, prostate, pancreas, intestine, bone marrow and placenta.

Hematopoietic stem cells, which may also referred to as adult tissue stem cells, include stem cells obtained from blood or bone marrow tissue of an individual at any age or from cord blood of a newborn individual.

Methods of isolating adult tissue stem cells are known in the arts and include, for example, those disclosed by Alison, M. R. [Tissue-based stem cells: ABC transporter proteins take center stage. J Pathol. (2003) 200(5): 547-50], Cal, J. et al., [Identifying and tracking neural stem cells. Blood Cells Mol Dis. (2003) 31(1): 18-27] and Collins, A. T. et al., [Identification and isolation of human prostate epithelial stem cells based on alpha(2)beta(1)-integrin expression. J Cell Sci. (2001) 114(Pt 21): 3865-72].

Generally, isolation of adult tissue stem cells is based on the discrete location (or niche) of each cell type included in the adult tissue, i.e., the stem cells, the transit

amplifying cells and the terminally differentiated cells [Potten, C. S. and Morris, R. J. (1988) Epithelial stem cells in-vivo. J. Cell Sci. Suppl. 10, 45-62]. Thus, an adult tissue such as, for example, prostate tissue is digested with Collagenase and subjected to repeated unit gravity centrifugation to separate the epithelial structures of the prostate (e.g., organoids, acini and ducts) from the stromal cells. Organoids are then disaggregated into single cell suspensions by incubation with Trypsin/EDTA (Life Technologies, Paisley, UK) and the basal, CD44-positive, stem cells are isolated from the luminal, CD57-positive, terminally differentiated secretory cells, using anti-human CD44 antibody (clone G4426; Pharmingen, Becton Dickinson, Oxford, UK) labeling and incubation with MACS (Miltenyi Biotec Ltd, Surrey, UK) goat anti-mouse IgG microbeads. The cell suspension is then applied to a MACS column and the basal cells are eluted and re-suspended in WAJC 404 complete medium [Robinson, E. J. et al. (1998) Basal cells are progenitors of luminal cells in primary cultures of differentiating human prostatic epithelium Prostate 37, 149-160].

Since basal stem cells can adhere to basement membrane proteins more rapidly than other basal cells [Jones, P. H. et al. (1995) Stem cell patterning and fate in human epidermis. Cell 60: 83-93; Shinohara, T., et al. (1999) beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc. Natl. Acad. Sci. USA 96: 5504-5509] the CD44 positive basal cells are plated onto tissue culture dishes coated with either type I collagen (52 μg/ml), type IV collagen (88 μg/ml) or laminin 1 (100 μg/ml; Biocoat.RTM., Becton Dickinson) previously blocked with 0.3 % bovine serum albumin (fraction V, Sigma-Aldrich, Poole, UK) in Dulbecco's phosphate buffered saline (PBS; Oxoid Ltd, Basingstoke, UK). Following 5 minutes, the tissue culture dishes are washed with PBS and adherent cells, containing the prostate tissue basal stem cells are harvested with trypsin-EDTA.

According to one embodiment, the stem cells utilized by the present invention are bone marrow (BM)-derived stem cells including hematopoietic, stromal or mesenchymal stem cells (Dominici, M et al., 2001. Bone marrow mesenchymal cells: biological properties and clinical applications. J. Biol. Regul. Homeost. Agents. 15: 28-37). BM-derived stem cells may be obtained from iliac crest, femora, tibiae, spine, rib or other medullar spaces.

Of the above described BM-derived stem cells, mesenchymal stem cells are the formative pluripotent blast cells, and as such are preferred for use with the present invention. Mesenchymal stem cells give rise to one or more mesenchymal tissues (e.g., adipose, osseous, cartilaginous, elastic and fibrous connective tissues, myoblasts) as well as to tissues other than those originating in the embryonic mesoderm (e.g., neural cells) depending upon various influences from bioactive factors such as cytokines. Although such cells can be isolated from embryonic yolk sac, placenta, umbilical cord, fetal and adolescent skin, blood and other tissues (e.g. liver, intestine, brain), their abundance in the BM far exceeds their abundance in other tissues and as such isolation from BM is presently preferred.

According to another embodiment, the stem cells comprise mesenchymal stem cells.

Methods of isolating, purifying and expanding adult stem cells including mesenchymal stem cells (MSCs) are known in the arts and include, for example, those disclosed by Caplan and Haynesworth in U.S. Pat. No. 5,486,359 and Jones E. A. et al. [Jones E. A. et al. (2002) Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells, Arthritis Rheum. 46(12): 3349-60].

Once the precursor beta cells or stem cells are obtained, the cells may be dispersed into a single cell suspension (e.g. by the addition of trypsin or by trituration) and are typically cultured (e.g. in cell medium such as CMRL-1066, available from e.g. Cellgro, Mediatech, Inc.). It will be appreciated that the cells may be grown in a serum free medium or on a matrix overlay with Matrigel. Additionally or alternatively, additional factors may be added to the cell culture medium which support expansion/differentiation of beta cells or which inhibit apoptosis of beta cells, such factors include but are not limited to, growth factors [e.g. fibroblast growth factor (FGF), hepatocyte growth factor (HGF)], hormones [e.g. gastrin, glucagon-like peptide-1 (GLP-1)] and/or insulinotropic agents (e.g. nicotinamide).

Furthermore, the precursor beta cells of the present invention express elevated levels of miR-7 which are higher than that of mature functioning beta cells which secret insulin in response to glucose.

The level is preferably determined in reference to a control cell (being a fully differentiated and functioning beta cell, which secretes insulin in response to glucose stimulation e.g. for normal glucose homeostasis).

According to a specific embodiment a de-differentiated state is when the level of miR-7 in the tested cell is at least 50 %, 40 %, 30 %, 20 %, 10 % or lower than that of the control cell.

As is mentioned hereinabove, following isolation and culturing, the beta cells (e.g. precursor beta cells) or stem cells of the present invention are contacted with an agent for downregulating (i.e. decreasing) an activity or expression of miR-7 in such cells.

According to the present teachings, downregulation of the activity or expression microRNA-7 (miR-7) in pancreatic beta cells results in cell differentiation and in an increase in insulin content in these cells (see Example 3 of the Examples section which follows). The increase in insulin levels can be a result of an increase in insulin transcription and/or post transcriptional control and/or increase in insulin translation and/or post-translational control. The increase in insulin content in the pancreatic beta cells according to the present teachings may also result from enhanced insulin storage and/or retarding insulin breakdown.

As used herein, the term "miR-7" refers to the microRNA (miRNA) molecule acting as post-transcriptional regulator. Exemplary miR-7 polynucleotide sequences are set forth in SEQ ID NOs: 21-26 and by GenBank accession nos. NR_029605.1, NR_029606.1 or NR_029607.1.

MicroRNAs are typically processed from pre-miR (pre-microRNA precursors). Pre-miRs are a set of precursor miRNA molecules transcribed by RNA polymerase III that are efficiently processed into functional miRNAs, e.g., upon transfection into cultured cells. A Pre-miR can be used to elicit specific miRNA activity in cell types that do not normally express this miRNA, thus addressing the function of its target by down regulating its expression in a "gain of (miRNA) function" experiment. Pre-miR designs exist to all of the known miRNAs listed in the miRNA registry (see below) and can be readily designed for any research.

Thus, the miR-7 of the present teachings may bind, attach, regulate, process, interfere, augment, stabilize and/or destabilize any target thereof. Such a target can be

any molecule, including, but not limited to, DNA molecules, RNA molecules and polypeptides, such as but not limited to, transcription factors such as Pax6.

It will be appreciated that the miR-7 of the present invention is part of, involved in and/or is associated with an insulin transcription pathway. MiR-7 can thus be identified via various databases including for example the micro-RNA registry (http://wwwdotsangerdotacdotuk/Software/Rfam/mirna/indexdotshtml).

Downregulation of miR-7 can be effected on the genomic and/or the transcript level using a variety of molecules which interfere with transcription (e.g., RNA silencing agents, Ribozyme, DNAzyme and antisense).

Following is a list of agents capable of downregulating expression level and/or activity of miR-7.

Nucleic acid agents that downregulate miR-7 activity include, but are not limited to, a target mimic, a micro-RNA resistant gene and a miRNA inhibitor.

The target mimic or micro-RNA resistant target is essentially complementary to the microRNA provided that one or more of following mismatches are allowed:

(a) a mismatch between the nucleotide at the 5' end of the microRNA and the corresponding nucleotide sequence in the target mimic or micro-RNA resistant target;

(b) a mismatch between any one of the nucleotides in position 1 to position 9 of the microRNA and the corresponding nucleotide sequence in the target mimic or micro-RNA resistant target; or

(c) three mismatches between any one of the nucleotides in position 12 to position 21 of the microRNA and the corresponding nucleotide sequence in the target mimic or micro-RNA resistant target provided that there are no more than two consecutive mismatches.

The target mimic RNA is essentially similar to the target RNA modified to render it resistant to miRNA induced cleavage, e.g. by modifying the sequence thereof such that a variation is introduced in the nucleotide of the target sequence complementary to the nucleotides 10 or 11 of the miRNA resulting in a mismatch.

Alternatively, a microRNA-resistant target may be implemented. Thus, a silent mutation may be introduced in the microRNA binding site of the target gene so that the DNA and resulting RNA sequences are changed in a way that prevents microRNA

binding, but the amino acid sequence of the protein is unchanged. Thus, a new sequence can be synthesized instead of the existing binding site, in which the DNA sequence is changed, resulting in lack of miRNA binding to its target.

According to a specific embodiment, the target mimic or micro-RNA resistant target is linked to the promoter naturally associated with the pre-miRNA recognizing the target gene and introduced into the cell. In this way, the miRNA target mimic or micro-RNA resistant target RNA will be expressed under the same circumstances as the miRNA and the target mimic or micro-RNA resistant target RNA will substitute for the non-target mimic/micro-RNA resistant target RNA degraded by the miRNA induced cleavage.

Non-functional miRNA alleles or miRNA resistant target genes may also be introduced by homologous recombination to substitute the miRNA encoding alleles or miRNA sensitive target genes.

Recombinant expression is effected by cloning the nucleic acid of interest (e.g., miRNA, target gene, silencing agent, etc.) into a nucleic acid expression construct under the expression of a promoter.

In other embodiments of the invention, synthetic single stranded nucleic acids are used as miRNA inhibitors. A miRNA inhibitor is typically between about 17 to 25 nucleotides in length and comprises a 5' to 3' sequence that is at least 90 % complementary to the 5' to 3' sequence of a mature miRNA. In certain embodiments, a miRNA inhibitor molecule is 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length, or any range derivable therein. Moreover, a miRNA inhibitor has a sequence (from 5' to 3') that is or is at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.1, 99.2, 99.3, 99.4, 99.5, 99.6, 99.7, 99.8, 99.9 or 100 % complementary, or any range derivable therein, to the 5' to 3' sequence of a mature miRNA, particularly a mature, naturally occurring miRNA.

According to an embodiment, peptide nucleic acids oligonucleotide analogues (PNA ON) are used as miRNA inhibitors. Such miRNA inhibitors have been described in detail in Torres et al., *Nucleic Acids Research* (2011) 1–16, incorporated herein by reference.

The miRNA inhibitors may be contacted with the cells using transient or stable transfection techniques. Thus, the miRNA inhibitors may be part of an expression vector, as described hereinbelow

According to one embodiment, downregulating the expression of a microRNA is effected by the use of a nucleic acid sequence which specifically binds and downregulates the expression of the microRNA. A nucleic acid sequence which may be used in accordance with the present invention may be purchased from any manufacturer, as for example, from Genecopoeia (miArrest, microRNA vector based inhibitors).

According to another embodiment, there is provide an isolated polynucleotide comprising a nucleic acid sequence for downregulating an expression of miR-7 or a precursor thereof.

Exemplary polynucleotides which may be used in accordance with the present invention to downregulate the expression of miR-7 include, but are not limited to, those set in SEQ ID NOs: 36-41.

Downregulation of miR-7 can also be achieved by RNA silencing. As used herein, the phrase "RNA silencing" refers to a group of regulatory mechanisms [e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression] mediated by RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including plants, animals, and fungi.

As used herein, the term "RNA silencing agent" refers to an RNA which is capable of inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g., the full translation and/or expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include noncoding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include dsRNAs such as siRNAs, miRNAs and shRNAs. In one embodiment, the RNA silencing agent is capable of inducing RNA interference. In another embodiment, the RNA silencing agent is capable of mediating translational repression.

Downregulation of miR-7 can be effected by using an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding miR-7.

Design of antisense molecules which can be used to efficiently downregulate miR-7 must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which specifically binds the designated mRNA within cells in a way which inhibits translation thereof.

The prior art teaches of a number of delivery strategies which can be used to efficiently deliver oligonucleotides into a wide variety of cell types [see, for example, Luft J Mol Med 76: 75-6 (1998); Kronenwett et al. Blood 91: 852-62 (1998); Rajur et al. Bioconjug Chem 8: 935-40 (1997); Lavigne et al. Biochem Biophys Res Commun 237: 566-71 (1997) and Aoki et al. (1997) Biochem Biophys Res Commun 231: 540-5 (1997)].

In addition, algorithms for identifying those sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of structural alterations in both the target mRNA and the oligonucleotide are also available [see, for example, Walton et al. Biotechnol Bioeng 65: 1-9 (1999)].

Such algorithms have been successfully used to implement an antisense approach in cells. For example, the algorithm developed by Walton et al. enabled scientists to successfully design antisense oligonucleotides for rabbit beta-globin (RBG) and mouse tumor necrosis factor-alpha (TNF alpha) transcripts. The same research group has more recently reported that the antisense activity of rationally selected oligonucleotides against three model target mRNAs (human lactate dehydrogenase A and B and rat gp130) in cell culture as evaluated by a kinetic PCR technique proved effective in almost all cases, including tests against three different targets in two cell types with phosphodiester and phosphorothioate oligonucleotide chemistries.

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an in vitro system were also published (Matveeva et al., Nature Biotechnology 16: 1374 - 1375 (1998)].

MiR-7 antisense agents include, but are not limited to, antisense molecules which target and inhibit miR-7 described in detail in Cheng A.M. et al., *Nucleic Acids Research* 2005 33(4):1290-1297, incorporated herein by reference, and anti-miRNA

oligos available from e.g. IDT (Integrated DNA Technologies, Inc, Israel) and also available from Exicon (miRCURY LNA™ microRNA Inhibitors, for more details see http://wwwdotexiqondotcom/microrna-knockdown).

It will be appreciated that the microRNA antisense agents (e.g. anti-miRNA oligos) of the present invention may also comprise chemical modifications, molecular modifications and/or the addition of moieties, e.g. a cholesterol moiety (e.g. antagomirs). Such molecules have been previously described in e.g. Krützfeldt J. et al., Nature (2005) 438:685-9 and Lennox and Behlke: Gene Therapy (2011) REVIEW: Chemical modification and design of anti-miRNA oligonucleotides, pg. 1–10.

According to a specific embodiment, the agent for downregulating an activity or expression of miR-7 is an antagomir. An exemplary antagomir which may be used according to the present teachings include Anti-miR-7 antagomir (2'OH)-chl (SEQ ID NO: 19).

Downregulation of miR-7 can also be effected by RNA interference. RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla. Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence

complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex.

Another method of evading the interferon and PKR pathways in mammalian systems is by introduction of small inhibitory RNAs (siRNAs) either via transfection or endogenous expression.

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 18-30 basepairs) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is theorized to result from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC.

It has been found that position of the 3'-overhang influences potency of an siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose et al., 2005). This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

The strands of a double-stranded interfering RNA (e.g., an siRNA) may be connected to form a hairpin or stem-loop structure (e.g., an shRNA). Thus, as mentioned the RNA silencing agent of the present invention may also be a short hairpin RNA (shRNA).

The term "shRNA", as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The number of nucleotides in the loop is a number between and including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides

in the loop. Examples of oligonucleotide sequences that can be used to form the loop include 5'-UUCAAGAGA-3' (Brummelkamp, T. R. et al. (2002) Science 296: 550) and 5'-UUUGUGUAG-3' (Castanotto, D. et al. (2002) RNA 8:1454). It will be recognized by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

According to another embodiment the RNA silencing agent may be a miRNA. miRNAs are small RNAs made from genes encoding primary transcripts of various sizes. They have been identified in both animals and plants. The primary transcript (termed the "pri-miRNA") is processed through various nucleolytic steps to a shorter precursor miRNA, or "pre-miRNA." The pre-miRNA is present in a folded form so that the final (mature) miRNA is present in a duplex, the two strands being referred to as the miRNA (the strand that will eventually basepair with the target) The pre-miRNA is a substrate for a form of dicer that removes the miRNA duplex from the precursor, after which, similarly to siRNAs, the duplex can be taken into the RISC complex. It has been demonstrated that miRNAs can be transgenically expressed and be effective through expression of a precursor form, rather than the entire primary form (Parizotto et al. (2004) Genes & Development 18:2237-2242 and Guo et al. (2005) Plant Cell 17:1376-1386).

Unlike, siRNAs, miRNAs bind to transcript sequences with only partial complementarity (Zeng et al., 2002, Molec. Cell 9:1327-1333) and repress translation without affecting steady-state RNA levels (Lee et al., 1993, Cell 75:843-854; Wightman et al., 1993, Cell 75:855-862). Both miRNAs and siRNAs are processed by Dicer and associate with components of the RNA-induced silencing complex (Hutvagner et al., 2001, Science 293:834-838; Grishok et al., 2001, Cell 106: 23-34; Ketting et al., 2001, Genes Dev. 15:2654-2659; Williams et al., 2002, Proc. Natl. Acad. Sci. USA 99:6889-6894; Hammond et al., 2001, Science 293:1146-1150; Mourlatos et al., 2002, Genes Dev. 16:720-728). A recent report (Hutvagner et al., 2002, Sciencexpress 297:2056-2060) hypothesizes that gene regulation through the miRNA pathway versus the siRNA pathway is determined solely by the degree of complementarity to the target transcript. It is speculated that siRNAs with only partial identity to the mRNA target will function

in translational repression, similar to an miRNA, rather than triggering RNA degradation.

Synthesis of RNA silencing agents suitable for use with the present invention can be effected as follows. First, the miR-7 mRNA sequence is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as demonstrated for GAPDH wherein siRNA directed at the 5' UTR mediated about 90 % decrease in cellular GAPDH mRNA and completely abolished protein level (wwwdotambiondotcom/techlib/tn/91/912.html).

Second, potential target sites are compared to an appropriate genomic database (e.g., human, mouse, rat etc.) using any sequence alignment software, such as the BLAST software available from the NCBI server (www.ncbi.nlm.nih.gov/BLAST/). Putative target sites which exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

It will be appreciated that the RNA silencing agent of the present invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

In some embodiments, the RNA silencing agent provided herein can be functionally associated with a cell-penetrating peptide." As used herein, a "cell-penetrating peptide" is a peptide that comprises a short (about 12-30 residues) amino acid sequence or functional motif that confers the energy-independent (i.e., non-endocytotic) translocation properties associated with transport of the membrane-permeable complex across the plasma and/or nuclear membranes of a cell. The cell-penetrating peptide used in the membrane-permeable complex of the present invention preferably comprises at least one non-functional cysteine residue, which is either free or derivatized to form a disulfide link with a double-stranded ribonucleic acid that has been modified for such linkage. Representative amino acid motifs conferring such properties are listed in U.S. Pat. No. 6,348,185, the contents of which are expressly incorporated herein by reference. The cell-penetrating peptides of the present invention preferably include, but are not limited to, penetratin, transportan, pIsl, TAT(48-60), pVEC, MTS, and MAP.

mRNAs to be targeted using RNA silencing agents include, but are not limited to, those whose expression is correlated with an undesired phenotypic trait. Exemplary mRNAs that may be targeted are those that encode truncated proteins i.e. comprise deletions. Accordingly the RNA silencing agent of the present invention may be targeted to a bridging region on either side of the deletion. Introduction of such RNA silencing agents into a cell would cause a down-regulation of the mutated protein while leaving the non-mutated protein unaffected.

