Specific Reprogramming of Alpha Cells to Insulin-Producing Cells by Short Glucagon Promoter-Driven Pdx1 and MafA

Ping Guo (✉ pguo@sprivail.org)

Steadman Philippon Research Institute

Ting Zhang

Children's Hospital of Pittsburgh

Aiping Lu

Steadman Philippon Research Institute

Chiyo Shiota

Vanderbilt University

Matthieu Huard

Steadman Philippon Research Institute

Kaitlyn Whitney

Steadman Philippon Research Institute

Johnny Huard

Steadman Philippon Research Institute

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Abstract

Endogenous reprogramming of pancreas-derived non-beta cells into insulin-producing cells is a promising approach to treat type 1 diabetes (T1D). One strategy that has yet to be explored is the specific delivery of insulin-producing essential genes, Pdx1 and MafA, to pancreatic alpha cells to reprogram the cells into insulin-producing cells in an adult pancreas. In this study, we utilized an alpha cell-specific glucagon (GCG) promoter to drive Pdx1 and MafA transcription factors to reprogram alpha cells to insulin-producing cells in chemically induced and autoimmune diabetic mice. Our results showed that a combination of a short glucagon-specific promoter with AAV serotype 8 can be used to successfully deliver Pdx1 and MafA into alpha cells in the mouse pancreas. Pdx1 and MafA expression specifically in alpha cells was also able to correct hyperglycemia in both induced and autoimmune diabetic mice. With this technology, targeted gene specificity and reprogramming were accomplished with an alpha-specific promoter combined with an AAV-specific serotype and provide an initial basis to develop a novel therapy for the treatment of T1D.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease caused by an immunological response against pancreatic beta cells that leads to severe insulin deficiency. In developing and mature pancreases, the pancreatic and duodenal homeobox 1 (Pdx1) transcription factor is essential for beta cell maturation, beta cell proliferation and function (1), while the v-maf musculoaponeurotic fibrosarcoma oncogene family protein A (MafA) transcription factor binds to the insulin promoter to regulate insulin expression and beta cell metabolism (2). In T1D, reduced Pdx1 and MafA expression in dysfunctional beta cells results in permanent glucose intolerance and eventual beta cell apoptosis (3). An alternative treatment modality to daily insulin injections that targets insulin-deficient beta cells is pancreatic islet cell transplantation (4); however, due to the lack of donor organs, this procedure is highly selective, and only a small number of T1D patients are treated with islet cell transplantation. Stem cell therapy is also currently practiced for the treatment of T1D but also faces many challenges (5). Although stem cell and islet cell transplantation modalities are current options to treat T1D, there is a significant appeal to utilize mature alpha cells that are native to the pancreas instead of transplanted cells to re-establish insulin production in the pancreas.

Alpha cells are a primary source of endocrine cells of pancreatic islets that are of the same origin as beta cells in the pancreas. Our group and others in the field found that the islet centers are predominately occupied by proliferative alpha cells in the absence of insulin-producing beta cells and following the loss of lymphocytic infiltration (6-8). Thus, generating new beta cells by reprogramming other native islet cell types in an adult pancreas through genetic modification is a viable approach. Ectopic expression of a combination of three key pancreatic beta cell transcription factors, Pdx1, neurogenin 3 (Ngn3) and MafA, has been shown to reprogram adult mouse pancreatic acinar cells into insulin-producing beta-like cells (9, 10). MafA was found to both potentiate the ability of Pdx1 to induce β -cell formation from Ngn3-positive endocrine precursors and enable Pdx1 to produce β -cells from α -cells(11). Moreover, co-overexpression of

these three genes has been shown to convert Sox9-positive liver cells into insulin-producing cells (12). Adeno-associated viral (AAV) vectors can deliver long-term expression of transgenes up to 4.5 kb in length (13) and have been found to be more efficient than adenoviral and lentiviral vectors in transducing pancreatic cells (14-16). Among the AAV vectors, serotype 8 has been shown to have the highest transduction efficiency in mouse islet endocrine cells (14-17). It has previously showed AAV8 with Cytomegalovirus (CMV) promoter driven Pdx1 and MafA enable α -cell to β -cells transdifferentiation(18). These results further support the use of AAV serotype 8 (AAV8) for targeted delivery of Pdx1 and MafA.

Here, we investigated an alpha cell GCG promotor to drive the transcription factors Pdx1 and MafA via an AAV serotype 8 vector to reprogram non-beta cells into insulin-producing cells and reverse beta cells in autoimmune T1D. With this gene delivery technique, we investigated specific pancreatic alpha cell expression of the Pdx1 and MafA genes by AAV8 mediation to determine whether gene transduction can normalize blood glucose levels and whether reprogrammed insulin-producing alpha cells can resist attack from immune cells in mice with induced or autoimmune diabetes. Considering the potential clinical applications of this technology, the human GCG promoter that drives the Pdx1 and MafA genes was evaluated in this study for future translation.