Exemplary miR-7 silencing agents include, but are not limited to, Anti-miR™ miRNA Inhibitors available from Ambion Inc. for inhibition of miR-7 (for more details see https://productsdotappliedbiosystemsdotcom/ab/en/US/adirect/ab?cmd=ABAntiPremiRNAKeywordSearch).

Another agent capable of downregulating miR-7 is a DNAzyme molecule capable of specifically cleaving an mRNA transcript or DNA sequence of miR-7. DNAzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 1997;943:4262) A general model (the "10-23" model) for the DNAzyme has been

proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 199; for rev of DNAzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)].

Examples of construction and amplification of synthetic, engineered DNAzymes recognizing single and double-stranded target cleavage sites have been disclosed in U.S. Pat. No. 6,326,174 to Joyce et al. DNAzymes of similar design directed against the human Urokinase receptor were recently observed to inhibit Urokinase receptor expression, and successfully inhibit colon cancer cell metastasis in-vivo (Itoh et al, 20002, Abstract 409, Ann Meeting Am Soc Gen Ther wwwdotasgtdotorg). In another application, DNAzymes complementary to bcr-ab1 oncogenes were successful in inhibiting the oncogenes expression in leukemia cells, and lessening relapse rates in autologous bone marrow transplant in cases of CML and ALL.

Another agent capable of downregulating miR-7 is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding miR-7. Ribozymes are being increasingly used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch et al., Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific somatic mutations in genetic disorders [Welch et al., Clin Diagn Virol. 10:163-71 (1998)]. Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as other firms have demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV)

RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - WEB home page).

Ribozymes specific for targeting miR-7 can be designed as was previously described by Suryawanshi, H. et al. [Supplementary Material (ESI) for Molecular BioSystems, The Royal Society of Chemistry 2010, incorporated herein by reference].

An additional method of regulating the expression of a miR-7 gene in cells is via triplex forming oligonuclotides (TFOs). Recent studies have shown that TFOs can be designed which can recognize and bind to polypurine/polypirimidine regions in double-stranded helical DNA in a sequence-specific manner. These recognition rules are outlined by Maher III, L. J., et al., Science,1989;245:725-730; Moser, H. E., et al., Science,1987;238:645-630; Beal, P. A., et al, Science,1992;251:1360-1363; Cooney, M., et al., Science,1988;241:456-459; and Hogan, M. E., et al., EP Publication 375408. Modification of the oligonuclotides, such as the introduction of intercalators and backbone substitutions, and optimization of binding conditions (pH and cation concentration) have aided in overcoming inherent obstacles to TFO activity such as charge repulsion and instability, and it was recently shown that synthetic oligonucleotides can be targeted to specific sequences (for a recent review see Seidman and Glazer, J Clin Invest 2003;112:487-94).

In general, the triplex-forming oligonucleotide has the sequence correspondence:

oligo	3'--A	G	G	T
duplex	5'--A	G	C	T
duplex	3'--T	C	G	A

However, it has been shown that the A-AT and G-GC triplets have the greatest triple helical stability (Reither and Jeltsch, BMC Biochem, 2002, Sept12, Epub). The same authors have demonstrated that TFOs designed according to the A-AT and G-GC rule do not form non-specific triplexes, indicating that the triplex formation is indeed sequence specific.

Thus for any given sequence in the miR-7 regulatory region a triplex forming sequence may be devised. Triplex-forming oligonucleotides preferably are at least 15, more preferably 25, still more preferably 30 or more nucleotides in length, up to 50 or 100 bp.

Transfection of cells (for example, via cationic liposomes) with TFOs, and formation of the triple helical structure with the target DNA induces steric and functional changes, blocking transcription initiation and elongation, allowing the introduction of desired sequence changes in the endogenous DNA and resulting in the specific downregulation of gene expression. Examples of such suppression of gene expression in cells treated with TFOs include knockout of episomal supFG1 and endogenous HPRT genes in mammalian cells (Vasquez et al., Nucl Acids Res. 1999;27:1176-81, and Puri, et al, J Biol Chem, 2001;276:28991-98), and the sequence- and target specific downregulation of expression of the Ets2 transcription factor, important in prostate cancer etiology (Carbone, et al, Nucl Acid Res. 2003;31:833-43), and the pro-inflammatory ICAM-1 gene (Besch et al, J Biol Chem, 2002;277:32473-79). In addition, Vuyisich and Beal have recently shown that sequence specific TFOs can bind to dsRNA, inhibiting activity of dsRNA-dependent enzymes such as RNA-dependent kinases (Vuyisich and Beal, Nuc. Acids Res 2000;28:2369-74).

Additionally, TFOs designed according to the abovementioned principles can induce directed mutagenesis capable of effecting DNA repair, thus providing both downregulation and upregulation of expression of endogenous genes (Seidman and Glazer, J Clin Invest 2003;112:487-94). Detailed description of the design, synthesis and administration of effective TFOs can be found in U.S. Patent Application Nos. 2003 017068 and 2003 0096980 to Froehler et al, and 2002 0128218 and 2002 0123476 to Emanuele et al, and U.S. Pat. No. 5,721,138 to Lawn.

Expressing the miR-7 downregulating agents of the present invention in beta cells (e.g. precursor beta cells) or stem cells may be effected using expression constructs encoding the miR-7 downregulating agents and capable of expressing same in the beta cells or stem cells.

The nucleic acid construct (also referred to herein as an "expression vector") of the present invention typically includes additional sequences which render this vector suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). In addition, typical cloning vectors may also contain a transcription and translation initiation sequence, transcription and translation terminator and a polyadenylation signal.

Eukaryotic promoters typically contain two types of recognition sequences, the TATA box and upstream promoter elements. The TATA box, located 25-30 base pairs upstream of the transcription initiation site, is thought to be involved in directing RNA polymerase to begin RNA synthesis. The other upstream promoter elements determine the rate at which transcription is initiated.

Enhancer elements can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Enhancers are active when placed downstream or upstream from the transcription initiation site. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for the present invention include those derived from polyoma virus, human or murine cytomegalovirus (CMV), the long term repeat from various retroviruses such as murine leukemia virus, murine or Rous sarcoma virus and HIV. See, Enhancers and Eukaryotic Expression, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 1983, and Bell MP et al., J Immunol. (2007) 179(3):1893-900, both of which are incorporated herein by reference.

In the construction of the expression vector, the promoter is preferably positioned approximately the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

Polyadenylation sequences can also be added to the expression vector in order to increase the efficiency of the miR-7 downregulating agents' mRNA translation. Two distinct sequence elements are required for accurate and efficient polyadenylation: GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream. Termination and polyadenylation signals that are suitable for the present invention include those derived from SV40.

In addition to the elements already described, the expression vector of the present invention may typically contain other specialized elements intended to increase the level of expression of cloned nucleic acids or to facilitate the identification of cells that carry the recombinant DNA. For example, a number of animal viruses contain DNA sequences that promote the extra chromosomal replication of the viral genome in

permissive cell types. Plasmids bearing these viral replicons are replicated episomally as long as the appropriate factors are provided by genes either carried on the plasmid or with the genome of the host cell.

The vector may or may not include a eukaryotic replicon. If a eukaryotic replicon is present, then the vector is amplifiable in eukaryotic cells using the appropriate selectable marker. If the vector does not comprise a eukaryotic replicon, no episomal amplification is possible. Instead, the recombinant DNA integrates into the genome of the engineered cell, where the promoter directs expression of the desired nucleic acid.

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

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

Expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses can be also used. SV40 vectors include pSVT7 and pMT2. Vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A$^+$, pMTO10/A$^+$, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene

into the infected cell. Thus, the type of vector used by the present invention will depend on the cell type transformed. The ability to select suitable vectors according to the cell type transformed is well within the capabilities of the ordinary skilled artisan and as such no general description of selection consideration is provided herein. For example, bone marrow cells can be targeted using the human T cell leukemia virus type I (HTLV-I) and kidney cells may be targeted using the heterologous promoter present in the baculovirus Autographa californica nucleopolyhedrovirus (AcMNPV) as described in Liang CY et al., (Arch Virol. 2004;149:51-60).

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

Introduction of nucleic acids by viral infection offers several advantages over other methods such as lipofection and electroporation, since higher transfection efficiency can be obtained due to the infectious nature of viruses.

As illustrated in Example 2 and Table 4, hereinbelow, miR-7 has multiple target genes. Thus, insulin content may be increased by upregulating in beta cells or stem cells expression of a miR-7 target gene.

Thus, there is provided a method of increasing insulin content in beta cells or stem cells, the method comprising expressing in the beta cells or stem cells a target gene of miR-7.

According to an embodiment, the target gene of miR-7 comprises epidermal growth factor receptor (EGFR), insulin-degrading enzyme (IDE), insulin receptor substrate 2 (IRS2), Kruppel-like factor 4 (KLF4), GLI family zinc finger 3 (GLI3), insulin receptor substrate 1 (IRS1), Sp1 transcription factor (SP1), O-linked N-

acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) (OGT), insulin-like growth factor 1 receptor (IGF1R) and one cut homeobox 2 (ONECUT2).

As used herein, the term "epidermal growth factor receptor (EGFR)" refers to the cell surface protein that binds to specific ligands [e.g. epidermal growth factor (EGF) and transforming growth factor α (TGFα)]. An exemplary EGFR is set forth in NP_005219.2, NP_958439.1, NP_958440.1, NP_958441.1 and in SEQ ID NO: 43 or SEQ ID NO: 42 encoding same.

As used herein, the term "insulin-degrading enzyme (IDE)" refers to the zinc-binding protease which cleaves multiple short polypeptides (e.g. insulin, glucagon, amylin, bradykinin, and kallidin). An exemplary IDE is set forth in NP_001159418.1, NP_004960.2 and in SEQ ID NO: 45 or SEQ ID NO: 44 encoding same.

As used herein, the term "insulin receptor substrate 1 (IRS1)" refers to the protein which is phosphorylated by insulin receptor tyrosine kinase and is involved in transmitting signals from the insulin and insulin-like growth factor-1 (IGF-1) receptors to intracellular pathways. An exemplary IRS1 is set forth in NP_005535.1 and in SEQ ID NO: 53 or SEQ ID NO: 52 encoding same.

As used herein, the term "insulin receptor substrate 2 (IRS2)" refers to the cytoplasmic signaling molecule that mediates effects of polypeptides (e.g. insulin, insulin-like growth factor 1, cytokines) by acting as a molecular adaptor between diverse receptor tyrosine kinases and downstream effectors. An exemplary IRS2 is set forth in NP_003740.2 and in SEQ ID NO: 47 or SEQ ID NO: 46 encoding same.

As used herein, the term "Kruppel-like factor 4 (KLF4)" refers to transcriptional activator or repressor [also known as gut-enriched Krüppel-like factor (GKLF)]. An exemplary KLF4 is set forth in NP_004226.3 and in SEQ ID NO: 49 or SEQ ID NO: 48 encoding same.

As used herein, the term "GLI family zinc finger 3 (GLI3)" refers to the zinc finger protein typically characterized as a DNA-binding transcription factor. An exemplary GLI3 is set forth in NP_000159.3 and in SEQ ID NO: 51 or SEQ ID NO: 50 encoding same.

As used herein, the term "Sp1 transcription factor (SP1)" refers to the zinc finger transcription factor that binds to GC-rich motifs of many promoters. An exemplary SP1

is set forth in NP_001238754.1, NP_003100.1, NP_612482.2 and in SEQ ID NO: 55 or SEQ ID NO: 54 encoding same.

As used herein, the term "O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) (OGT)" refers to the glycosyltransferase that catalyzes the addition of a single N-acetylglucosamine in O-glycosidic linkage to serine or threonine residues. An exemplary OGT is set forth in NP_858058.1, NP_858059.1 and in SEQ ID NO: 57 or SEQ ID NO: 56 encoding same.

As used herein, the term "insulin-like growth factor 1 receptor (IGF1R)" refers to the receptor which binds insulin-like growth factor. An exemplary IGF1R is set forth in NP_000866.1 and in SEQ ID NO: 59 or SEQ ID NO: 58 encoding same.

As used herein, the term "one cut homeobox 2 (ONECUT2)" refers to the transcription factors, which are typically characterized by a cut domain and an atypical homeodomain. An exemplary ONECUT2 is set forth in NP_004843.2 and in SEQ ID NO: 61 or SEQ ID NO: 60 encoding same.

Upregulation of a protein of a target gene of miR-7 of the present invention [e.g. epidermal growth factor receptor (EGFR), insulin-degrading enzyme (IDE), insulin receptor substrate 2 (IRS2), Kruppel-like factor 4 (KLF4), GLI family zinc finger 3 (GLI3), insulin receptor substrate 1 (IRS1), Sp1 transcription factor (SP1), O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) (OGT), insulin-like growth factor 1 receptor (IGF1R) and one cut homeobox 2 (ONECUT2)] can be effected at the genomic level (*i.e.*, activation of transcription via promoters, enhancers, regulatory elements), at the transcript level (*i.e.*, correct splicing, polyadenylation, activation of translation) or at the protein level (*i.e.*, post-translational modifications, interaction with substrates and the like).

Following is a list of agents capable of upregulating the expression level and/or activity of a protein of a target gene of miR-7 (e.g. EGFR, IDE, IRS2, KLF4, GLI3, IRS1, SP1, OGT, IGF1R and ONECUT2).

Upregulating expression of a polypeptide encoded by the target gene of miR-7 may be an exogenous polynucleotide sequence designed and constructed to express at least a functional portion of the protein of a target gene of miR-7. Accordingly, the

exogenous polynucleotide sequence may be a DNA or RNA sequence encoding a target gene molecule, capable of increasing insulin content in a beta cell or stem cell.

As used herein, the phrase "polypeptide" encompasses a naturally occurring polypeptide which is comprised solely of natural amino acid residues or synthetically prepared polypeptides, comprised of a mixture of natural and modified (non-natural) amino acid residues as described hereinabove.

The phrase "functional portion" as used herein refers to part of the target gene protein (*i.e.*, a polypeptide) which exhibits functional properties of the enzyme such as binding to a substrate. Thus, for example, a functional portion of EGFR comprises the kinase domain and C-terminal sequence for docking of signaling molecules and the functional portion of IGF1R comprises the tyrosin kinase domain.

To express exogenous target genes of miR-7 (e.g. EGFR, IDE, IRS2, KLF4, GLI3, IRS1, SP1, OGT, IGF1R or ONECUT2) in mammalian cells, a polynucleotide sequence encoding a target genes of miR-7 (e.g. EGFR, IDE, IRS2, KLF4, GLI3, IRS1, SP1, OGT, IGF1R or ONECUT2) is preferably ligated into a nucleic acid construct suitable for mammalian cell expression. Such a nucleic acid construct includes a promoter sequence for directing transcription of the polynucleotide sequence in the cell e.g., beta cell or stem cell-specific promoter in a constitutive or inducible manner.

It will be appreciated that the nucleic acid construct of some embodiments of the invention can also utilize homologues of target genes of miR-7 (e.g. EGFR, IDE, IRS2, KLF4, GLI3, IRS1, SP1, OGT, IGF1R or ONECUT2) which exhibit the desired activity (*i.e.*, increase in insulin content). Such homologues can be, for example, at least 80 %, at least 81 %, at least 82 %, at least 83 %, at least 84 %, at least 85 %, at least 86 %, at least 87 %, at least 88 %, at least 89 %, at least 90 %, at least 91 %, at least 92 %, at least 93 %, at least 94 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 % or 100 % identical to SEQ ID NO: 42 (EGFR); SEQ ID NO: 44 (IDE); SEQ ID NO: 46 (IRS2); SEQ ID NO: 48 (KLF4); SEQ ID NO: 50 (GLI3); SEQ ID NO: 52 (IRS1); SEQ ID NO: 54 (SP1); SEQ ID NO: 56 (OGT); SEQ ID NO: 58 (IGF1R); and SEQ ID NO: 60 (ONECUT2), as determined using the BestFit software of the Wisconsin sequence analysis package, utilizing the Smith and Waterman algorithm, where gap weight equals 50, length weight equals 3, average match equals 10 and average mismatch equals -9.

Constitutive promoters suitable for use with some embodiments of the invention are promoter sequences which are active under most environmental conditions and most types of cells such as the cytomegalovirus (CMV) and Rous sarcoma virus (RSV).

Nucleic acid construct suitable for use according to the present teachings are described in further detail hereinabove.

An agent capable of upregulating a protein of a target gene of miR-7 (e.g. EGFR, IDE, IRS2, KLF4, GLI3, IRS1, SP1, OGT, IGF1R and ONECUT2) may also be any compound which is capable of increasing the transcription and/or translation of an endogenous DNA or mRNA encoding the protein of a target gene of miR-7 (e.g. EGFR, IDE, IRS2, KLF4, GLI3, IRS1, SP1, OGT, IGF1R and ONECUT2) and thus increasing endogenous activity thereof. Thus, for example, Glucagon-like peptide-1 (GLP-1, e.g. as set forth in NP_002045.1) or Gastric inhibitory polypeptide (GIP, e.g. as set forth in NP_004114.1) may be used to upregulate expression of IRS1 and IRS2.

Measuring insulin content following expression of the target gene of miR-7 may be carried out using any method known in the art and as described in further detail hereinabove.

It will be appreciated that downregulation of miR-7 or expression of a target gene of miR-7 in pancreatic beta cells (e.g. precursor beta cells) or stem cells may further lead to proliferation or differentiation of these cells. Alternatively, downregulation of miR-7 (or expression of a target gene of miR-7) may be used to enhance differentiation or proliferation of pancreatic alpha cells. Such cells may be used as experimental models for further investigation of these cell types.

According to one embodiment, there is provided an isolated population of cells generated according to the above described methods.

According to another embodiment, there is provided an isolated population of cells comprising an exogenous agent for downregulating an activity or expression of mirR-7, wherein the cells secrete insulin.

For *ex-vivo* therapy, beta cells (e.g. precursor beta cells) or stem cells are preferably treated with the agent of the present invention (as detailed in further detail hereinabove), following which they are administered to the subject in need thereof.

Administration of the *ex-vivo* treated cells of the present invention (e.g. mature beta cells, precursor beta cells or stem cells) can be effected using any suitable route of

introduction, such as intravenous, intraperitoneal, intra-kidney, intra-gastrointestinal track, subcutaneous, transcutaneous, intramuscular, intracutaneous, intrathecal, epidural, and rectal. According to presently preferred embodiments, the *ex-vivo* treated cells of the present invention may be introduced to the individual using intravenous, intra-kidney, intra-gastrointestinal track, and/or intraperitoneal administration.

The beta cells or stem cells may be obtained from any autologous or non-autologous (i.e., allogeneic or xenogeneic) donor. For example, cells may be isolated from a human cadaver or from a human pancreatic cell donor. Alternatively, cells may be obtained from any xenogeneic donor (e.g. porcine origin).

Beta cells or stem cells of xenogeneic origing (e.g. porcine) are preferably obtained from a source which is known to be free of zoonoses, such as porcine endogenous retroviruses. Similarly, human-derived beta cells or stem cells are preferably obtained from substantially pathogen-free sources.

Since non-autologous cells are likely to induce an immune reaction when administered to the body several approaches have been developed to reduce the likelihood of rejection of non-autologous cells. These include either suppressing the recipient immune system or encapsulating the non-autologous cells in immunoisolating, semipermeable membranes before transplantation. Alternatively, cells may be uses which do not express xenogenic surface antigens, such as those developed in transgenic pigs.