Materials & Methods

Human GCG Promoter

The human glucagon gene is over 85% similar to the mouse, rat, bovine and hamster glucagon genes (19-23). In fact, the TATA box and CAAT box are located at 577 to 581 and 535 to 538 promoter regions, respectively, and have the ability to drive gene expression. Length variations of human GCG promoters (US patent US20170087254A1) were screened, and the 648 bp human GCG promoter was selected to drive gene expression for the *in vivo* experiments. We and other groups have reported that AAV stereotypes 6 & 8 are similar in function and able to facilitate transduction of pancreatic cells (16, 24, 25). Our group has previously reported that combining the sox9 promoter and AAV serotype 6 was able to deliver the green fluorescent protein (GFP) and Cre genes specifically to pancreatic ductal cells and was used for pancreatic ductal cell lineage tracing (24). Based on this finding, the AAV serotype tissue tropism with a specific GCG promoter together, using viral pancreatic ductal infusion, is an achievable *in vivo* gene delivery method of Pdx1 and MafA and labeling method of GFP+ pancreatic cells. In this experiment, the authors first designed and constructed a double-stranded AAV containing a human proglucagon short promoter driving GFP, AAV-GCG-GFP and AAV-GCG-Cre to specifically target alpha cells in wild-type (WT) mouse pancreas and ROSA26 tomato reporter mouse pancreas, respectively. Then, the GCG promoter driving Pdx1 and MafA was constructed to generate a recombinant viral vector with a GFP reporter gene, AAV8-GCG-Pdx1-MafA-GFP (AAV8-GCG-PM). This recombinant virus construct was then infused into the pancreatic ducts of mice through the common bile/pancreatic duct to tag alpha cells. After confirming AAV8 and GCG promoter specificity, we then infused the virus into the pancreatic ducts of beta cell toxic chemical, Alloxan (ALX) induced diabetic mice and autoimmune diabetic mice

(NOD/ShiLt mice) when diabetes at the early-onset stage (blood glucose at 200 mg/dl, lower than 250 mg/dl).

Chemicals and Antibodies

Alloxan monohydrate (ALX) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-insulin antibody was purchased from DAKO (Cat. No. IR00261, North America, Carpinteria, CA, USA). Glucagon-specific antibody was purchased from Fisher Scientific (Cat. No. PIPA583352, Pittsburgh, PA, USA). Mouse anti-glucagon antibody was purchased from Millipore Sigma (Cat. No. G2654, St. Louis, MO, USA). Ki67 antibody was purchased from Cell Signaling Technology (Cat. No. 12202S, Danvers, MA), and Alexa 488-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-mouse and Cy5-conjugated donkey anti-guinea pig antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Antibodies against Pdx1, MafA and CD45 were purchased from Abcam (Cambridge, MA, USA).

Design of the AAV8-GCG-Pdx1-MafA Gene Expression Vector

The pAAV-CMV-GFP plasmid was modified with the human 648 bp GCG promoter to replace the CMV promoter. The Pdx1 and MafA gene cassettes were added to the front of the GFP gene (**Figure 2A**). The GCG promoter drives Pdx1 and MafA open reading frames (ORFs), and the GFP tag was connected with 2A peptide sequences, pAAV-GCG-Pdx1-P2A-MafA-T2A-GFP (short form: pAAV-GCG-PM). For cleavage of the stop codon flanking the tomato red reporter gene, the GFP sequence in the pAAV-GCG-GFP vector was replaced with iCre ORFs (24, 26).

Purification of AAV Serotype 8 Vector and Determination of Transgene Expression

The generation and purification of AAV was performed with triple plasmid PEI transfection and PEG/(NH₄)₂SO₄ aqueous two-phase partitioning, as previously described (14, 27). Determination of transgene expression was also performed using an established protocol, as previously described (24). Briefly, virus-mediated GFP or Pdx1-MafA cassette gene transfer assays were performed *in vitro* in a 12-well culture plate with alpha-TC1 clone 9 (ATCC CRL-2350) cells. Approximately 10 μ L of the purified AAV-GCG-GFP (titer was 3×10^{12} genome copy particle GCP/ml) or AAV-GCG-Pdx1-MafA (titer 2.3×10^{12} GCP/ml) virus was added to each well. At 72 hours post infection, GFP cell fluorescence was visualized under a microscope (ZEISS Microscopy, Jena, GER) with a 525 nm filter. For gene expression, the alpha cell line alpha-TC1 clone 9 were infected once with AAV-GCG-GFP or AAV-GCG-PM for 72 hours. The cells were then harvested at 4 °C and lysed for RNA or protein extraction. Approximately 10 μ g of protein lysates were separated on a 10% SDS polyacrylamide gel and transferred onto a nitrocellulose