Encapsulation techniques are generally classified as microencapsulation, involving small spherical vehicles, and macroencapsulation, involving larger flat-sheet and hollow-fiber membranes (Uludag, H. et al. (2000). Technology of mammalian cell encapsulation. Adv Drug Deliv Rev *42*, 29-64).

Methods of preparing microcapsules are known in the art and include for example those disclosed in: Lu, M. Z. et al. (2000). Cell encapsulation with alginate and alpha-phenoxycinnamylidene-acetylated poly(allylamine). Biotechnol Bioeng *70*, 479-483; Chang, T. M. and Prakash, S. (2001) Procedures for microencapsulation of enzymes, cells and genetically engineered microorganisms. Mol Biotechnol *17*, 249-260; and Lu, M. Z., et al. (2000). A novel cell encapsulation method using photosensitive poly(allylamine alpha-cyanocinnamylideneacetate). J Microencapsul *17*, 245-521.

For example, microcapsules are prepared using modified collagen in a complex with a ter-polymer shell of 2-hydroxyethyl methylacrylate (HEMA), methacrylic acid (MAA), and methyl methacrylate (MMA), resulting in a capsule thickness of 2-5 μm. Such microcapsules can be further encapsulated with an additional 2-5 μm of ter-polymer shells in order to impart a negatively charged smooth surface and to minimize plasma protein absorption (Chia, S. M. et al. (2002). Multi-layered microcapsules for cell encapsulation. Biomaterials *23*, 849-856).

Other microcapsules are based on alginate, a marine polysaccharide (Sambanis, A. (2003). Encapsulated islets in diabetes treatment. Diabetes Thechnol Ther *5*, 665-668), or its derivatives. For example, microcapsules can be prepared by the polyelectrolyte complexation between the polyanions sodium alginate and sodium cellulose sulphate and the polycation poly(methylene-co-guanidine) hydrochloride in the presence of calcium chloride.

It will be appreciated that cell encapsulation is improved when smaller capsules are used. Thus, for instance, the quality control, mechanical stability, diffusion properties, and in vitro activities of encapsulated cells improved when the capsule size was reduced from 1 mm to 400 μm (Canaple, L. et al. (2002). Improving cell encapsulation through size control. J Biomater Sci Polym Ed *13*, 783-96). Moreover, nanoporous biocapsules with well-controlled pore size as small as 7 nm, tailored surface chemistries, and precise microarchitectures were found to successfully immunoisolate microenvironments for cells (See: Williams, D. (1999). Small is beautiful: microparticle and nanoparticle technology in medical devices. Med Device Technol *10*, 6-9; and Desai, T. A. (2002). Microfabrication technology for pancreatic cell encapsulation. Expert Opin Biol Ther *2*, 633-646).

Examples of immunosuppressive agents which may be used in conjunction with the *ex-vivo* treatment include, but are not limited to, methotrexate, cyclophosphamide, cyclosporine, cyclosporin A, chloroquine, hydroxychloroquine, sulfasalazine (sulphasalazopyrine), gold salts, D-penicillamine, leflunomide, azathioprine, anakinra, infliximab (REMICADE.sup.R), etanercept, TNF.alpha. blockers, a biological agent that targets an inflammatory cytokine, and Non-Steroidal Anti-Inflammatory Drug (NSAIDs). Examples of NSAIDs include, but are not limited to acetyl salicylic acid, choline magnesium salicylate, diflunisal, magnesium salicylate, salsalate, sodium

salicylate, diclofenac, etodolac, fenoprofen, flurbiprofen, indomethacin, ketoprofen, ketorolac, meclofenamate, naproxen, nabumetone, phenylbutazone, piroxicam, sulindac, tolmetin, acetaminophen, ibuprofen, Cox-2 inhibitors and tramadol.

According to another embodiment of the present invention, treating a medical condition associated with an insulin deficiency is effected by administering to the subject the agent *per se* for downregulating an activity or expression of miR-7.

For *in-vivo* therapy, the agent (as detailed in further detail hereinabove) is administered to the subject as is or as part of a pharmaceutical composition.

According to one embodiment, expression vectors are used for in-vivo expression of the miR-7 downregulating agents (i.e. in-vivo therapy).

As specified in further detail above, any mammalian expression vectors may be used for in-vivo therapy. Moreover, the expression vectors may comprise any additional sequences which render the vectors suitable for in-vivo expression agents in pancreatic beta cells (e.g. promoters, enhancers etc.).

Typically, recombinant viral vectors are useful for *in-vivo* expression of miR-7 downregulating agents since they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

Thus, the *ex-vivo* treated beta cells or stem cells or the miR-7 downregulating agent of the present invention can be administered to the individual *per se* or as part of a pharmaceutical composition which also includes a physiologically acceptable carrier.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the agent accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

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

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, inrtaperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of

the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients

may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuos infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (miR-7 downregulating agent) effective to prevent, alleviate or ameliorate symptoms of a disorder (e.g., insulin related disease) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide ample levels of the active ingredient which are sufficient to induce or suppress the biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment

lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc. The dosage and timing of administration will be responsive to a careful and continuous monitoring of the individual changing condition.

It will be appreciated that animal models exist by which the agents of the present invention may be tested prior to human treatment. For example, Type I diabetes models include, pancreatectomy in dogs, spontaneous rodent models (e.g. BBDP rats and the NOD mice). Type II diabetes models and obese animal models include, db/db (diabetic) mice, Zucker diabetic fatty (ZDF) rats, sand rats (Psammomys obesus) and obese rhesus monkeys.

Regardless of the above, the *ex-vivo* treated beta cells or stem cells or the miR-7 downregulating agent of the present invention are administered at an amount selected to avoid unwanted side-effects associated with elevated concentrations thereof.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

The agents of the invention can be suitably formulated as pharmaceutical compositions which can be suitably packaged as an article of manufacture. Such an

article of manufacture comprises a label for use in treating an insulin related disease (e.g. diabetes), the packaging material packaging a pharmaceutically effective amount of the beta cells or stem cells or the miR-7 downregulating agent.

It will be appreciated that each of the agents or compositions of the present invention may be administered in combination with other known treatments, including but not limited to, insulin including short-acting insulin [e.g. lispro (Humalog) or aspart (NovoLog)] and longer acting insulin [e.g. Neutral Protamine Hagedorn (NPH), Lente, glargine (Lantus), detemir, or ultralente] and oral medication for control of blood sugar levels e.g. sulfonylurea or biguanide [metformin Glucophage)].

The agents or compositions of the present invention may be administered prior to, concomitantly with or following administration of the latter.

In order to test treatment efficacy, the subject may be evaluated by physical examination as well as using any method known in the art, as for example, by finger stick blood glucose test, fasting plasma glucose test, oral glucose tolerance test, glycosylated hemoglobin or hemoglobin A1c, body mass index (BMI) and the like.

As used herein the term “about” refers to ± 10 %.

The terms "comprises", "comprising", "includes", "including", “having” and their conjugates mean "including but not limited to".

The term “consisting of means “including and limited to”.

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as

individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

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

GENERAL MATERIALS AND EXPERIMENTAL PROCEDURES

Animals

Mice were housed and handled in accordance with protocols approved by the Institutional Animal Care of Weizmann Institute of Science Ethics Committee. Conditional miR-7 transgenic mice were generated as previously described [Srinivas et al. (2001) BMC Dev Biol (1) 4]. Briefly, a 500 bp fragment flanking the miR-7-1a gene was cloned into the Rosa26 locus downstream of the PGK promoter and a transcriptional STOP cassette and upstream of IRES-EGFP-polyadenylation signal. Correct homologous recombination onto the ROSA26 locus was identified by southern blot analysis to embryonic stem cell colonies (129/SvEv). Scanning 209 colonies identified 29 positive colonies and the mouse line was derived through blastocyst injection (C57BL6/J background). Rosa-miR-7 mice were crossed to a Pdx1-Cre transgene and mated to homozygousity. Other mouse strains used in this study were Ngn3-CreER, serving as Ngn3 nulls [previously described by Wang et al., *Dev. Biol.* (2010) 339: 26-37] and a Pax6 null allele [previously described by St-Onge et al., *Nature* (1997) 387: 406-409].

Organ culture

Dorsal pancreatic rudiments of E12.5 ICR mouse embryos were dissected from the adjacent mesenchyme, using a tungsten needle. The explants were cultured in M199 medium supplemented with 10 % fetal bovine serum (GIBCO), 2 mM L-glutamine, 100 U/mL penicillin/streptomycin. Individual explants were plated in 30 μl inverted 'hanging drops' on a 35-mm Petri dish cover (NUNC), with medium containing either antagomirs (Dharmacon) or cholesterol conjugated miRNA mimics (IDT) at 1 μM. The exact sequences of the oligos are depicted in Table 1, below. Explants were further grown for up to 48 hr at 37 °C with a 5 % CO_2 in a humidified incubator. BrdU (3 μg/ml) was added to the medium 1 hour before harvest for analysis of proliferation.

Table 1: Sequences of primers and oligos

Gene name	Forward Primer	Reverse Primer
Pax6	AACAACCTGCCTATGCAA CC (SEQ ID NO: 1)	ACTTGGACGGGAACTGACAC (SEQ ID NO: 2)
Insulin	CCTGTTGGTGCACTTCCTAC (SEQ ID NO: 3)	TGCAGTAGTTCTCCAGCTGG (SEQ ID NO: 4)
Glucagon	AAACCAAGATCACTGACAAGA AATAGGT (SEQ ID NO: 5)	TTTGAAATTGTACATCCCAA GTGAC (SEQ ID NO: 6)
Ghrelin	CAGGCTCCAGCTTCCTGA (SEQ ID NO: 29)	GTGGCTGCAGTTTAGCTGGT (SEQ ID NO: 30)
Pdx1	TTCCCGAATGGAACCGAGC (SEQ ID NO: 7)	GTAGGCAGTACGGGTCCTCT (SEQ ID NO: 8)
Pax4	ACCTCATCCCAGGCCTATCTC (SEQ ID NO: 9)	TGAGGAGGAAGCCACAGGA A (SEQ ID NO: 10)
Cpa1	CAGATCGGCAGCACCTTTGAA (SEQ ID NO: 11)	GACCCACTCCCTGGAATGGA (SEQ ID NO: 12)
Ptf1a	TCCCATCCCCTTACTTTGATGA (SEQ ID NO: 13)	GTAGCAGTATTCGTGTAGCT GG (SEQ ID NO: 14)
Gapdh	TGGCAAAGTGGAGATTGTTGCC (SEQ ID NO: 15)	AAGATGGTGATGGGCTTCCC G (SEQ ID NO: 16)
Hprt	CTGGTTAAGCAGTACAGCCCCA AA (SEQ ID NO: 17)	TGGCCTGTATCCAACACTTC GAGA (SEQ ID NO: 18)
Anti-miR-7 antagomir (2'OH)-chl	ApsCpsAACAAAAUCACUAGUC UUpsCpsCpsAps-Chol (SEQ ID NO: 19)	
Anti-miR-122 antagomir (2'OH)-chl	ApsCpsAAACACCAUUGUCACAC UpsCpsCpsAps-Chol (SEQ ID NO: 20)	
MafB	CGTCCTTCCTCCCTCTAGCTC (SEQ ID NO: 31)	ACTCCCTGTCCCTGCCATG (SEQ ID NO: 32)
Arx	CAGCATTTGGCAGGCTCT (SEQ ID NO: 33)	AGGATGTTGAGCTGCGTGAG (SEQ ID NO: 34)
siPAx6	ACCAUGAUCGACAAGAUUUGCCAT (SEQ ID NO: 35)	

Pancreas histology and quantification analysis

Immunofluorescence of paraffin sections was carried out as previously described [Melkman-Zehavi et al. EMBO J. (2011) 30(5):835-45]. Whole-explants staining was carried out as previously described [Kredo-Russo, S. and Hornstein, E. (2011) Methods Mol Biol, 732, 89-97]. The primary antibodies used were: rabbit anti-Pax6 (1:300, Covanc), guinea pig anti-insulin (1:200, Dako), rabbit anti-glucagon (1:200, Dako). Secondary antibodies used were: Cy2-Cy3- or Cy5-conjugated donkey anti-guinea pig, anti-mouse, and anti-rabbit IgG (1:200, Jackson ImmunoResearch). Nuclei were stained

with Dapi (1: 10,000, Molecular Probes). Whole-mount BrdU analysis that included a 2 hour DNase I treatment was carried out as previously described [Tkatchenko A.V., Biotechniques (2006) 40: 29-30, 32].

Fluorescent confocal images were captured with a Zeiss LSM 510 microscope, using an optical depth of 1 μm, with at least 6-8 optical sections at 5 μm intervals throughout the whole organ.

Morphometry of the explants was performed by quantification of the immunostained area from the entire explant sections from a minimum of three mutants and three wild-type matched littermates,. Total tissue area and total hormone-positive area, were calculated using 'Niss-elements' software (Nikon) [Garofano et al. (1998) Diabetologia, 41, 1114-1120].

For cell number quantification at E15.5, hormone-positive cells were manually counted every fifth section throughout the whole pancreas anlagen. Data were the average number of cells/section in multiple sections and were analyzed for four or more individual animals per genotype. Cell number analysis of total hormone-positive cells in whole E12.5 explants was performed manually, by counting cells in six stacked z-section confocal images, spanning the whole explants.

Quantitative PCR for miRNA and mRNA

Extraction of total RNA was carried out by the miRNeasy Mini Kit (QIAGEN). Synthesis of mRNA cDNA was created using an oligo d(T) primer (Promega) and SuperScript II reverse transcriptase (Invitrogen). Synthesis of miRNA cDNA was created using Taqman MicroRNA qPCR Assays (Applied Biosystems). qPCR analysis was performed on LightCycler® 480 System (Roche) using Kapa™ SYBR® Green qPCR kit (Finnzymes). miRNA and mRNA levels were normalized to the expression of small RNAs (sno234 and U6) or mRNA (Gapdh and Hprt), respectively. Primer sequences are described in Table 1, above.

miRNA in situ hybridization

Paraffin sections of E15.5 pancreata were hybridized with DIG-labeled LNA probes (Exiqon) overnight at 48 °C (miR-7) or 54 °C (U6, control) as previously described [Pena et al. (2009) Nat Methods (6) 139-141] and developed with TSA kit (PerkinElmer) as previously described [Silahtaroglu et al. (2007) Nat Protoc (2) 2520-

2528]. When *in situ* hybridization was combined with immunoflourescence, primary antibody was added to the Anti-Dig-POD incubation (1:500 Roche).

Cell culture, luciferase reporter assay and western blotting

HEK-293T cells (American Type Culture Collection) and MIN6 cells were grown in Dullbecco's modified Eagle medium (DMEM) with 10 % FBS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin at 37 °C; 5 % CO2 in a humidified incubator. Experiments on MIN6 cells were performed between passages 18 to 28.

A 742 bp fragment of the mouse Pax6 3′UTR sequence (chr2 105536551-105537201) was subcloned into psiCHECK-2 Vector (Promega) and transfected into HEK-293T cells. Dual-Reporter luciferase assay was performed 48 hr later, according to the manufacturers' instructions (Promega).

miR-7 overexpression was achieved using expression vectors miRVec-miR-7 or miRVec control. miR-7 knockdown was carried out using oligos against miR-7 or against scrambled sequence, as negative control oligos (50 nM, Ambion), using Lipofectamine 2000 Reagent (Invitrogen).

For western blots, cellular lysate was subject to 10 % SDS-PAGE and immunoblotted with rabbit anti-Pax6 (1:5,000 Chemicon), mouse anti-GAPDH (1:10000, Ambion) and quantified with ImageJ software.

For analysis of insulin transcription, firefly luciferase reporter driven by the rat insulin promoter and an A20-Renilla luciferase construct were transfected using Lipofectamine 2000 Reagent Invitrogen) to MIN6 cells. Anti miR-7 oligo (100 nM) and Pax6 siRNA 10 nM) were from IDT;

Statistical Analysis

Analysis was performed using either Student's t-test or two-way ANOVA by the JMP software. Results were provided as mean ± SEM. The null hypothesis was rejected at the 0.05 level (**) or 0.01 (*).Gene Ontology analysis was performed using DAVID (as previously described by Dennis et al. *Genome Biol.* (2003) 4, P3].

EXAMPLE 1

miR-7 is expressed in the endocrine cells of the pancreas

To determine the spatial expression pattern of miR-7, the present inventors carried out *in situ* hybridization combined with immunefluorescent protein detection on

E12.5-E15.5 pancreatic sections, using a digoxigenin (DIG)-labeled LNA probe. At this time point, called 'secondary transition', many endocrine cells are generated within the pancreatic epithelium. Bright field analysis revealed that miR-7 expression was restricted to a subset of clustered epithelial cells at the "trunk" compartment of the branching pancreatic epithelium (Figures 1A-B). To identify in higher resolution what cell types expressed miR-7, the present inventors carried out fluorescence *in situ* hybridization. This experiment revealed clusters of miR-7 expressing cells (Figure 1C). The hybridization pattern was specific to miR-7 and could not be detected with a probe against the ubiquitously-expressed small RNA U6 or a scrambled miRNA sequence (Figures 1D-E). Fluorescence *in situ* hybridization combined with immunostaining of endocrine proteins demonstrated co-localization of miR-7 with insulin and glucagon in differentiating β- and α-cells, respectively (E13.5-E15.5; Figures 1F-H). miR-7 and Cpa1 expression domains were mutually exclusive at E15.5 (Figures 1I-K), as were miR-7 and Hnf1β at E14.5 (Figures 1L-N). These data indicate that miR-7 was not expressed in differentiated acinar or duct cells. To examine miR-7 expression in endocrine precursor cells, immunostaining of Ngn3 was performed. At E12.5, E13.5 and E14.5, miR-7 was colocalized with many Ngn3-positive cells (Figures 1O-T), suggesting that miR-7 was induced in newly born endocrine cells. Independent genetic support to this study came from the analysis of Ngn3-null pancreata. It was previously shown that Ngn3- deficient embryos completely lack endocrine hormone-producing cells [Gradwohl G. et al., *Proc. Natl. Acad. Sci. USA* (2000) 97: 1607-1611]. Consistent with this, the expression of endocrine markers, such as Pax6 and insulin, was downregulated in Ngn3-null pancreata (Figure 1U). As miR-7 expression was also abrogated in E14.5 Ngn3-null pancreas, the present inventors concluded that this miRNA is specifically expressed within the endocrine lineage. Furthermore, this regulation was specific to miR-7, as the expression of miR-17 and Let-7b was not changed (Figure 1U). Notably, miR-375, another pancreatic miRNA, was also downregulated in Ngn3-null pancreata, yet some residual expression was maintained, unlike miR-7 (Figure 1U). Altogether, this analysis revealed the endocrine-specific expression pattern of miR-7, wherein miR-7 is induced in Ngn3+ precursors and is maintained in the differentiated endocrine cells.

EXAMPLE 2

Pax6 is a miR-7 target

To identify potential miR-7 targets that play a role in pancreas development, two unbiased bioinformatic approaches were employed. First, 'gene ontology' (GO) was analyzed in terms related to miR-7 targets [DAVID, as previously described in Dennis G. et al., *Genome Biol.* (2003)4, P3]. Among the 237 predicted miR-7 targets [TargetScan as described in Lewis et al. (2005) *Cell*, 120, 15-20], the GO term 'Regulation of transcription' was found to be the most significantly enriched (52 genes, $P<2.35E^{-7}$). Intriguingly, within this list, Pax6 was the only established pancreatic transcription factor (see Tables 2-4, below, and Figure 2A). Furthermore, the binding site for miR-7 at the Pax6 mRNA 3' untranslated region (3' UTR) is predicted to be strong and conserved (Figure 2B),

Independently, an interaction map was built of miRNAs with the 3' UTRs of transcription factors that are known to control pancreas development, including Pdx1, Ngn3, Nkx2.2, Nkx6.1, MafB, Pax4, Pax6, Arx, Hnf1b and Hnf6 (for a comprehensive list see Tables 2-4, below). This approach provided a wealth of potential interactions, however, Pax6 was the only miR-7 predicted target. As Pax6 expression is known to be tightly regulated in many organs, the present inventors hypothesized that miR-7 may be a new endocrine regulatory gene upstream of Pax6.