membrane. The membrane was blocked with 5% milk-PBS for 1 hour at room temperature and probed with anti-GAPDH (Thermo Fisher Scientific, Waltham, MA, USA), and anti-Pdx1 and MafA antibodies were applied to probe the protein for 4 hours at room temperature (RT). Samples were washed three times with PBST and treated with anti-rabbit conjugated HRP secondary antibody. GAPDH, Pdx1 and MafA proteins in the blot were visualized by chemiluminescence and imaged with ChemiDoc (Bio-Rad, Hercules, CA, USA). Total RNA was isolated from GFP-positive cells after FACS sorting using an RNeasy mini kit (Qiagen, Inc., Hilden, GER) following the manufacturer’s instructions. RNA sample quality was analyzed using Genesys 10 UV (Thermo Electron Co., Waltham, MA, USA). RNA (1 µg) was treated with 0.5 units DNase I (amplification grade; Invitrogen, Waltham, MA, USA) for 5 minutes at 37°C and 1 µl of 0.5 M EDTA was added to stop the DNase reaction and then annealed with 50 ng random hexamer oligonucleotide. cDNA was synthesized using the superscript first strand synthesis system for real-time PCR (RT-PCR) following the manufacturer’s instructions. The cDNA products were diluted 20-fold before PCR amplification. The PCR primers that were used are described in **Table 1**.

Table 1. PCR primers

Name	Forward	Reverse	Product (bp)
Pdx1	5'-TTCCCGAATGGAACCGAGCCTG-3'	5'-TTTTCCTCGGGTTCCGCTGTGT-3'	139
MafA	5'-GCTTCAGCAAGGAGGAGGTCAT-3'	5'-TCTCGCTCTCCAGAATGTGCCG-3'	119
GAPDH	5'-GTTGTCTCCTGCGACTTCA-3'	5'-GGTGGTCCAGGGTTTCTTA-3'	184

Alloxan (ALX) T1D Induction Procedure

All murine procedures were approved by the Animal Research and Care Committee at the Children's Hospital of Pittsburgh and the University of Pittsburgh IACUC and University of Texas Science Center IACUC at Houston. The methods were performed in accordance with the approved guidelines and regulations. C57BL/6, NOD/ShiLt, and ROSA26 tdTomato red reporter (C56BL/6 background) mice were purchased from the Jackson Lab (Bar Harbor, ME, USA). The beta cell toxin ALX was created to induce diabetes by injection of ALX via the dorsal tail vein at a dosage of 65 mg/kg. The study was carried out in compliance with the Animal Research- Reporting of In Vivo Experiments (ARRIVE) guidelines.

Autoimmune diabetic mice

NOD/ShiLtJ female mice are more widely used than males due to the onset of insulin-dependent diabetes mellitus (IDDM) symptoms occurring earlier and with an incidence above 90% by 30 weeks of age (Jackson Lab NOD/ShiLt mice introduction). Female NOD/ShiLtJ mice were used for our autoimmune

diabetes therapy with AAV8-GCG-PM viral infusion at 16 weeks of age or older at the early onset of hyperglycemia (24, 25, 27-30).

AAV-Mediated Gene Transfection and Pancreatic Alpha Cell Specific Targeting In Vivo

In vivo viral gene transfer into adult C57BL/6 mice was performed at 10 weeks of age, as previously described (24). Briefly, for pancreatic ductal infusion, the mouse was anesthetized with the isoflurane anesthetic machine fitted out close to supply the mouse via inhalation, for GFP gene pancreatic ductal transformation, anesthetized 10-week-old C57BL/6 mice (n=8) were infused with 120 μ l of AAV8-GCG-GFP (titer 3.31×10^{12} GCP/ml) or 150 μ l AAV8-GCG-P-M (titer 2.35×10^{12} GCP/ml) through the common pancreatic/biliary duct at a rate of 10 μ l/minute. A clip was placed on the bile duct near the hilum of the liver to temporarily occlude the common hepatic duct, leading to specific perfusion of the pancreatic ducts. Once blood glucose levels reached 200 mg/dl, C57BL/6- or ALX-induced diabetic C57BL/6 and 16-week-old NOD/ShiLtJ female mice were used for AAV8-GCG-PM pancreatic ductal transformation and treatment for diabetes *in vivo*. The same infusion technique was applied to ROSA26 tomato reporter mice using the AAV8-GCG-Cre virus to confirm GCG promoter specificity and lineage tracing of transdifferentiating alpha cells in ALX-induced diabetic mice.