Table 2: Genes related to pancreas differentiation and their predicted targets

Predicted targeting miRNAs		
PicTar	**TargetScan-mouse**	**Gene name**
none conserved sites	none conserved sites	Pdx1/IPF1
miR-106b	none conserved sites	Ngn3 (neurogenin 3)
miR-106a		
miR-294		
miR-124a		
miR-20b		
miR-20a		
miR-291b-3p		
miR-291a-3p		
miR-17-5p		
miR-295		
miR-93		

mmu-miR-452	miR-133	NKX2-2
	miR-30a/30a-5p	
	miR-182	
	miR-17-5p/20/93/106/519	
	miR-26ab	
	miR-96	
	miR-374	
	miR-342	
	miR-377	
none conserved sites	No predictions (short 3'UTR)	NKX6-1
mmu-miR-130a	miR-148/152	MafB
mmu-miR-130b	miR-338/338-3p	
mmu-miR-188	miR-29abc	
mmu-miR-29a	miR-130/301	
mmu-miR-29b	miR-223	
mmu-miR-301	miR-155	
mmu-miR-29c	miR-203	
mmu-miR-338		
mmu-miR-148b		
mmu-miR-152		
mmu-miR-148a		
mmu-miR-199b		
mmu-miR-199a		
mmu-miR-485-3p		
mmu-miR-155		
mmu-miR-126-5p		
mmu-miR-223		
mmu-miR-192		
mmu-miR-186		
mmu-miR-365	miR-365	Pax6
mmu-miR-7	miR-7	
mmu-miR-7b	miR-129/129-5p	
mmu-miR-129-5p	miR-375	
mmu-miR-300	miR-196	
mmu-miR-450	miR-96	
mmu-miR-375	miR182	
none conserved sites	none conserved sites	Pax4
none conserved sites	miR-204	Arx
	miR-96	
	miR-132/212	
	miR-27ab	
	miR-300	
	miR-130/301	
	miR-139-5p	

none conserved sites	miR-200bc/429	HNF1b
	miR-375	
	miR-194	
	miR-24	
	miR-25/32/92/363/367	
none conserved sites	mmu-miR-320	Onecut1 (HNF6)

(Table 2, cont.)

Table 3: Gene Ontology analysis of predicted miR-7 targets

calculated Pvalue for term and corrected P values									
FDR	**Benjamini**	**Bonferroni**	**Fold Enrichment**	**Pop Total**	**Pop Hits**	**P-value**	**% of total predicted miR-7 targets**	**Count (nos. of genes in this term category)**	**Term**
3.77E-04	2.68E-04	2.68E-04	2.046948738	13588	2227	2.35E-07	24.3	52	**GO:0045449~regulation of transcription**
0.002706436	9.62E-04	0.00192331	3.273188102	13588	616	1.68E-06	10.7	23	GO:0006357~regulation of transcription from RNA polymerase II promoter
0.002859408	6.78E-04	0.00203191	3.691137521	13588	475	1.78E-06	9.3	20	GO:0045941~positive regulation of transcription
0.003662215	6.51E-04	0.002601657	3.473499696	13588	530	2.28E-06	9.8	21	GO:0010557~positive regulation of macromolecule biosynthetic process
0.004253361	6.05E-04	0.003020984	3.592808038	13588	488	2.65E-06	9.3	20	GO:0010628~positive regulation

									of gene expression
0.006460509	7.66E-04	0.00458508	3.848686074	13588	410	4.02E-06	8.4	18	GO:0010629~negative regulation of gene expression
0.006777121	6.88E-04	0.004809249	3.335063114	13588	552	4.22E-06	9.8	21	GO:0031328~positive regulation of cellular biosynthetic process
0.007079523	6.29E-04	0.00502331	2.077827132	13588	1772	4.41E-06	19.6	42	GO:0006350~transcription

(Table 3, cont.)

Table 4: miR-7 predicted targets under Gene Ontology Term "Regulation of Transcription" (mus musculus)

Regulation of transcription	
Gene Name	Gene ID
BCL6 co-repressor-like 1	453169
CCR4-NOT transcription complex, subunit 8	460864
CGG triplet repeat binding protein 1	451590
GATA binding protein 6	472801
GATA zinc finger domain containing 2B	470085
GLI-Kruppel family member GLI3	442692
Kruppel-like factor 12	452902
Kruppel-like factor 4 (gut)	439346
PHD finger protein 17	443767
PHD finger protein 21A	422523
RIKEN cDNA D930049A15 gene; zinc finger, MIZ-type containing 1	424115
SET domain containing (lysine methyltransferase) 8; predicted gene 8590	465940
additional sex combs like 1 (Drosophila)	468967
bromodomain and WD repeat domain containing 1	434997
catenin (cadherin associated protein), delta 1	424730
cone-rod homeobox containing gene	439284
dachshund 1 (Drosophila)	467246
early growth response 3	454142
estrogen-related receptor gamma	477434
eukaryotic translation initiation factor 2C, 1	467835
forkhead box N3	480126
helicase, lymphoid specific	477958
homeodomain interacting protein kinase 2	445147
inhibitor of growth family, member 5	430151
methyl CpG binding protein 2	426701

methyl-CpG binding domain protein 2	480586
nuclear factor I/B	479694
nuclear receptor subfamily 1, group H, member 2	445414
nuclear receptor subfamily 4, group A, member 3	457788
paired box gene 6	431410
poly (ADP-ribose) polymerase family, member 1	443346
purine rich element binding protein B	424173
ras responsive element binding protein 1	459023
retinoblastoma 1	427771
similar to PBX3a; pre B-cell leukemia transcription factor 3	460161
similar to Zinc finger and BTB domain containing 1; zinc finger and BTB domain containing 1	449895
similar to mKIAA0658 protein; cryptochrome 2 (photolyase-like)	422529
similar to mafG; v-maf musculoaponeurotic fibrosarcoma oncogene family, protein G (avian)	459027
sine oculis-related homeobox 4 homolog (Drosophila)	461708
special AT-rich sequence binding protein 1	424985
trans-acting transcription factor 1	481531
transcription factor 12	475056
transcription factor 4	482380
v-maf musculoaponeurotic fibrosarcoma oncogene family, protein K (avian)	470659
zinc fingers and homeoboxes 3	452440

(Table 4, cont.)

* Of note, Table 2 is a bioinformatic analysis of mRNAs encoding for proteins involved in pancreas development, with respect to conserved miRNA binding sites in their 3'UTRs. Predictions are based on TargetScans and PicTar. Table 3 is a gene Ontology (GO) analysis of miR-7 conserved targets, and Table 4 depicts the genes listed under the GO term "Regulation of Transcription", among them is Pax6.

To determine whether miR-7 directly targets Pax6 3'UTR the present inventors performed a heterologous reporter assay. The whole 3'UTR of Pax6 (742 bp) was cloned into a dual luciferase reporter vector and introduced into HEK-293T cells along with expression vector for miR-7 (miRvec-7) or a miRNA control vector (harboring a random and non-targeting miRNA-like sequence: 'Ctrl'). Overexpression of miR-7 significantly decreased luciferase activity relative to negative control (Figure 2C, pink bar; reduced to 62 %). The addition of an anti-miRNA oligo partially blocked this repression, supporting the functionality of the predicted miR-7 binding site. Moreover, when inventors introduced a 3'UTR mutant sequences, in which 6 nucleotides of the potential 'seed' binding-site were eliminated (marked in red, Figure 2B), miR-7-dependent

repression was completely abolished (Figure 2C). To determine whether miR-7 represses the expression of endogenouse Pax6, inventors transfected the beta cell line (MIN6) with miRvec-7. Overexpression of miR-7 decreased PAX6 protein levels to 60 % relative to control miRvec, as measured by western blots. Consistently, inhibition of miR-7 by anti-miR-7 oligos significantly upregulated PAX6 protein levels (Figures 2D-G). Taken together, these results indicate that Pax6 is a *bona-fide* target of miR-7 in beta cells.

Inventors next tested if miR-7 and its target Pax6 are co-expressed in the developmental context of endocrine differentiation. miR-7 *in situ* hybridization combined with Pax6 immunofluorescence revealed that miR-7 and Pax6 are indeed co-expressed in endocrine cell, allowing direct molecular interactions (Figures 2H-J). To quantify temporal dynamics of miR-7 and Pax6 expression, inventors performed qPCR on RNA extracted from E12.5-E15.5 pancreata. miR-7 levels increased in time, reaching maximal levels at E14.5. Intriguingly, inventors noted trend of reciprocal miR-7 and Pax6 expression in which miR-7 upregulation is associated with Pax6 downregulation (Figure 2K). Hence, miR-7-based attenuation may serve to progressively inhibit Pax6 expression levels as maturation of endocrine cells occurs.

EXAMPLE 3

miR-7 controls endocrine differentiation in explants culture

To determine the functional role of miR-7 in the endocrine lineage, inventors carried out gain- and loss- of function experiments in a primary pancreatic explant system (see schematic illustration in Figure 3A). E12.5 pancreatic buds were cultured for 48 hrs under defined *ex-vivo* conditions, providing a reliable model for normal development [as previously described by Kredo-Russo, S. and Hornstein, E. (2011), supra]. Inventors detected typical Ngn3, insulin and miR-7 expression that recapitulated *in-vivo* differentiation, including the expected differentiation of alpha and beta cells (Figure 4A-E).

To manipulate miR-7 expression in organ cultures, inventors used cholesterol-conjugated 2'-O-methyl (2'OMe) 'antagomirs' against miR-7 or miR-7 ‘mimic’ oligos (termed ‘miR-7 KD’ and ‘miR-7 OE’, respectively). As 'non-targeting' negative controls, inventors used antagomirs against the liver specific miR-122, which is not

expressed in the pancreas and miR-67-mimic, a nematode miRNA that is not expressed in vertebrates (termed 'Ctrl-KD or 'Ctrl-OE', respectively). First, inventors verified that a Cy-bound oligos is efficiently taken up by the explants (Figures 4F-H). Next, the functionality of miR-7 KD and miR-7 OE oligos was confirmed by co-transfecting them into HEK-293T with a miR-7 luciferase reporter that harbors multiple miR-7 binding sites on its 3'UTR [as previously described by Kefas et al. (2008) Cancer Res (68] 3566-3572]. Reporter luciferase activity was strongly suppressed by miR-7 overexpression, and this was reversed by co-transfectng miR-7 KD together with miR-7 OE oligos (Figure 4I). Inventors then used this system to study miR-7-Pax6 interactions in pancreatic explants.

In explants treated with miR-7 KD, Pax6 mRNA levels were up-regulated by 2.5 fold, relative to control (Figure 3B), whereas in explants treated with miR-7 OE, Pax6 mRNA levels were repressed to 60 %, relative to control (Figure 3M). Additionally, immunofluorescent analysis revealed reduction of PAX6 protein levels (Figures 4S-T). These results suggest functional regulation of Pax6 levels downstream of miR-7 in pancreas development.

Intriguingly, upon miR-7 KD, insulin and glucagon mRNAs levels were increased (insulin by 22 % and glucagon by 61 %, Figure 3C). Accordingly, an increase in insulin protein content was demonstrated by ELISA measurement in miR-7 KD explants, relative to control (Figure 3D). Furthermore, comprehensive morphometric analysis revealed a 20 % increase in the insulin-positive area and in the number of insulin-positive cells. Similarly, 40 % increase in the glucagon-positive area and in the number of glucagon-positive cells were found in miR-7 KD pancreata, relative to controls (Figures 3E-J, 3L and Figures 8A-J). However, Somatostatin expression in miR-7 KD was comparable with control (Figures 8A-J). To understand the potential causes for the increase in insulin and glucagon-positive cells, the proliferation capacity of endocrine cells was studied. The total numbers of BrdU-positive nuclei and the percentage of proliferating insulin-positive and glucagon-positive cells, measured by either Ki67 or BrdU, was comparable in miR-7 KD and control explants (Figures 9A-G). In addition, the numbers of Ngn3-positive progenitors, was not affected by miR-7 KD (Figures 8K-S) suggesting that the increase in insulin- and glucagon-positive cells

emerges neither from enhanced proliferation, nor from changes in the size of the Ngn3 precursor pool.

Although Pax6 positively regulates insulin and glucagon expression, it negatively regulates ghrelin expression and differentiation of ε-cells. Accordingly, miR-7 KD resulted in reduced numbers of ghrelin-positive cells (Figures 3E-J, 3L and Figures 8A-J) supporting the view that miR-7 knockdown acts upstream of Pax6 to promote differentiation into insulin- and glucagon-positive cells at the expense of ghrelin-positive cells.

Inventors next quantified the expression levels of a set of transcription factors that are essential for beta and alpha-cell differentiation. This analysis revealed upregulation of Arx, which is specifically expressed in alpha cells and of the beta cell factors, Pax4 and MafB in miR-7 KD explants. However, the levels of the exocrine markers Cpa1 and Ptf1a were unchanged (Figure 3K). Consistently, miR-7 OE resulted in downregulation of insulin and MafB mRNA levels, two genes that are directly controlled by Pax6, and in reduced beta cell mass (Figure 3N-R). However, glucagon and Arx mRNA levels were not significantly changed. Taken together, these findings revealed that miR-7 negatively control differentiation and maturation of hormone-producing cells.

EXAMPLE 4

Overexpression of miR-7 *in-vivo* reduced expression of endocrine genes

To examine the consequences of miR-7 overexpression *in-vivo*, inventors generated a conditional miR-7 transgenic mouse line. miR-7a-1 genomic sequence was inserted by homologous recombination into the ubiquitously-expressed Rosa26 locus (Figures 5A-B) as previously described by Srinivas et al. (supra). In this knock-in model, the expression of miR-7 is coupled to the expression of enhanced green fluorescent protein (EGFP) and both are conditionally blocked by a 'Neo-STOP' cassette, flanked by LoxP sites. Thus, miR-7 and EGFP expression are induced only in tissues that express a Cre transgene. To validate the inducible expression of miR-7, inventors isolated mouse embryonic fibroblasts (MEFs) from Rosa26-miR-7 mice. Upon introduction of Cre recombinase by adenoviral vector, the expression of miR-7 was

specifically up-regulated by 2 fold, whereas the level of an unrelated miRNA, miR-199, remained unchanged (Figure 5C).

To stimulate the production of miR-7 early in the pancreatic lineage, inventors crossed the Rosa26-miR-7 allele to a Pdx1-Cre transgene. Pdx1-Cre;Rosa26-miR-7 mice specifically expressed GFP protein in E13.5 pancreas cells (Figures 5D-E), while littermates that did not harbor the Pdx1-Cre transgene did not express GFP (termed 'Ctrl').

Inventors next characterized the Pdx1-Cre;Rosa26-miR-7 mice by qPCR of E15.5 pancreata. The expression of the mature endocrine cell markers, insulin and glucagon was downregulated (insulin to 72 %, glucagon to 68 %). Quantification of the expression of transcription factors showed a reduction in Arx (reduced to 63 %), Pax4 (reduced to 62 %), and in the miR-7 target gene Pax6 (reduced to 49 %, Figure 5F). Notably, Cpa1 and Ptf1a levels were unchanged, indicating that the exocrine lineage was unaffected by miR-7 mis-expression in Pdx1-Cre;Rosa26-miR-7 mice. Immunostaining for insulin and glucagon on E15.5 pancreas sections revealed a decrease in the expression of endocrine markers (Figures 5G-J). Therefore, when miR-7 was overexpressed in Pdx1-Cre;Rosa26-miR-7 embryos, Pax6 levels decreased and the expression of insulin and glucagon was downregulated, providing in-vivo evidence for control of Pax6 by miR-7.

To elucidate the genetic interactions of miR-7 and Pax6, a luciferase reporter, driven by the insulin promoter was used as previously described [Melkman-Zehavi et al., (2011), supra]. As the insulin promoter is directly activated by Pax6, it provides a model system for monitoring the effect of miR-7-Pax6 axis on insulin expression. Transfecting MIN6 cells with either siRNA against Pax6 ('siPax6') or miR-7 overexpression vector ('miR-7 OE'), resulted in downregulation of insulin promoter activity, relative to scrambled siRNA, or empty miRvec, respectively (Figure 5K). These results support the observed decrease in insulin mRNA expression by miR-7 OE in explants (Figure 3N) and in miR-7 transgenic model (Figure 5F). The combined effect of siPax6 together with miR-7 OE, significantly repressed the activity of the insulin promoter (Figure 5K). Furthermore, transfecting MIN6 cells with knockdown oligos against miR-7 ('miR-7 KD') resulted in upregulation of insulin promoter activity,

relative to scrambled oligos ('Ctrl KD'), consistent with insulin mRNA upregulation in miR-7 KD-treated explants (Figure 5L).

However, when inhibited simultaneously, siPax6 reversed the effect of miR-7 KD, suggesting that Pax6 mediates miR-7 regulation of insulin expression (Figures 5K-L). These results stress the epistatic relationship of miR-7 upstream of Pax6, in the control of insulin expression. Taken together, when miR-7 is overexpressed, Pax6 levels are decreased and the expression of is down-regulated, consistent with the data obtained from explant studies. Therefore, miR-7 negatively regulates Pax6 and downstream endocrine differentiation. Inventors next studied the consequences of a genetic manipulation in the miR-7 target, Pax6.

EXAMPLE 5

Pax6 haplo-insufficiency resembles miR-7 overexpression phenotype

To examine the outcome of Pax6 downregulation by an independent mechanism, inventors analyzed Pax6 heterozygous pancreata (termed 'het'). These mice were generated by replacing the start codon and the entire paired domain with a beta-galactosidase gene as previously described by St-Onge [see Figure 6A, St-Onge et al. (1997) *Nature* 387: 406-409]. qPCR analysis of mRNA, extracted from E15.5 Pax6 heterozygous pancreata showed a 50 % reduction in Pax6 mRNA levels relative to wild-type littermate controls (termed 'wt', Figure 6B). Immunostaining and quantification further revealed downregulation of protein expression at the cellular level (Figures 6C-E). While homozygous loss of Pax6 causes complete loss of insulin and of glucagon expression, here inventors show that in Pax6 heterozygote pancreata insulin and glucagon mRNA levels decreased by 65 % and 30 %, respectively (Figure 6F). In addition, MafB levels were slightly downregulated in Pax6 heterozygouse (20 % reduction), whereas Pdx1, Arx, Pax4 and Ptf1a remained unchanged relative to wt (Figure 6G). In addition, serial confocal quantification revealed a 22 % decrease in beta cells numbers, while alpha cell numbers remained unchanged (Figures 6H-J). These data revealed the sensitivity of MafB, insulin and glucagon expression to Pax6 levels. Moreover, Pax6 haplo-insufficiency phenocopied miR-7 overexpression, especially the predominant impact on insulin-expressing cells. Inventors next mapped miR-7 into the transcriptional network that specifies the endocrine cells.

EXAMPLE 6

miR-7 functions upstream of Pax6 and is dependent on Ngn3

To examine whether miR-7 and Pax6 reciprocally regulate each other, inventors quantified miR-7 levels in E15.5 Pax6 null pancreata by qPCR. miR-7 expression was not changed in Pax6 heterozygous or in homozygote null pancreata relative to wild-type littermate controls (Figure 7A). Therefore, inventors conclude that miR-7 is not controlled by Pax6. To examine how miR-7 is activated upstream of Pax6, inventors quantified miR-7 levels in E15.5 Ngn3-null pancreata. Intriguingly, miR-7 expression was completely abrogated in Ngn3-null pancreas relative to littermate controls (Figure 1F). This analysis demonstrated that miR-7 expression is dependent on the presence of Ngn3, providing a molecular mechanism for endocrine-specific expression of miR-7 (Figure 7B). In summary, the presented data suggest that miR-7 acts downstream of Ngn3 to limit Pax6 expression levels, allowing endocrine cell maturation and precise development of the endocrine pancreas.