Murine Hormone and Metabolic Assays

Murine blood glucose measurements were performed after a 2-hour fasting period using the tail-tip method to collect sufficient blood samples. The effects of the interventions on blood glucose levels were determined by the Optium EZ monitoring system (Abbott, Lake Bluff, IL, USA). Glucose tolerance testing (IPGTT) was performed as previously described (29). Briefly, ALX-induced or NOD/ShiLtJ mice that underwent overnight fasting (16 hours) were administered an intraperitoneal injection of glucose (2 gm/kg body weight). Blood glucose levels were measured at 10-, 15-, 30- and 120-minute time points. Serum triglycerides and total cholesterol were measured spectrophotometrically using an enzymatic assay kit (ABX Pentra A11A01640, Horiba-ABX, Montpellier, FRA). Serum free fatty acids were measured by the acyl-CoA synthase and acyl-CoA oxidase methods (NEFA-HR 434-91795, Wako Chemicals, Richmond, VA, USA). Serum β -hydroxybutyrate was quantified by an enzymatic assay (NEFA FA115, Randox Lab, Crumlin, UK). The biochemical parameters were determined using a Pentra 400 analyzer (Horiba-ABX, Montpellier, FRA) following the manufacturer's instructions.

Tissue Harvest and Immunohistochemistry

Harvesting of murine pancreases was carried out as described previously (24, 31). Briefly, mice were infused with 10 ml PBS, followed by 10 ml 4% paraformaldehyde (PFA). The pancreatic tissues were then harvested and fixed in 4% PFA for 2 h at 4 °C and then cryoprotected in 30% sucrose overnight before freezing. The pancreas was embedded in Tissue Tek OCT (Sakura Finetek, Torrance, CA, USA) and

immersed in liquid nitrogen for freezing. Frozen blocks were stored at -80°C until batch sectioning. Pancreatic tissue was cryosectioned ($5\text{ }\mu\text{m}$), blocked with 10% normal donkey serum (NDS) in 0.1% PBST for 45 minutes at RT, incubated with primary antibody in 5% NDS in 0.1% PBST at 4°C overnight, and washed with 0.1% PBST at RT three times for 15 minutes. The tissues were then incubated with a secondary antibody in 1% NDS in PBST at RT for 30 minutes, washed three times with 0.1% PBST at RT for 10 minutes, and counterstained with DAPI for 5 minutes. Tissues were then washed three times with 0.1% PBS and mounted on slides with aqua mounting media and a coverslip. For confocal microscopy, pancreatic tissue was sectioned in $10\text{ }\mu\text{m}$ and stained with insulin and glucagon antibodies, followed by treatment with fluorescent-conjugated secondary antibodies. The fluorescence was visualized with a Zeiss LSM710 confocal microscope by sequentially scanning at (excitation/emission) 488/505–530 nm (Cy2), 543/570(Cy3), and 633/650 nm (Cy5).

Quantification and Statistical Analysis

Sample size was determined according to the published literature (32). Immunohistochemistry quantification was performed on at least 5 sections that were $100\text{ }\mu\text{m}$ apart. At least 2000 cells were counted for each experimental condition. If the percentage of GFP-positive or tomato red cells was low, counting continued beyond 2000 cells until at least 50 positive cells were counted. All values are depicted as means $\pm$ S.E. In each condition, 5 mice were analyzed in all groups. RT-PCR data were analyzed from 5 samples from each condition. All data were statistically analyzed by 2-tailed Student's t-test. Statistical significance was considered when $p < 0.05$.

For *in vitro* experiments, each experimental condition entailed at least 5 repeats or 3 repeats. For *in vivo* experiments, 5 mice were used for each group. Alpha cell mass, beta cell mass, beta cell number and percentage of beta cells were quantified, as previously described (24). All data were statistically analyzed by one-way ANOVA with a Bonferroni correction, followed by Fisher's exact test. The χ -squared test with 1 degree of freedom was applied to compare observed and estimated data. All error bars represent standard deviation (SD). Significance is presented as * when $p < 0.05$ and # when $p < 0.01$. No significance was presented as NS. P and n values as well as statistical methods are described in the figure legends.

Results

Alpha cell number coincides with disease advancements in T1D mouse islets

We examined the pancreas of NOD/ShiLtJ mice based on blood glucose levels from early onset of the disease (between 200 and 250 mg/dl) to the advanced stage (600 mg/dl, glucometer highest point). The alpha cells were located at the periphery of the islet in the NOD/ShiLtJ mouse when blood glucose levels before the onset (normal level of blood glucose $< 200\text{ mg/dl}$) (**Figure 1B**), which was similar to wild-type mice (**Figure 1A**). Interestingly, the alpha cells began to concentrate at the center of the islet when the blood glucose level increased (**Figure 1C to 1F**). These results confirmed a previous finding that

alpha cells predominately occupy the islet center where beta cells recede (6-8, 18, 26). This observation also validated that the alpha cell population is preserved at the time of early onset and in the late stage of autoimmune diabetes. Alpha cell proliferation was seen in NOD mice islets (**Figure 1H to I**). The proliferated alpha cells, Ki67 and glucagon double positive cells, were found more in 300mg/dl islets of NOD mice (**Figure 1H**), but more non-glucagon positive cells (ki67 positive and glucagon negative) were seen in high level of hyperglycemia (**Figure 1J**). Alpha cell proliferation was higher at diabetes pathological initiate period when blood glucose levels go up and beta cells disappear in the NOD islets (**Figure 1K**),