Taken together, the present inventors have discovered a miR-7-based mechanism that controls and refines Pax6 levels in the endocrine pancreas, through a conserved binding site in Pax6 mRNA 3'UTR. This mechanism enables correct insulin and glucagon expression and proper beta and alpha cell differentiation. The present inventors showed that miR-7 is expressed uniquely in the endocrine lineage and that its expression is up-regulated between mouse gestational ages E12.5 - E15.5. Intriguingly, despite high expression levels, miR-7 serves an inhibitory factor in endocrine cell maturation. For example, miR-7 knockdown led to upregulation of endocrine markers. Conversely, overexpression of miR-7 repressed endocrine gene expression, mainly insulin. While miR-7 appears as an inhibitor of final differentiation, more likely it controls Pax6 levels in a temporal fashion, allowing higher levels of the transcription factor first and dampening its expression later (see model in Figure 10). The present inventors have further demonstrated that miR-7 expression is dependent on Ngn3.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all

such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. A method of ex-vivo increasing insulin content in beta cells or stem cells, the method comprising contacting said beta cells or stem cells with an agent for downregulating an activity or expression of miR-7, thereby increasing said insulin content in said beta cells or stem cells.

2. An isolated population of cells generated according to the method of claim 1.

3. An isolated population of cells comprising an exogenous agent for downregulating an activity or expression of miR-7, wherein said cells secrete insulin.

4. A pharmaceutical composition comprising the isolated population of cells of claims 2 or 3 and a pharmaceutically acceptable carrier or diluent.

5. Use of the isolated population of cells of claims 2 or 3 for the manufacture of a medicament identified for treating a medical condition associated with an insulin deficiency.

6. The isolated population of cells of claims 2 or 3 for treating a medical condition associated with an insulin deficiency.

7. A method of treating a medical condition associated with an insulin deficiency in a subject in need thereof, the method comprising administering to the subject the isolated population of cells of claims 2 or 3, thereby treating the medical condition associated with the insulin deficiency.

8. A method of treating a medical condition associated with an insulin deficiency in a subject in need thereof, the method comprising administering to the subject an agent for downregulating an activity or expression of miR-7, thereby treating the medical condition associated with the insulin deficiency.

9. The method of claim 1, wherein said stem cells comprise embryonic stem cells.

10. The method of claim 1, wherein said stem cells comprise human pluripotent stem cells.

11. The method of claim 1, wherein said stem cells comprise mesenchymal stem cells.

12. The method of claim 1, wherein said beta cells comprise precursor beta cells.

13. The method of claim 12, wherein said precursor beta cells comprise de-differentiated beta cells.

14. The method of claim 13, wherein said de-differentiated beta cells comprise induced pluripotent stem cells generated from beta cells.

15. The method of claim 12, wherein said precursor beta cells comprise transdifferentiated liver cells.

16. The method of claim 1, wherein said beta cells comprise mature beta cells.

17. The method of claims 1 or 8, or isolated population of cells of claim 3, wherein said agent is a polynucleotide agent.

18. The method of claims 1 or 8, or isolated population of cells of claim 3, wherein said agent is an antagomir.

19. The use of claim 5, isolated population of cells of claim 6, or method of claims 7 or 8, wherein said medical condition associated with an insulin deficiency comprises diabetes mellitus.

20. The method of claims 7 or 8, wherein said subject is a human subject.

21. A method of increasing insulin content in beta cells or stem cells, the method comprising expressing in said beta cells or stem cells a target gene of miR-7 selected from the group consisting of epidermal growth factor receptor (EGFR), insulin-degrading enzyme (IDE), Kruppel-like factor 4 (KLF4), GLI family zinc finger 3 (GLI3), insulin receptor substrate 1 (IRS1), Sp1 transcription factor (SP1), O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) (OGT), insulin-like growth factor 1 receptor (IGF1R) and one cut homeobox 2 (ONECUT2), thereby increasing said insulin content in said beta cells or stem cells.

22. The method of claim 21, being effected ex-vivo.

23. The method of claim 21, being effected in-vivo.

24. A nucleic acid construct comprising a nucleic acid sequence encoding a target gene of miR-7 selected from the group consisting of epidermal growth factor receptor (EGFR), insulin-degrading enzyme (IDE), Kruppel-like factor 4 (KLF4), GLI family zinc finger 3 (GLI3), insulin receptor substrate 1 (IRS1), Sp1 transcription factor (SP1), O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) (OGT), insulin-like growth factor 1 receptor (IGF1R) and one cut homeobox 2 (ONECUT2) wherein said target gene of miR-7 is under a transcriptional regulation of a cis-acting regulatory element.

25. The nucleic acid construct of claim 24, wherein said cis-acting regulatory element is a beta cell or stem cell specific promoter.

26. An isolated population of beta cells or stem cells exogenously expressing a target gene of miR-7 selected from the group consisting of epidermal growth factor receptor (EGFR), insulin-degrading enzyme (IDE), Kruppel-like factor 4 (KLF4), GLI family zinc finger 3 (GLI3), insulin receptor substrate 1 (IRS1), Sp1 transcription factor (SP1), O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase) (OGT), insulin-like growth factor 1 receptor (IGF1R) and one cut homeobox 2 (ONECUT2).

27. An isolated population of cells generated according to the method of claim 22.

28. A pharmaceutical composition comprising the isolated population of cells of claim 26 or 27 and a pharmaceutically acceptable carrier or diluent.

29. The isolated population of cells of claim 26 or 27 for treating a medical condition associated with an insulin deficiency.

30. A method of treating a medical condition associated with an insulin deficiency in a subject in need thereof, the method comprising administering to the subject the isolated population of cells of claim 26 or 27, thereby treating the medical condition associated with the insulin deficiency.

31. The method of claim 21 or isolated population of beta cells or stem cells of claim 26, wherein said stem cells comprise embryonic stem cells.

32. The method of claim 21 or isolated population of beta cells or stem cells of claim 26, wherein said stem cells comprise human pluripotent stem cells.

33. The method of claim 21 or isolated population of beta cells or stem cells of claim 26, wherein said stem cells comprise mesenchymal stem cells.

34. The method of claim 21 or isolated population of beta cells or stem cells of claim 26, wherein said beta cells comprise precursor beta cells.

35. The method or isolated population of beta cells or stem cells of claim 34, wherein said precursor beta cells comprise de-differentiated beta cells.

36. The method or isolated population of beta cells or stem cells of claim 35, wherein said de-differentiated beta cells comprise induced pluripotent stem cells generated from beta cells.

37. The method or isolated population of beta cells or stem cells of claim 34, wherein said precursor beta cells comprise transdifferentiated liver cells.

38. The method of claim 21 or isolated population of beta cells or stem cells of claim 26, wherein said beta cells comprise mature beta cells.

39. The isolated population of cells of claim 29 or the method of claim 30, wherein said medical condition associated with an insulin deficiency comprises diabetes mellitus.

40. The method of claim 30, wherein said subject is a human subject.

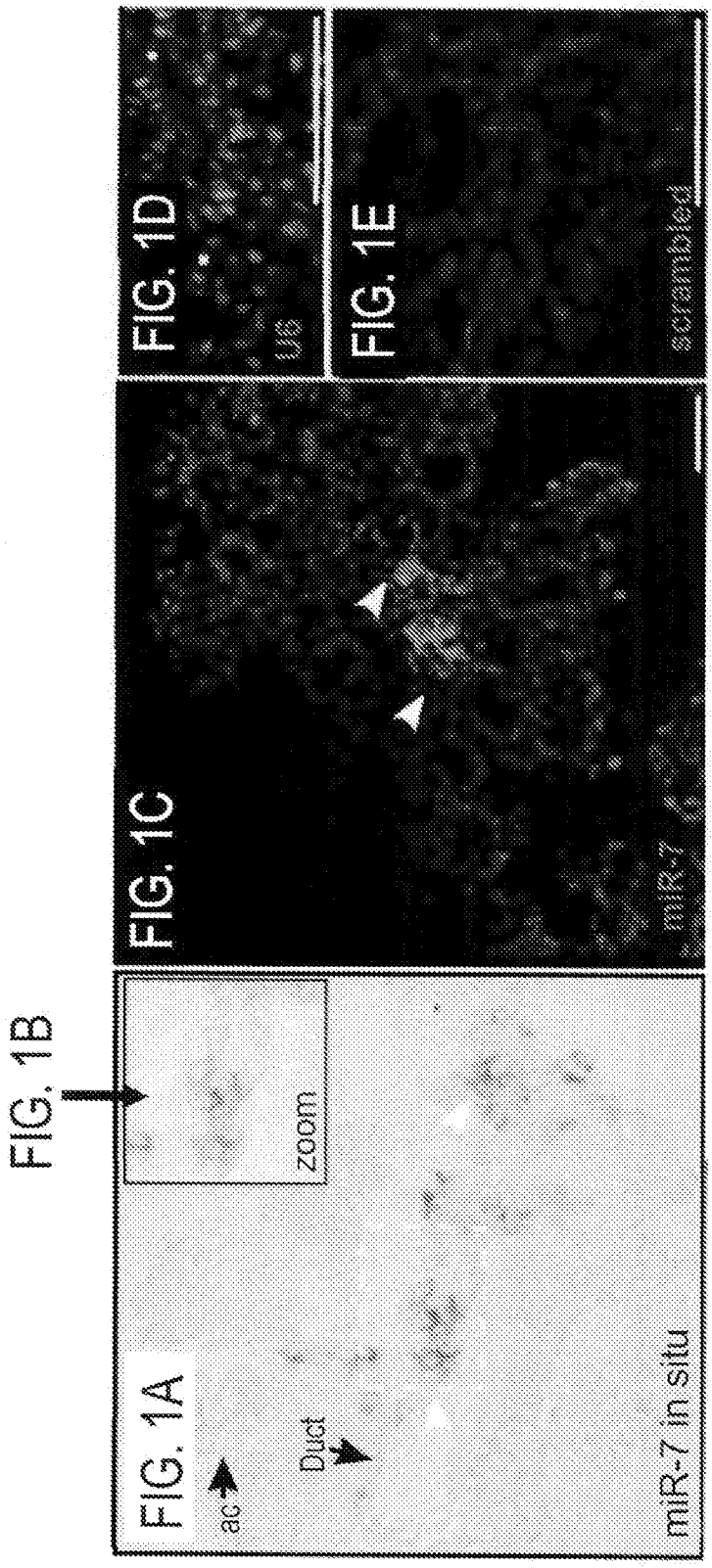


FIG. 1A
ac
Duct
zoom
miR-7 in situ
FIG. 1B
FIG. 1C
miR-7
FIG. 1D
us
FIG. 1E
scrambled

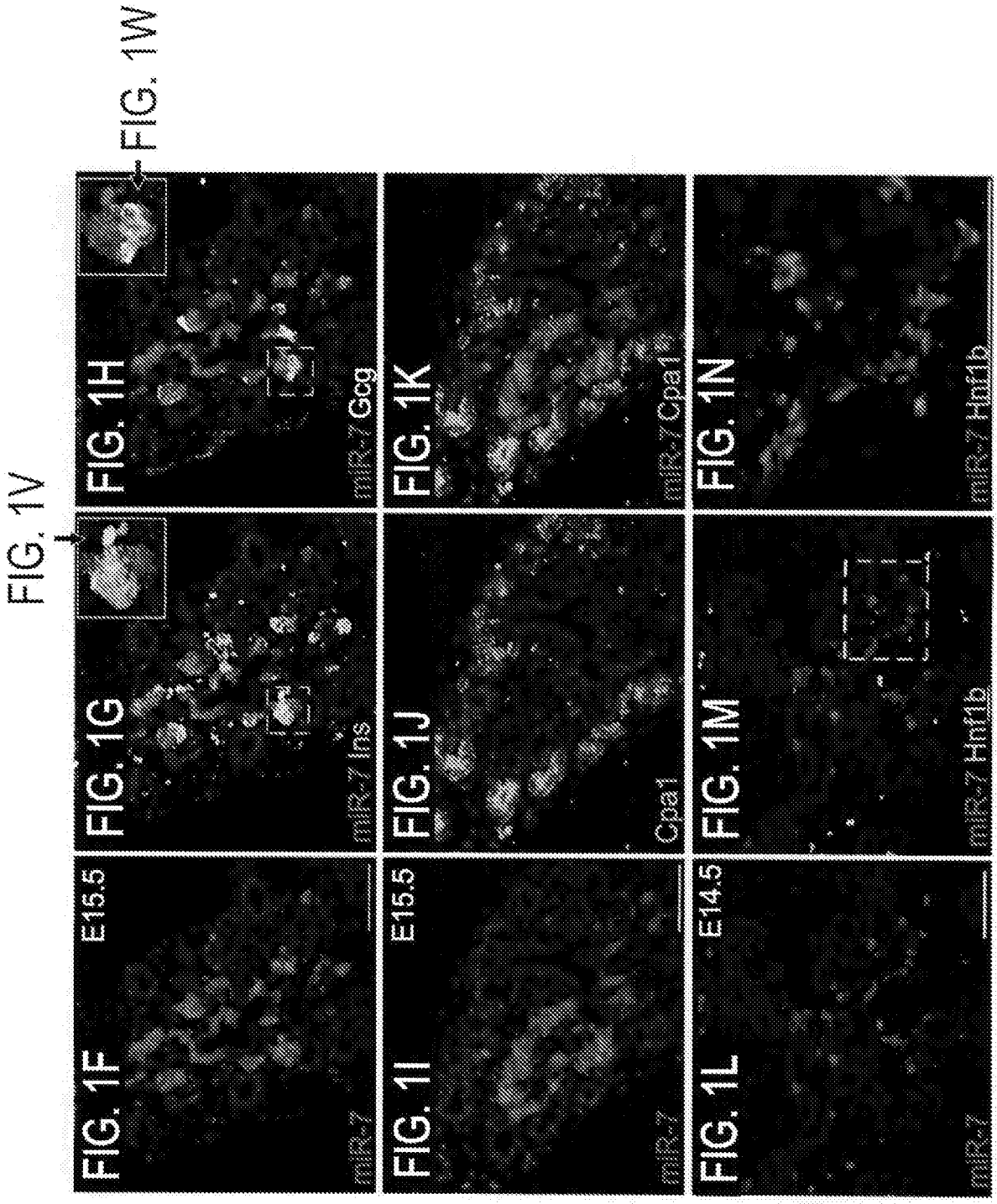


FIG. 1V
FIG. 1W
FIG. 1F
E15.5
miR-7
FIG. 1G
miR-7 Ins
FIG. 1H
miR-7 Gcg
FIG. 1I
E15.5
miR-7
FIG. 1J
Cpa1
FIG. 1K
miR-7 Cpa1
FIG. 1L
E14.5
miR-7
FIG. 1M
miR-7 Hnf1b
FIG. 1N
miR-7 Hnf1b

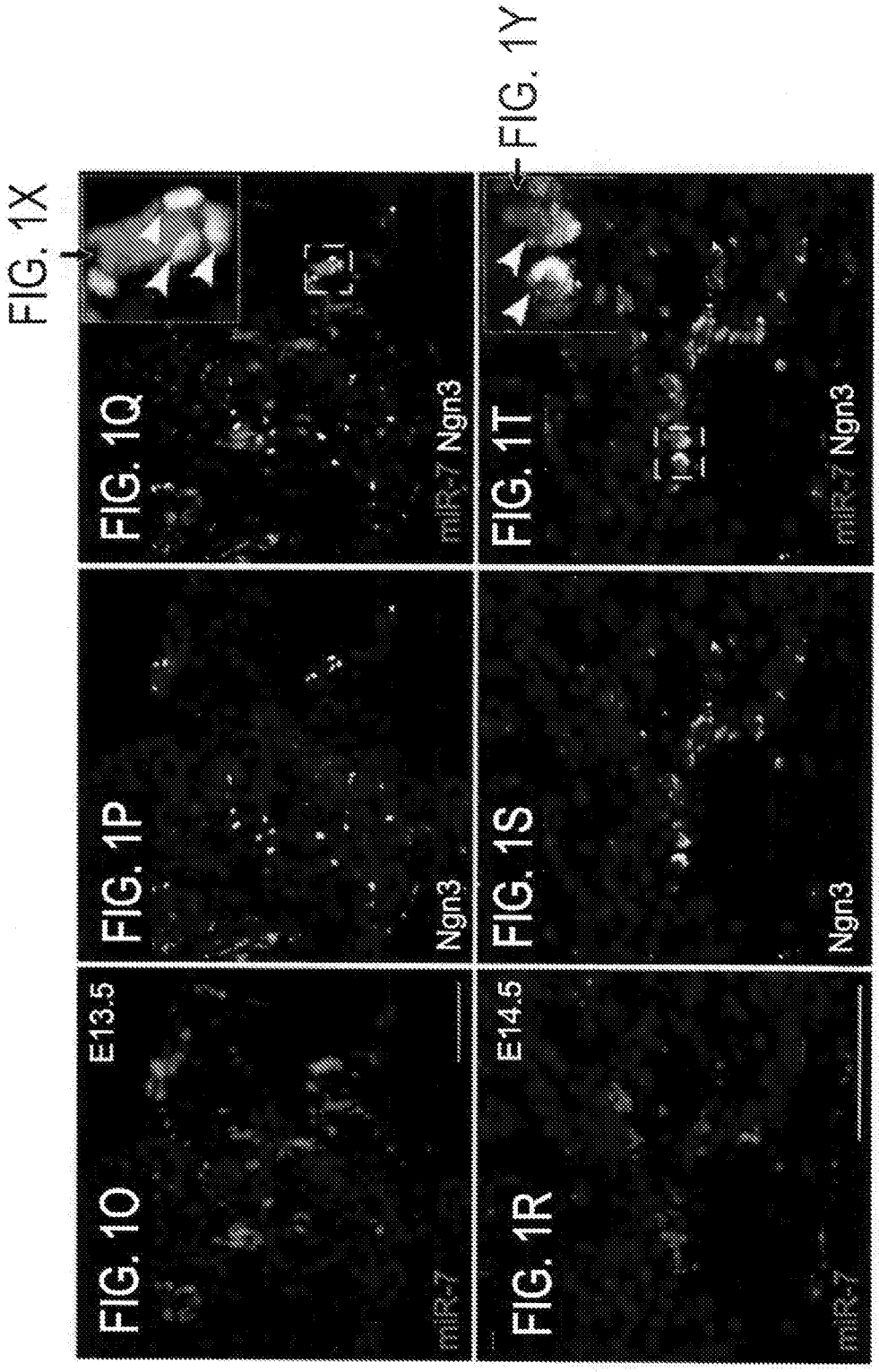


FIG. 1X
FIG. 1Y
FIG. 1O
E13.5
miR-7
FIG. 1P
Ngn3
FIG. 1Q
miR-7 Ngn3
FIG. 1R
E14.5
miR-7
FIG. 1S
Ngn3
FIG. 1T
miR-7 Ngn3