AAV8-GCG-Pdx1-MafA vector construction and GCG promoter drive gene expression specificity

To deliver Pdx1 and MafA transcription factors to alpha cells in the early onset of diabetes, human GCG promoters were used to first determine which size GCG promoter combination with AAV8 vector measured and compared to effectively facilitate transduction in specific alpha cells. To assemble and purify the viruses, the AAV-GCG-GFP vector was constructed by modifying the pAAV-CMV-GFP vector with the GCG promoters and replacing the CMV promoter (**Figure 2A**), following a previously described method (14, 27). We first measured gene expression in a GCG-activated alpha cell line, alpha TC1 clone 9 cells, to determine the activation of the GCG promoter in these cell populations. First, alpha TC cells that were infected with purified AAV8-GCG-GFP and AAV8-GCG-Pdx1-MafA-GFP respectively, were used as a confirmatory experiment and were up to 95% GFP⁺ only 3 days after viral infection. In the confirmatory experiment, the Pdx1 and MafA genes measured by Western blotting were present in alpha TC cells. The virus generated from clone 2 was used for the experiments. Then, we determined that the Pdx1 and MafA genes were expressed in alpha TC1 clone 9 cells 7 days following infection. More than 80% of alpha cells were visualized to be GFP positive under fluorescence microscope (**Figure 2B**). Pdx1 and MafA proteins measured by Western blotting were highly expressed from clone 2 virus (purified from three different plasmid clones) that infected alpha TC1 clone 9 cells (**Figure 2C**). We performed alpha cell reprogramming experiment by AAV virus infusion when alpha cell increasing in the islets as the schematically shows (**Figure 2D**).

AAV8-GCG viral gene delivery leads to specific targeting in pancreatic alpha cells in vivo

With confirmed specificity and gene expression *in vitro*, we then performed pancreatic ductal infusion with the virus. After AAV8-GCG-PM viral infusion in the pancreas of C57BL/6 wild-type (WT) mice and AAV8-GCG-Cre viral infusion in the pancreas of ROSA26 tomato reporter mice, we observed more than 96% GFP (**Figures 3A & 3B**) and 94% tomato red (**Figure 4A**) expression in glucagon-positive alpha cells. There are approximal 7% uncertain insulin and glucagon positive cells were assigned to beta cells after confocal microscope scan. These double positive cells are matched with the observation in human normal and T1D islets by other group(33). Furthermore, in the pancreas infused with AAV8-GCG-

GFP virus, very few GFP-positive cells were outside of the islets compared to the AAV8-CMV-GFP virus-infused pancreas (**Figure 3A**). mRNA expression of Pdx1 and MafA from two AAV8-GCG-PM virus infused mice was confirmed by RT-PCR (**Figure 3C**). Alpha cells transduced with AAV8-GCG-Cre virus proliferated, and some insulin-positive cells were red and glucagon positive, indicating that the cells transdifferentiated to insulin-producing cells post viral infusion in the ALX-induced diabetic pancreas (**Figure 4B**). The specificity of targeting was confirmed by confocal microscope sequentially scanning (Supplementary **Figure 1**).

Delivery of the Pdx1 and MafA genes into pancreatic alpha cells by AAV viral infusion in the pancreas of ALX-induced diabetic mice

To address whether the human GCG promoter drives Pdx1 and MafA to normalize autoimmune NOD/ShiLtJ diabetic mice, we first tested beta-cell toxin alloxan (ALX)-injected female C57BL/6J mice that developed diabetes at one week. The mice were sacrificed at 7, 21, 35 and 45 days after AAV8-GCG-PM delivery. After sectioning and staining with anti-insulin and glucagon antibodies, pancreatic tissue in the ALX-induced diabetic mice contained very few GFP-positive cells outside of islets 7 days following viral infusion (**Figure 5Ci to I**). Pdx1 and MafA expression was confirmed by RT-PCR after fluorescence-activated cell sorting (FACS) of GFP-positive cells from AAV8-GCG-PM 7 days post viral infusion (*data not shown*).