FIG. 1U

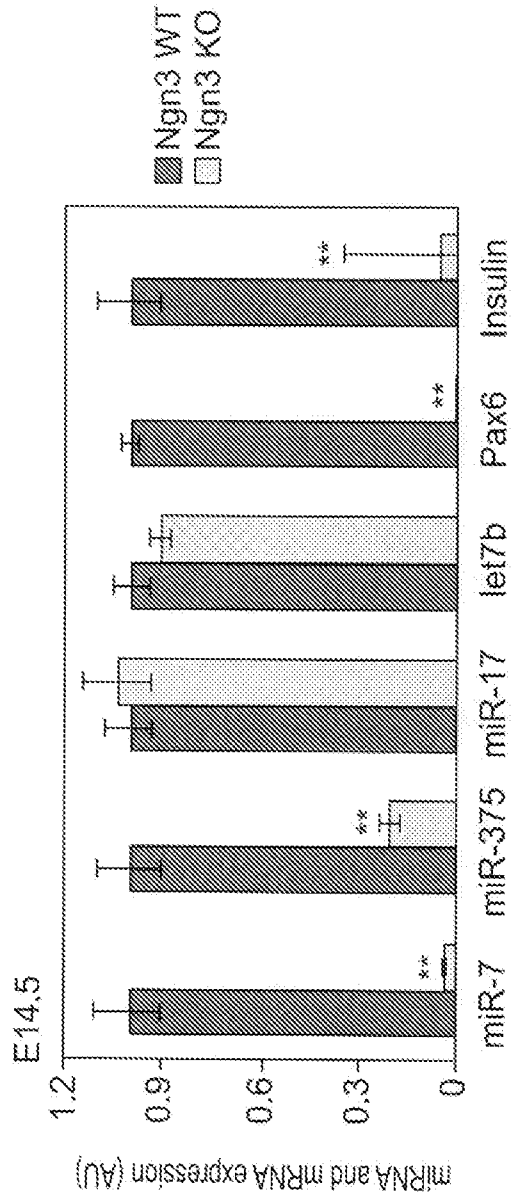
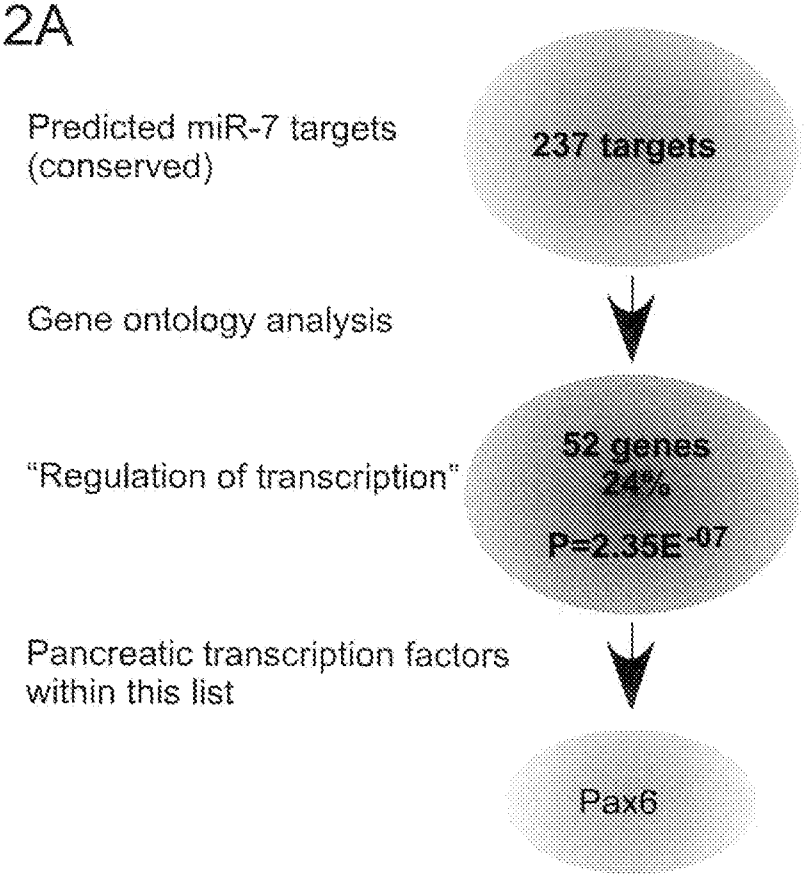

Ngn3 WT
Ngn3 KO
E14.5
miRNA and mRNA expression (AU)
1.2
0.9
0.6
0.3
0
miR-7
miR-375
miR-17
let7b
Pax6
Insulin
**

FIG. 2A


Predicted miR-7 targets (conserved)
237 targets
Gene ontology analysis
"Regulation of transcription"
52 genes
24%
P=2.35E-07
Pancreatic transcription factors within this list
Pax6

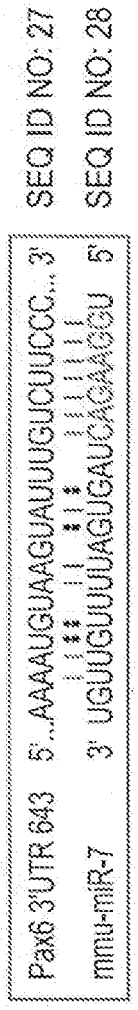


FIG. 2B

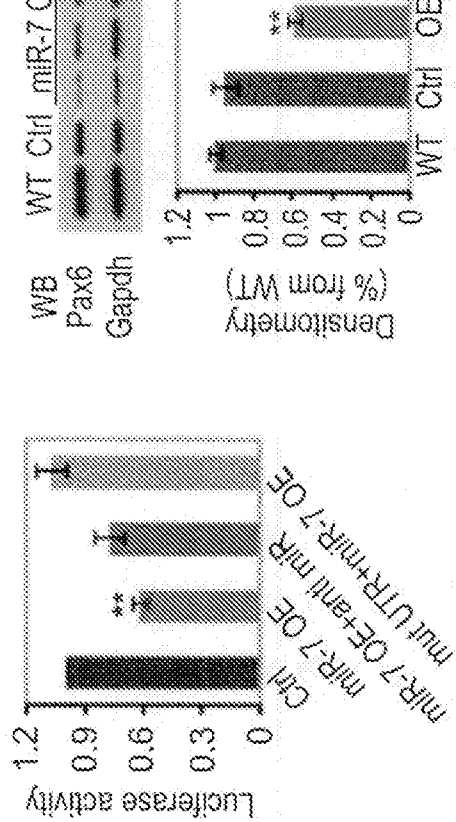


FIG. 2C

FIG. 2D

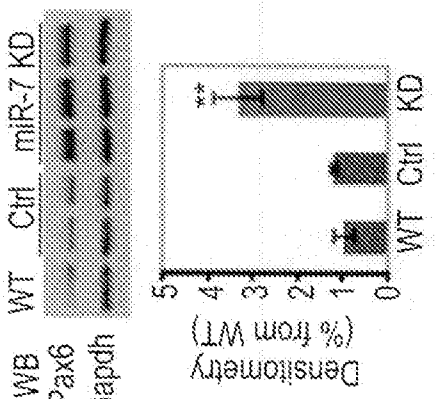


FIG. 2E

FIG. 2F

FIG. 2G

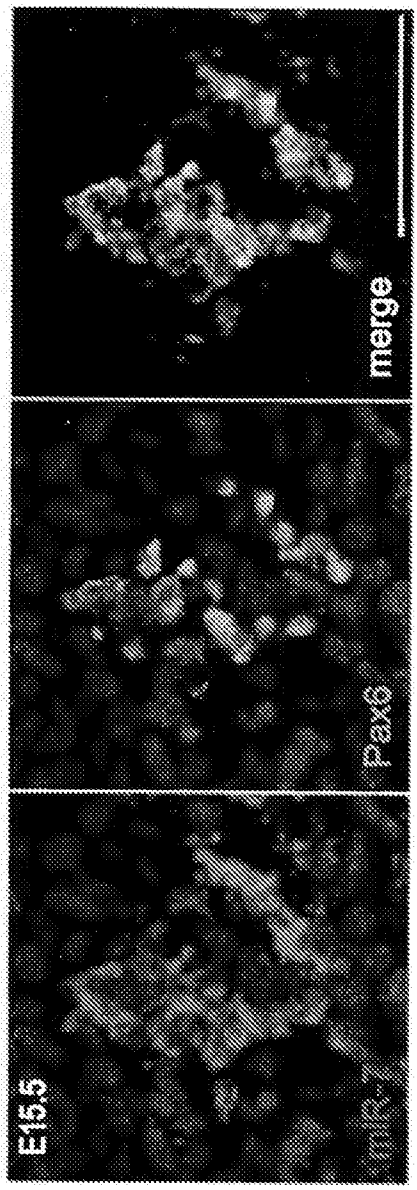


FIG. 2H FIG. 2I FIG. 2J

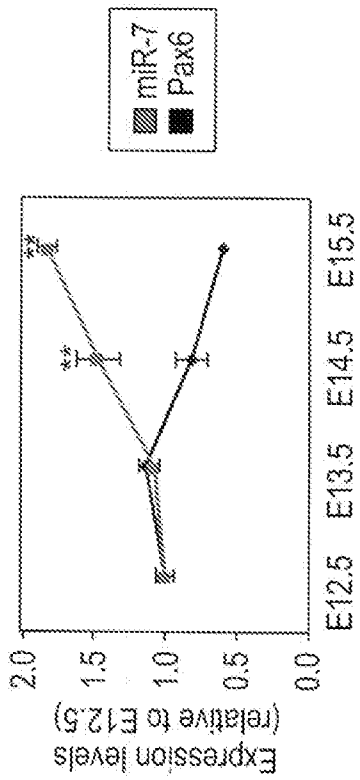


FIG. 2K

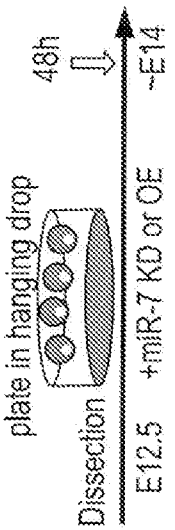


FIG. 3A

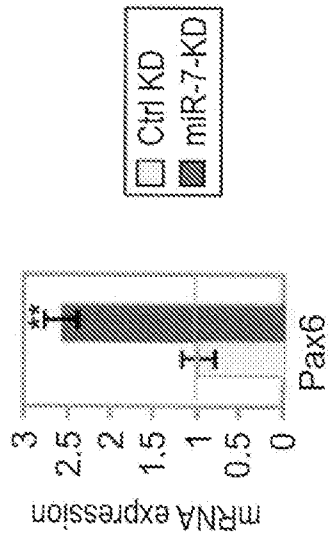


FIG. 3B

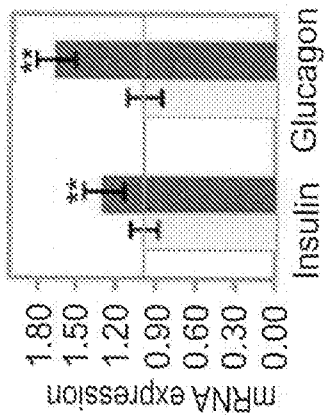


FIG. 3C

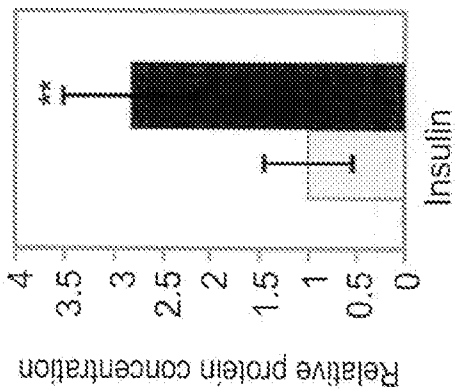


FIG. 3D

FIG. 3F FIG. 3H FIG. 3J

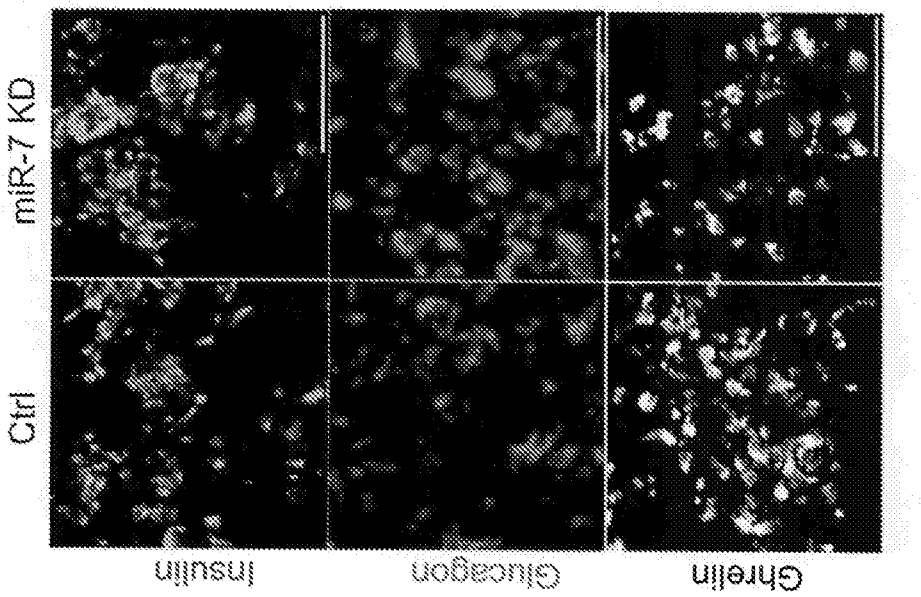


FIG. 3E FIG. 3G FIG. 3I

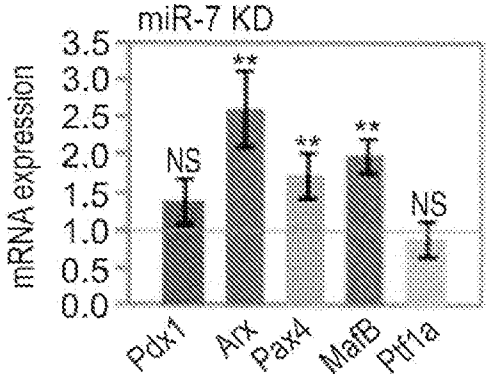

miR-7 KD
mRNA expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NS
**
**
**
NS
Pdx1
Arx
Pax4
MafB
Ptf1a

FIG. 3K

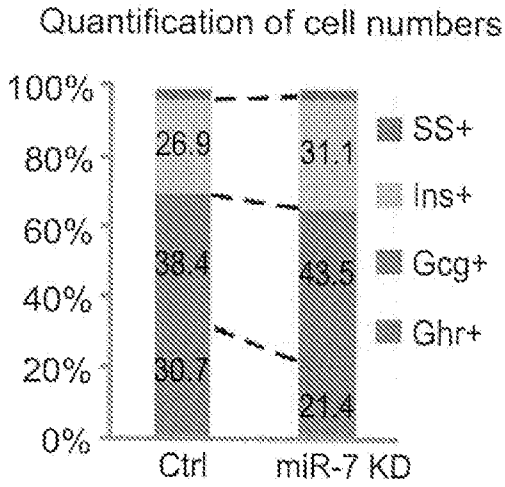

Quantification of cell numbers
100%
80%
60%
40%
20%
0%
26.9
38.4
30.7
31.1
43.5
21.4
SS+
Ins+
Gcg+
Ghr+
Ctrl
miR-7 KD

FIG. 3L

FIG. 3M

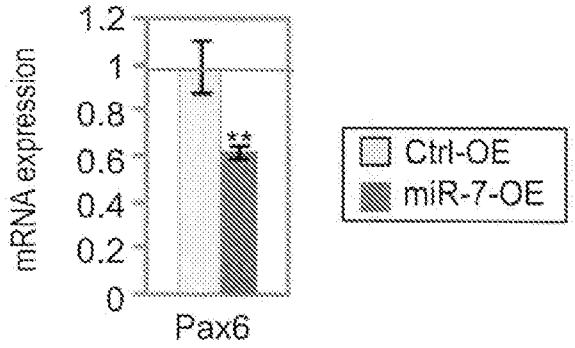


FIG. 3N

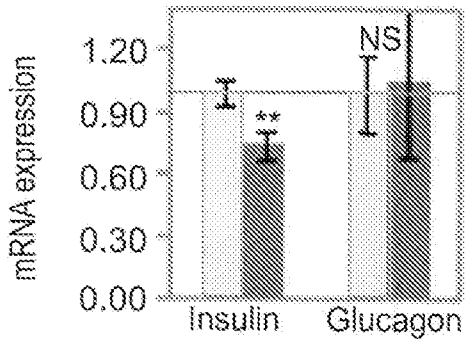
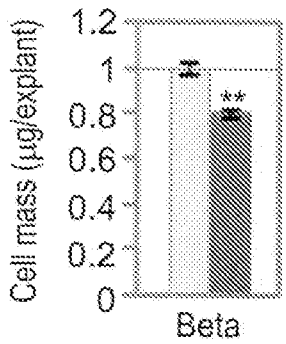