Blood glucose normalizes by AAV8-GCG-PM viral infusion in Alloxan (ALX)-induced diabetic mice

After viral infusion, the blood glucose and IPGTT were tested at 4 weeks, and the blood glucose levels were monitored for more than 6 weeks. As we had anticipated, the blood glucose levels normalized in the AAV8-GCG-PM viral infused group compared to the AAV8-GCG-GFP group (**Figure 5A**). Glucose tolerance in the AAV8-GCG-PM-infused ALX-induced mice dramatically improved compared to that in the AAV8-GCG-GFP group 4 weeks after viral infusion (**Figure 5B**). Immunohistochemistry of glucagon and insulin showed that beta cell mass significantly increased in ALX-induced mice receiving AAV8-GCG-PM compared to mice that received AAV8-GCG-GFP at 4 weeks after viral infusion (**Figure 5D**). Additionally, the beta cell mass reached more than 65% of that of WT mice (no ALX, no virus) (**Figure 5D**). A significant change in alpha cell mass compared to AAV8-GCG-GFP infused mice is demonstrated in **Figure 5D**, * $p < 0.01$. Immunohistochemistry showed a lower alpha cell mass in 6-week-old ALX+AAV8-GCG-PM mice (**Figures 5C i to l**) than in ALX+AAV8-GCG-GFP mice (**Figures 5C e to h**), similar with normal mice (non ALX treated) targeted with GFP (**Figure 5C a to i**). These results demonstrated that AAV8-GCG-PM rescued blood glucose levels 3 weeks after viral infusion in ALX-induced mice (**Figure 5**). Thus, these results demonstrated that intraductal infusion of AAV-GCG-PM normalized blood glucose levels in ALX-induced diabetic mice.

Blood glucose normalizes by AAV8-GCG-PM viral infusion in autoimmune (NOD/ShiLtJ) diabetic mice

Next, we examined whether AAV8-GCG-PM-transformed alpha cells would be recognized by an autoimmune diabetic immune system in NOD/ShiLt mice. The NOD/ShiLt mice were treated with a single dose of ductal infusion with the AAV8-GCG-PM virus early after the onset of hyperglycemia, when the blood glucose of the mice had surpassed 200 mg/dl. With 150 μ l virus (10^{12} genome copy particle [GCP]/ml) infusion, blood glucose levels were monitored, and IPGTT was performed following the same procedures as the ALX-induced diabetic mice, as described above. After monitoring blood glucose levels for more than 7 months, blood glucose levels normalized in the AAV8-GCG-PM viral infused group compared to the AAV8-GCG-GFP group in NOD/ShiLt female mice (**Figure 6A**). Immunohistochemistry of glucagon and insulin demonstrated that AAV-GCG-PM rescued blood glucose levels at 5 weeks after viral infusion (**Figure 6B**), but AAV-GCG-GFP not (**Figure 6B a to f**). Pdx1 and MafA expression was detected by RT-PCR from FACS-sorted GFP-positive cells harvested from the islets of AAV8-GCG-PM virus-infused NOD mice but was very low in control mice (*data not shown*). These results demonstrate that AAV8 combined with a human GCG promoter can drive Pdx1 and MafA in alpha cells more efficiently and specifically than the AAV8 CMV promoter-driven approach. Furthermore, the mice receiving control AAV-GCG-GFP showed a continuous increase in blood glucose levels and died within 6 weeks (**Figure 6A**). Immunohistochemical staining showed that the NOD/ShiLt mice that received AAV-GCG-PM had a significantly greater insulin-positive cell mass (**Figure 6B e to l**) as a basis for their normalized blood glucose. The increased beta cell mass was maintained at approximately 70%, which is comparable to the normal glycemia (NG) pancreas and maintained until the end of the experiment (**Figure 6D**). IPGTT showed that glucose tolerance was better than that of the pancreas infused with AAV GCG GFP virus (**Fig. 6C**). We observed that beta cell mass was increased at 4 weeks (* $p < 0.01$) (**Fig. 6D**) and maintained until the end of the 30-week experiment. There were no surviving AAV8-GCG-GFP-infused NOD/ShiLt mice after 6 weeks (**Fig. 6D**). We also observed concentrations of triglycerides, free fatty acids and β -hydroxybutyrate in the serum of AAV8-GCG-PM virus-infused mice with normalized insulinemia (**Figure 6E**). To this end, insulinitis (based on CD45 staining for immune cells) also decreased (**Figure 7**). Normalized blood glucose levels were maintained for 7 months, with very few immune cells found in the islet centers.

Discussion

Alpha cells are endocrine cells in mammalian islets of the pancreas, which comprise 20% of the human islet (34). Alpha cells play an important role in regulating glucose homeostasis in human daily activities by synthesizing and secreting the hormone glucagon to elevate glucose levels in the blood (34). Further evidence has demonstrated that the role of glucagon appears to be vital for hyperglycemia to arise in T1D (35, 36). Interestingly, the alpha cell population is preserved in the early and late stages of autoimmune diabetes. We and other groups have reported that alpha cell mass increases through proliferation when beta cell numbers diminish in islet centers during the development of diabetes, while

blood glucose levels begin to surge (6-8, 18, 26). Thus, a more efficient approach to treat T1D is reprogramming alpha cells to insulin-producing cells at the early onset of diabetes, increasing the transformed alpha cell number (following transformation) and proliferation.

T1D diabetic islets have been shown to dramatically decrease in functioning beta cells that coincide with the remaining alpha cells occupying the islet centers (3-5); however, the mass of alpha cells is also affected by invading immune cells in progressive T1D. This interesting phenomenon was observed in our experiment (**Figure 1**). Based on this finding, we designed a therapeutic strategy to infect native pancreatic alpha cells using an AAV8-GCG-PM viral delivery method that requires beta cell essential genes (Pdx1 and MafA) to reprogram alpha cells into insulin-producing cells. Following disease development, alpha cells proliferate, and virally infected alpha cells carrying the Pdx1 and MafA genes coincide to proliferate, which makes reprogramming more effective (**Figure 2D Schematic**) and prevents the development of severe disease. In animal experiments, a common approach is to target glucagon-secreting alpha cells *in vivo* by crossing mice expressing Cre recombinase under the glucagon promoter (Gcg-Cre mice) with loxp reporter mice, which have a loxP site transcriptional STOP sequence upstream of the fluorescent protein gene sequence (26, 37). However, these methods are time consuming due to breeding and limited to mouse strains. We demonstrated that a combination of the sox9 promoter and AAV serotype 6 is able to deliver the GFP gene in pancreatic ductal cells (24). Based on this finding that the combination of AAV serotype tissue tropism with a specific gene promoter, together with ductal infusion of the virus, leads to successful *in vivo* gene delivery and labeling of pancreatic ducts, we could be able to deliver a reporter gene to target alpha cells and deliver Pdx1 and MafA genes specific to alpha cells. First, we tested beta-cell toxin alloxan (ALX)-injected C57BL/6J mice, which developed diabetes one week after degradation of the beta cells of pancreatic islets (18). We found that ALX induced diabetes with a modest blood glucose increase but a significant increase in alpha-cell mass, which is consistent with our previous observations (18). The AAV8 virus carrying Pdx1-MafA genes was delivered into mouse pancreatic ducts of ALX-induced diabetic C57BL/6J mice by retrograde infusion of 150 µl of AAV8-GCG-PM (24, 25, 27-30). From our results, we found that the short GCG promoter and AAV8 combination was the best for targeting mouse alpha cells. These results demonstrated that the human short GCG promoter is specific and sufficient to drive gene expression *in vivo*. Here, we demonstrated that AAV8 in combination with the human alpha cell promoter is accomplished by delivering transgenes in pancreatic alpha-cells *in vivo* and that the Pdx1 and MafA genes under the human alpha cell promoter are able to normalize T1D mice either in chemically induced or autoimmune-induced diabetic mice. We first showed that Pdx1 and MafA expression *in vivo* was able to correct hyperglycemia in both ALX-induced diabetes and autoimmune diabetic NOD/ShiLtJ mice, implying that beta cell-like reprogramming occurred in alpha cells. Among all AAV serotypes, we found that 8 and 6 were the best for infecting mouse pancreatic cells (24, 25). We chose the AAV serotype 8 vector since we found that serotype 8 had a better infection efficiency in mouse islet cells than serotype 6 (25). AAV serotype 6 infects mouse pancreatic duct cells, while serotype 8 does not, but duct cells were not the focus of the current study. Both serotypes infect mouse acinar cells

well; however, combining the human GCG promoter increases tighter control of the tropism of AAV8, which could specifically infect alpha cells.

We designed and constructed various sizes of the human GCG promoter for assembly in the pAAV vector combined with the GFP reporter gene. Based on our previous work, we found that glucagon regulates its own synthesis by autocrine signaling (38) and infected the alpha TC1 clone 9 cell line with the AAV8-GCG-GFP virus and AAV8-GCG-Pdx1-MafA. We found that GFP was expressed in alpha TC1 clone 9 cells 3 days after infection. After infusion into murine pancreases, we observed specific GFP⁺ expression in mouse alpha cells with little overlap in insulin-positive cells but almost no GFP expression in acinar cells. In the AAV GCG promoter-driven Cre virus used to infuse into the pancreas of ROSA26 tomato reporter mice, we observed 85 to 90% Cre cleaved red fluorescence in the nuclei of alpha cells. This result indicates that the GCG promoter drives the Cre gene to determine the function of cleaving the loxp sites in the alpha cells of ROSA26 tomato reporter mice, which further confirms the GCG promoter specificity. Even though this short human GCG is only 648 bp, after analyzing the results of a BLAST search, we found that the promoter sequence is 83% and 82% similar to the mouse and rat promoters, respectively, in GenBank. Our experimental results indicated that the short GCG promoter is sufficient to drive gene expression and with specificity for identifying alpha cells in the mouse pancreas.