FIG. 3O



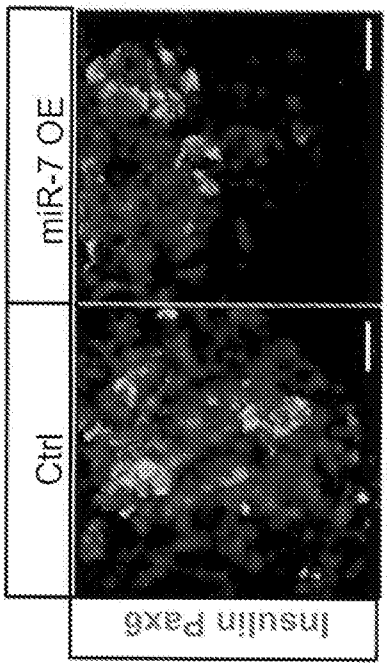


FIG. 3P

FIG. 3Q

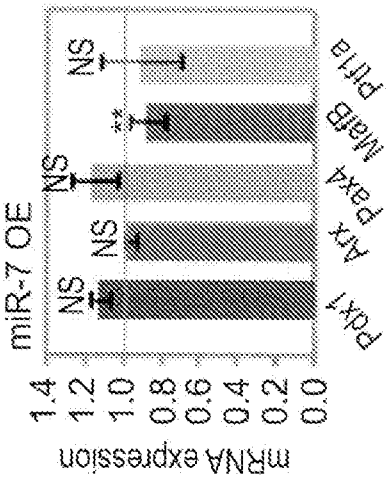


FIG. 3R

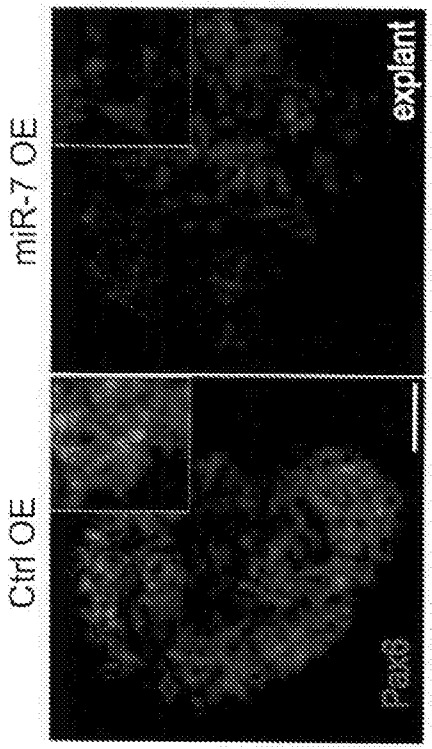


FIG. 3T

FIG. 3S

FIG. 4A

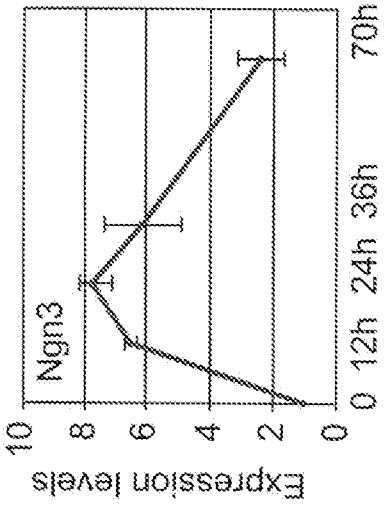


FIG. 4B

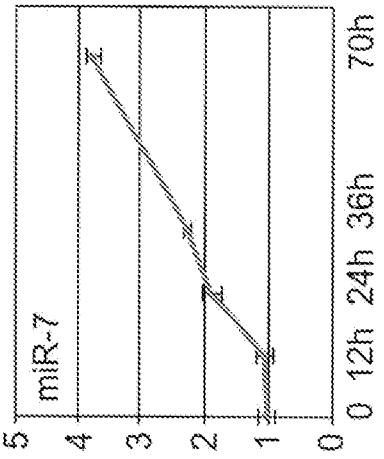


FIG. 4C

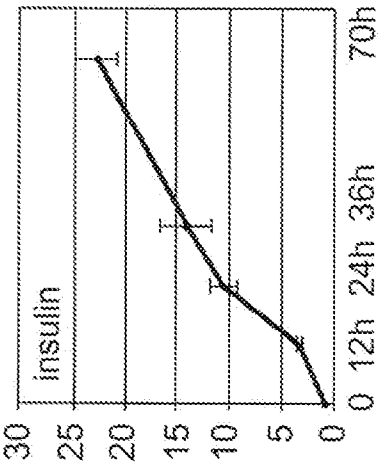


FIG. 4D

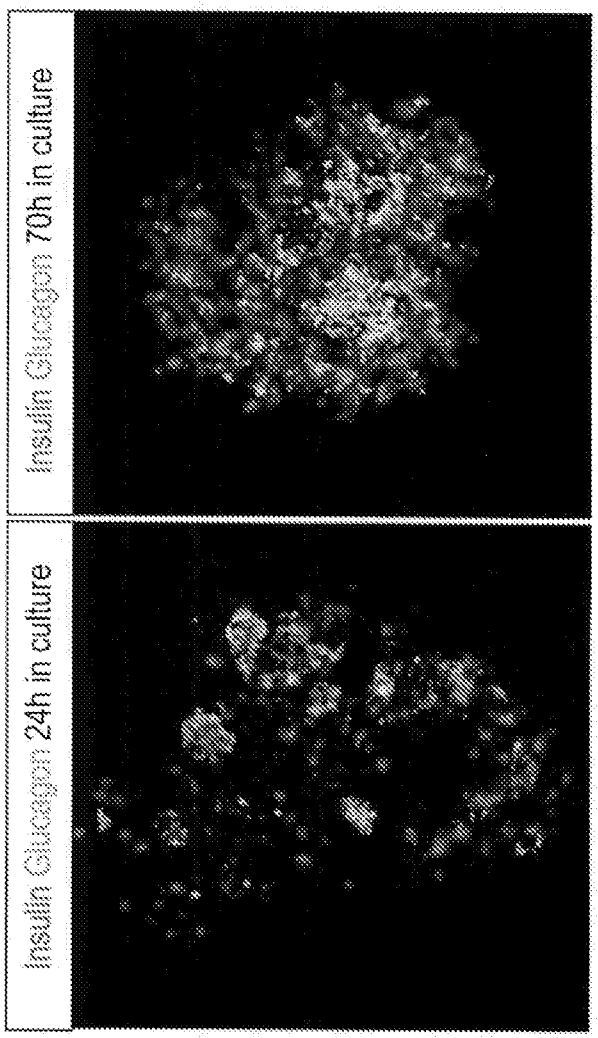


FIG. 4E

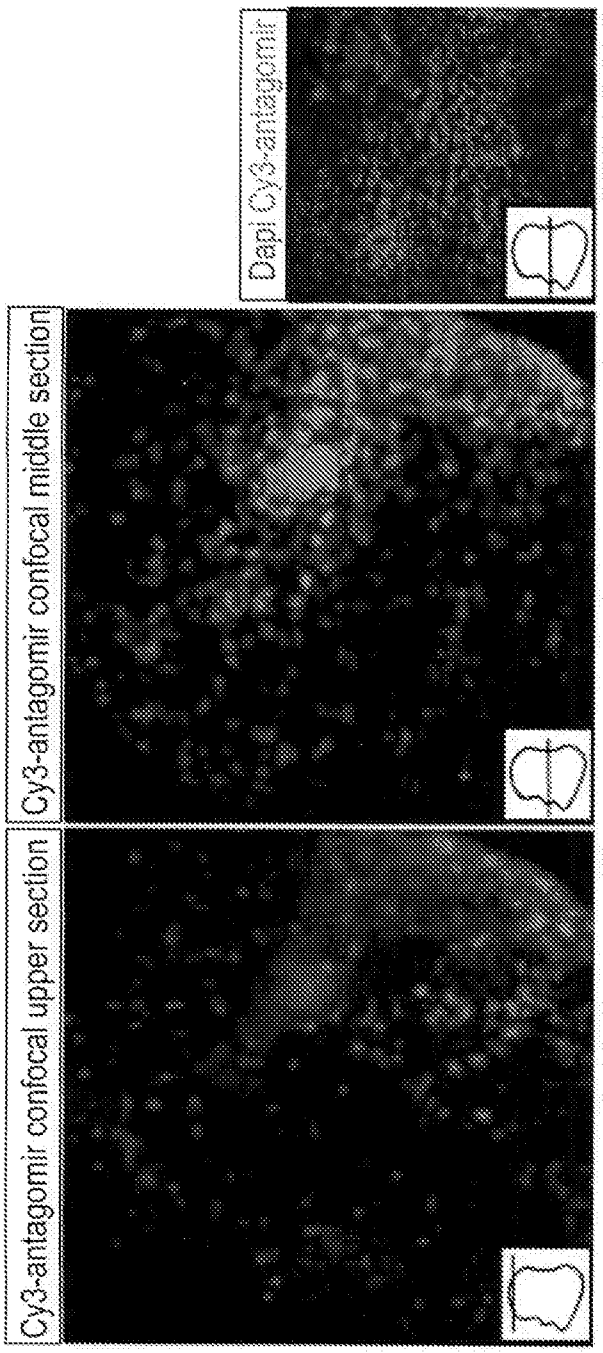


FIG. 4F

FIG. 4G

FIG. 4H

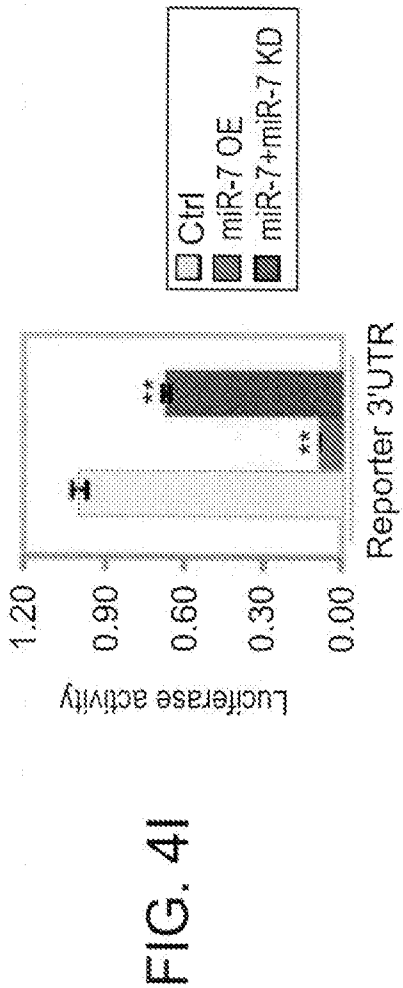


FIG. 4I

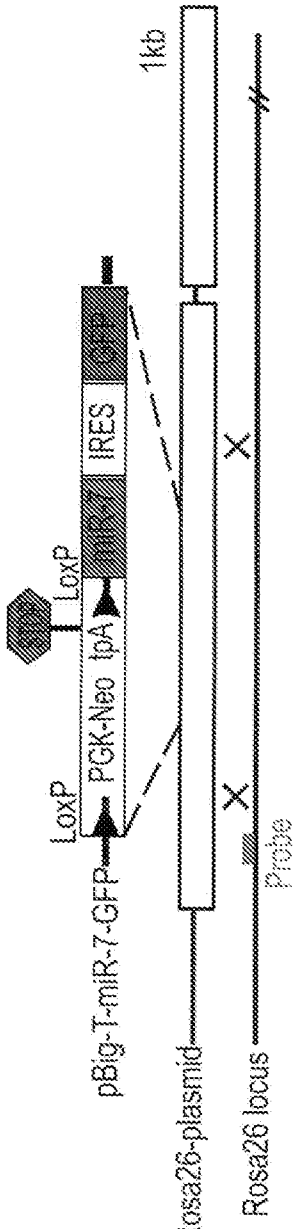


FIG. 5A

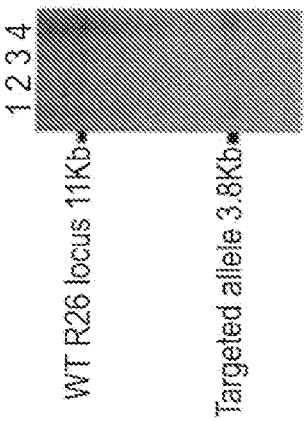


FIG. 5B

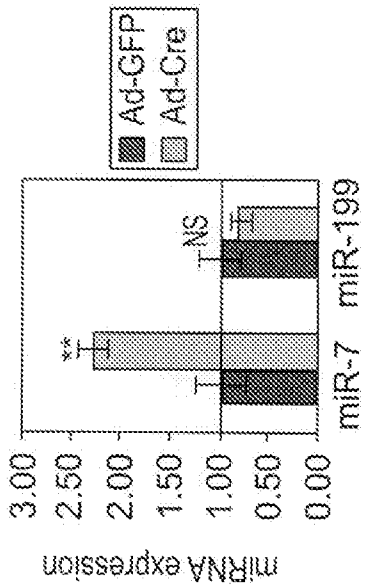


FIG. 5C

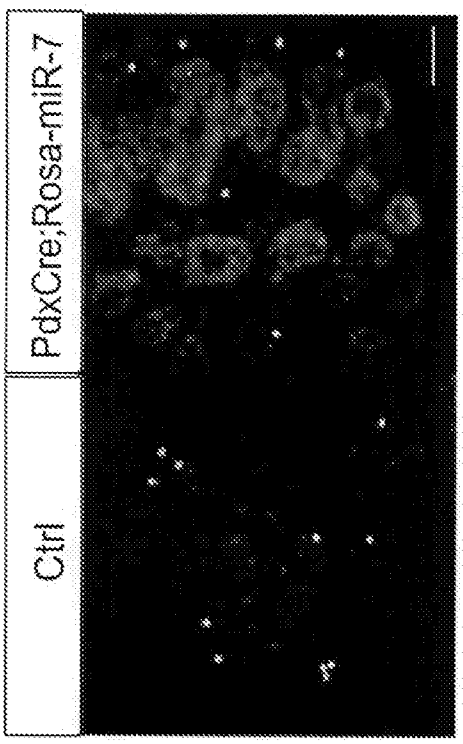


FIG. 5D

FIG. 5E

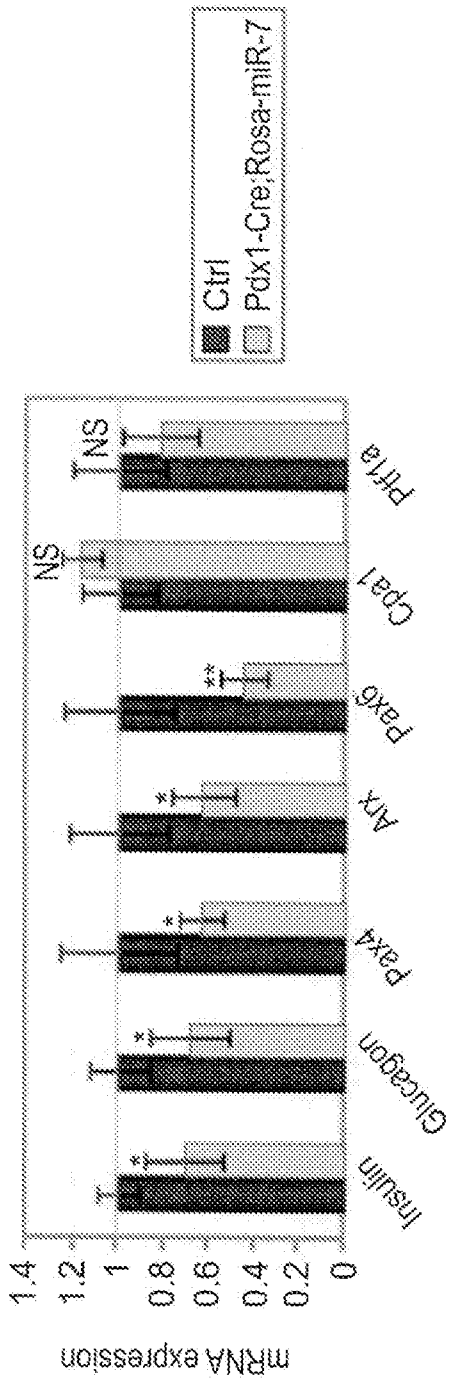


FIG. 5F

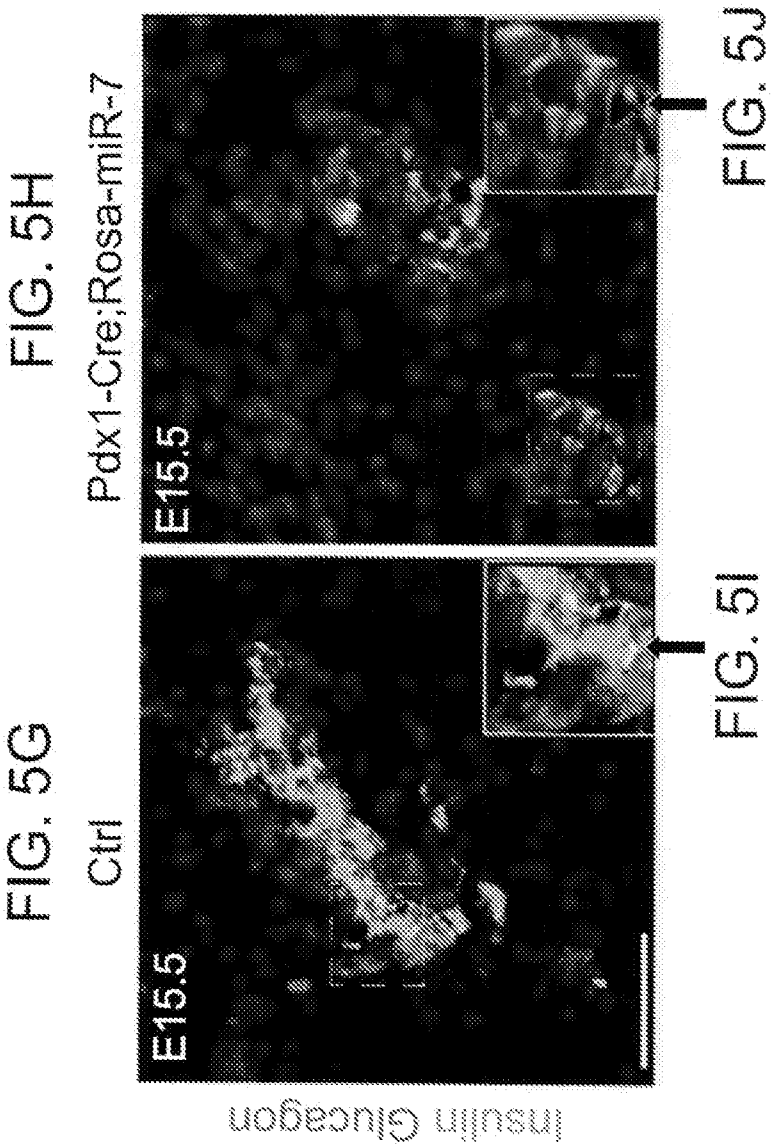


FIG. 5H
Pdx1-Cre;Rosa-miR-7
E15.5
FIG. 5J
FIG. 5G
Ctrl
E15.5
FIG. 5I
Insulin Glucagon

FIG. 5K

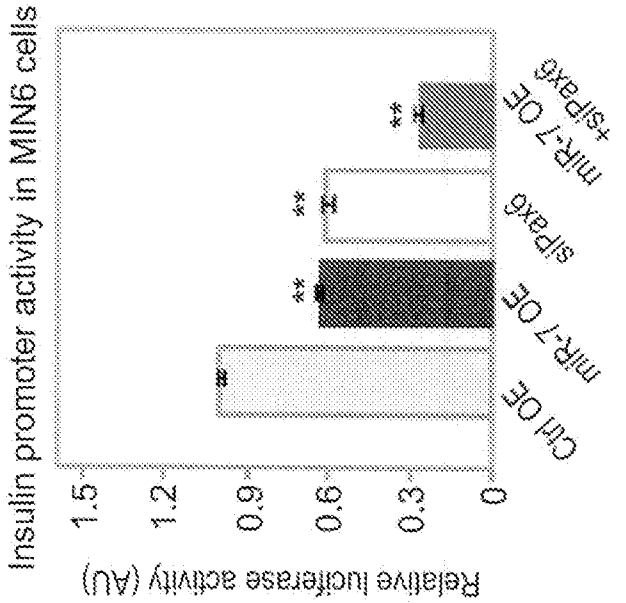
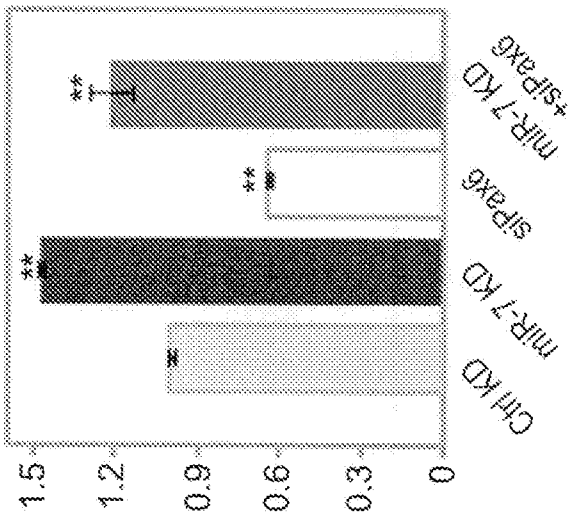

Insulin promoter activity in MIN6 cells
Relative luciferase activity (AU)
1.5
1.2
0.9
0.6
0.3
0
Ctrl OE
miR-7 OE
siPaxb
miR-7 OE +siPaxb
**
**
**

FIG. 5L


1.5
1.2
0.9
0.6
0.3
0
Ctrl KD
miR-7 KD
siPaxb
miR-7 KD +siPaxb
**
**
**

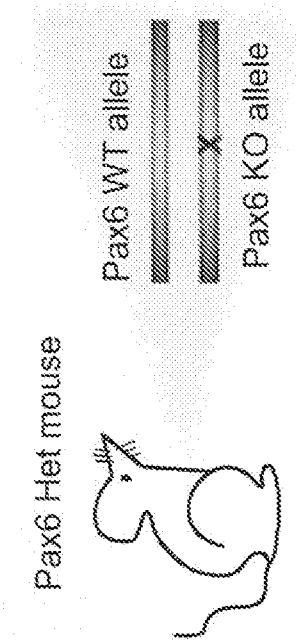


FIG. 6A

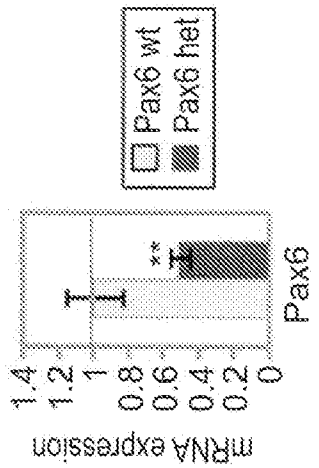


FIG. 6B

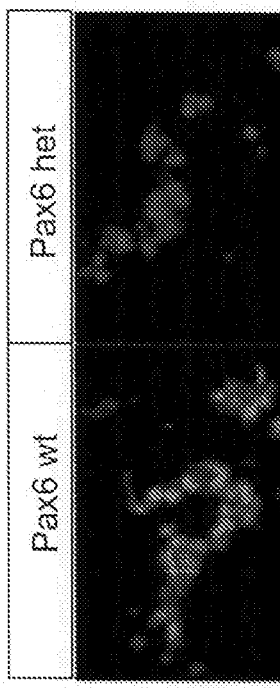


FIG. 6C

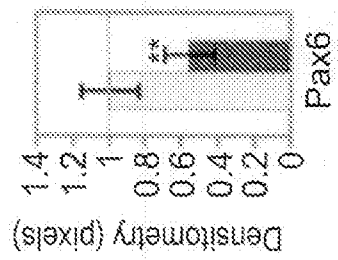


FIG. 6E

FIG. 6D

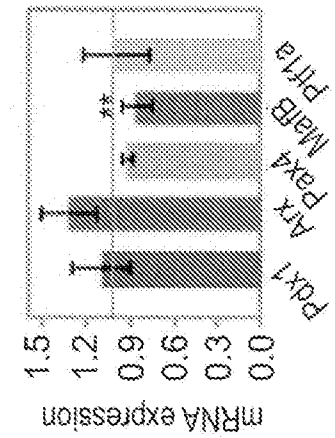


FIG. 6G

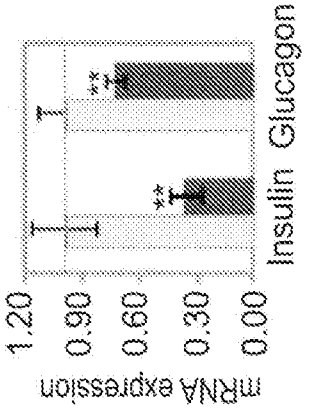


FIG. 6F

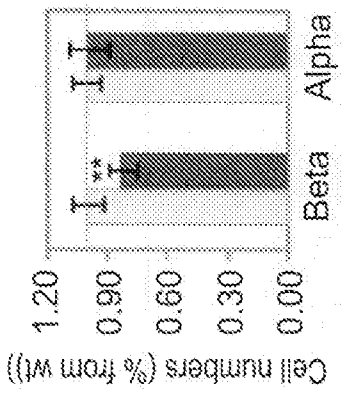


FIG. 6J

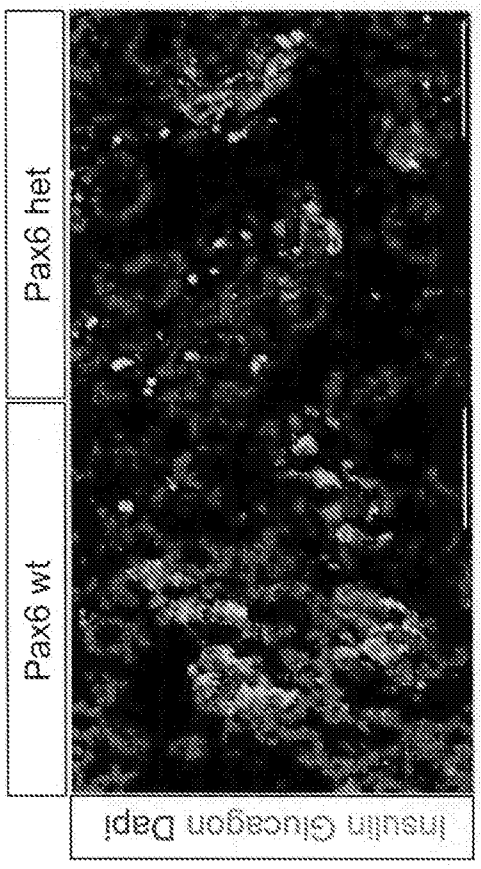


FIG. 6H FIG. 6I

FIG. 7A

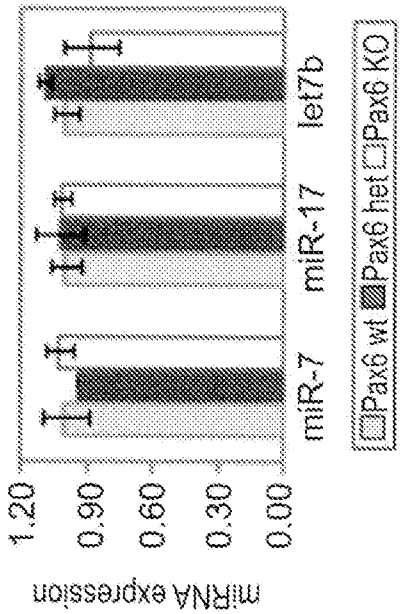


FIG. 7B

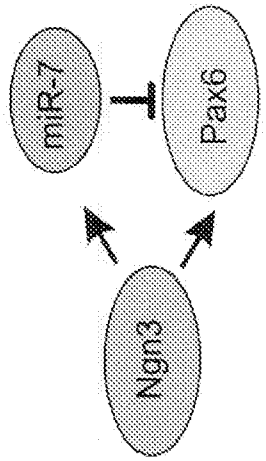
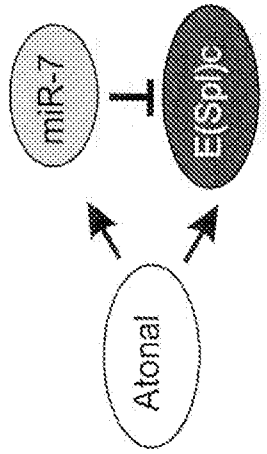


FIG. 7C



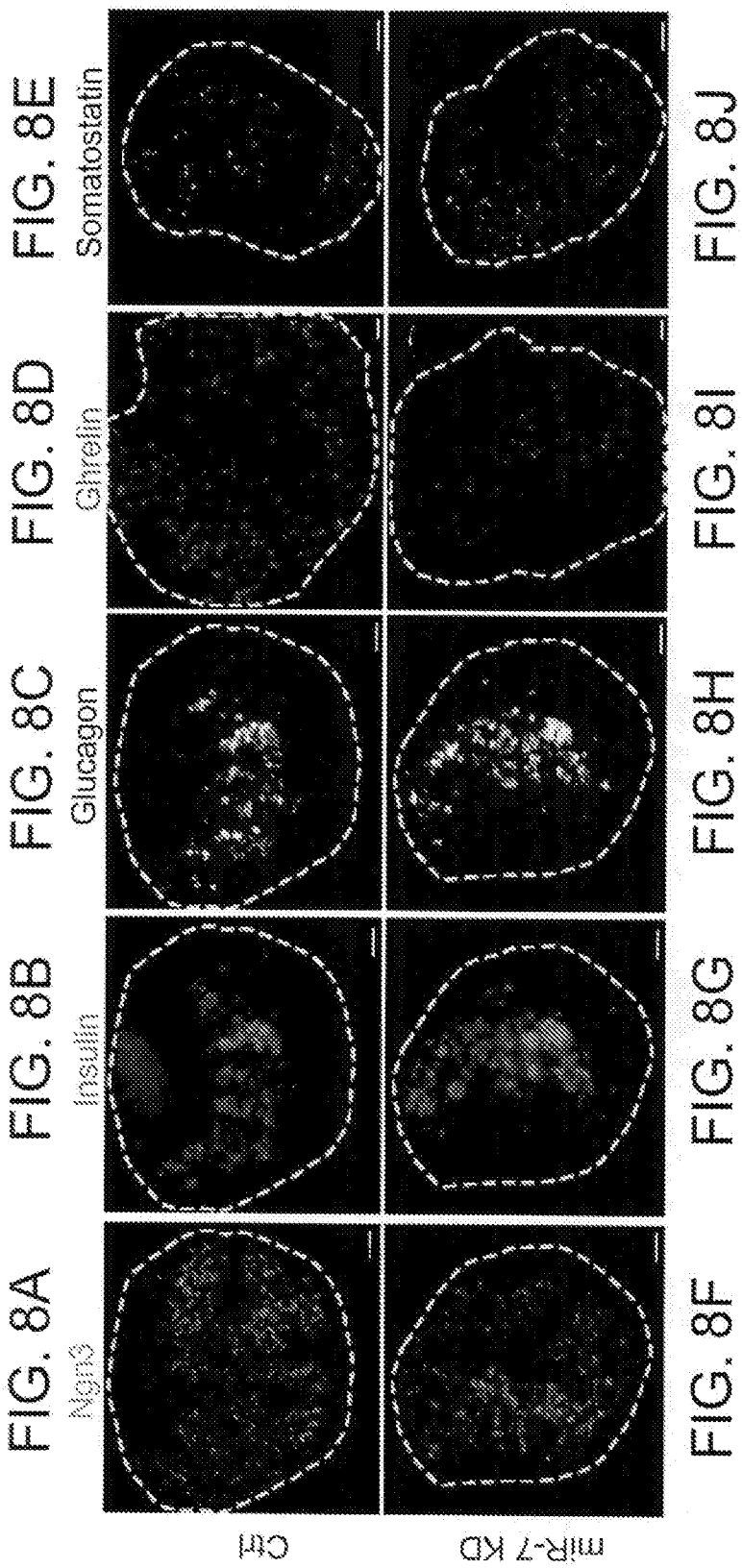


FIG. 8A
Ngn3
FIG. 8B
Insulin
FIG. 8C
Glucagon
FIG. 8D
Ghrelin
FIG. 8E
Somatostatin
FIG. 8F
FIG. 8G
FIG. 8H
FIG. 8I
FIG. 8J
Ctrl
miR-7 KD

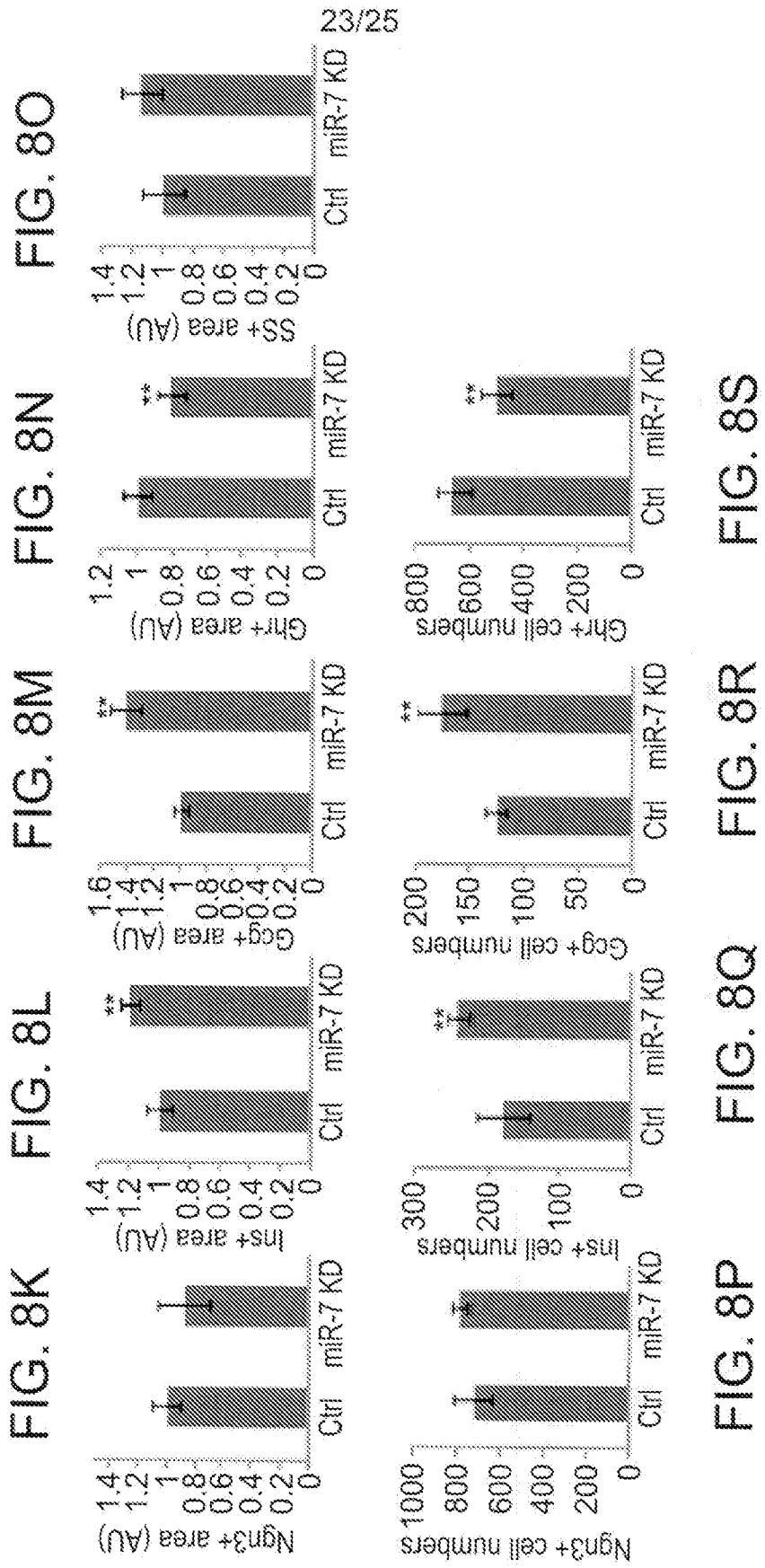


FIG. 8K
Ngn3+ area (AU)
FIG. 8L
Ins+ area (AU)
FIG. 8M
Gcg+ area (AU)
FIG. 8N
Ghr+ area (AU)
FIG. 8O
SS+ area (AU)
FIG. 8P
Ngn3+ cell numbers
FIG. 8Q
Ins+ cell numbers
FIG. 8R
Gcg+ cell numbers
FIG. 8S
Ghr+ cell numbers
Ctrl
miR-7 KD

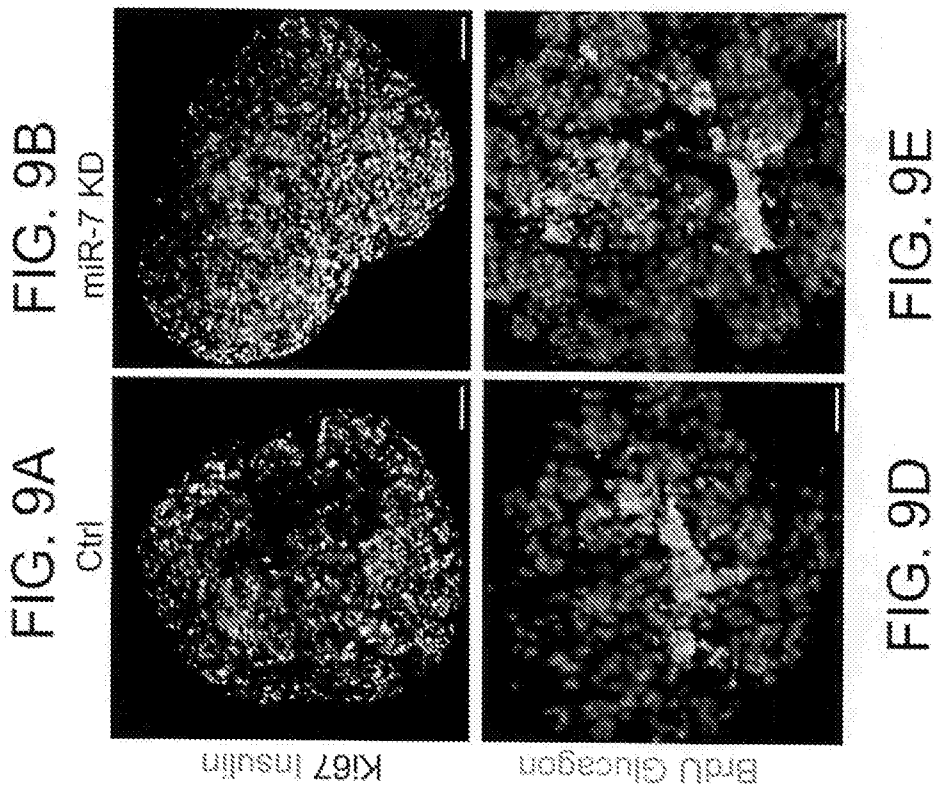
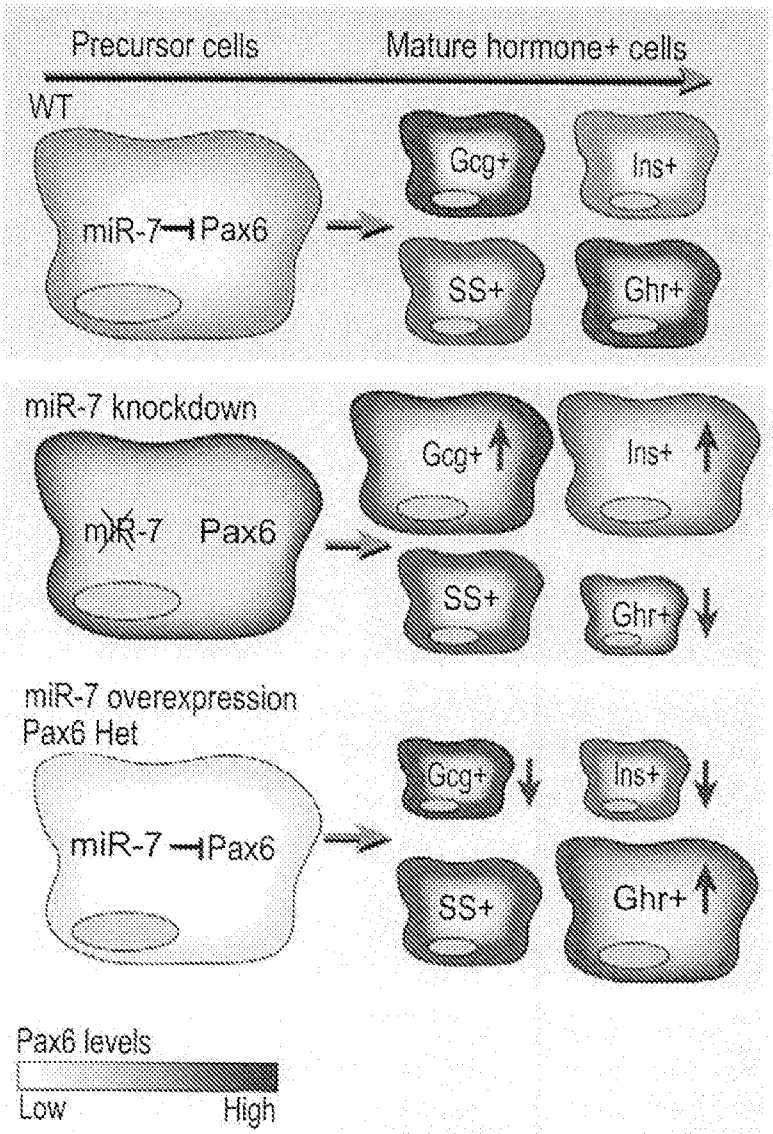


FIG. 9A
Ctrl
FIG. 9B
miR-7 KD
Ki67 Insulin
FIG. 9D
BrdU Glucagon
FIG. 9E
FIG. 9C
%double ki67+ins+cells
3.5
3
2.5
2
1.5
1
0.5
0
Ctrl
miR-7 KD
FIG. 9F
%double BrdU+Gcg+ cells
5
4
3
2
1
0
Ctrl
miR-7 KD
FIG. 9G
Total BrdU+ cells
1000
500
0
Ctrl
miR-7 KD

FIG. 10


Precursor cells
Mature hormone+ cells
WT
miR-7
Pax6
Gcg+
Ins+
SS+
Ghr+
miR-7 knockdown
miR-7
Pax6
Gcg+
Ins+
SS+
Ghr+
miR-7 overexpression
Pax6 Het
miR-7
Pax6
Gcg+
Ins+
SS+
Ghr+
Pax6 levels
Low
High