In human T1D islets of diabetes patients, islet cells are proliferated at the early onset of diabetes(33). As we observed and other groups reported that alpha cells are proliferated at early onset of diabetes and dominating in islet following the disease development, with the GCG promoter we successful, at this appropriate diabetes early onset stage, to deliver insulin producing essential transcription factors Pdx1 and MafA specific in alpha cells and reprogram the infected alpha cells into insulin producing cells. The time to trigger alpha cells to reprogram into insulin-producing cells is critical for the treatment of diabetes, especially for NOD/ShiLt mice, as they give impetus to diabetes very quickly once disease onset. The overexpression of Pdx1 and MafA in NOD/ShiLt islets completely protected against the development of spontaneous autoimmune diabetes. Additionally, in contrast to their diabetic NOD/ShiLt with AAV8-GCG-GFP control, NOD-AAV8 GCG Pdx1 and MafA animals had normal levels of serum triglycerides, free fatty acids, and beta-hydroxybutyrate, indicating that they have normal energy metabolism. AAV8 combined with GCG promoter-mediated Pdx1 and MafA gene transfer to the pancreas protected NOD/ShiLt mice against the development of autoimmune diabetes, as evidenced by the significant reduction in the incidence of spontaneous diabetes in the animals that received the therapeutic vector. Our results demonstrated that two transcription factors, Pdx1 and MafA, assembled in an AAV8 viral vector with a human GCG promoter through a retrograde infusion technique are able to specifically reprogram pancreatic alpha cells into insulin-producing beta-like cells, and blood glucose levels in autoimmune NOD/ShiLt diabetic mice can be normalized. AAV8-GCG-Pdx1-MafA-treated mice also showed preservation of beta-cell mass and normal levels of circulating insulin. Here, we showed that Pdx1 and MafA expression under the GCG promoter driven *in vivo* was able to correct hyperglycemia in both ALX-induced diabetes and in autoimmune diabetic NOD/ShiLt mice, signifying that beta cell-like reprogramming was occurring, and this is not suppression of the GCG promoter in alpha cells, as we saw glucagon expression is still normal, as AAV is not a genome integration virus. We noticed that few beta

cells (less than 7%) are infected with the virus or insulin and glucagon double positive in WT islets, however, due to the beta cells are no longer expressed of insulin when diabetes occurs; therefore, the viral infection of beta cells in WT islet may not happen in the diabetes islets. Whether Pdx1-MafA expression in T1D beta cells can alleviate diabetes development we need further study to design insulin promoter drive Pdx-MafA in the future.

With this cell reprogramming, we did not observe significant immune cells in the AAV-GCG-Pdx1-MafA-infused islets in NOD/ShiLt mice. We currently do not know the reason, but the possibility is that ductal viral infusion with AAV GCG promoter-driven Pdx1 and MafA altered the autoimmunity of NOD/ShiLt mice, leading to extended survival of neogenic insulin cells. It is also possible that AAV8-GCG-Pdx1-MafA-infected alpha cells are not immune cells recognized, as they do not produce mature insulin at the preprogramming stage. Once the alpha cells reprogrammed into insulin-producing cells, they maintained glucagon production, which may confuse the immune system of NOD/ShiLt mice. In addition, the GCG promoter is significantly different from the insulin promoter; for example, the transcription factor ATF4 binds to the insulin promoter, causing beta cell dysfunction and apoptosis (39, 40), and activated ATF4 regulates cell immunity (41), which may play an attractive role in immune cell homing to islets. The mechanisms by which Pdx1 and MafA reprogram alpha cells into insulin-producing cells with protective action on transgenic islets or after AAV8-GCG-Pdx1 and MafA-mediated gene delivery have not been fully elucidated. The next research aim was to establish stable Pdx1 and MafA gene integration into the alpha cell glucagon gene locus to further study the mechanism of alpha to beta transdifferentiation. We previously demonstrated that using AAV serotype 8 with CMV promoter driving Pdx1 and MafA success reprogramed alpha cells into insulin producing cells and escaped immune cells attacked for four months(18). As the CMV promoter is a broad activation strong promoter although it has serotype 8 tropism tight controlled expression in islet cells when beta cells disappeared in diabetic islets, it still has leaky expression in other islet cells and acinar cells. Here, we modified the AAV construct with GCG promoter replaced CMV promoter to drive Pdx1 and MafA expression in diabetic mice islets, specifically in aloha cells that we observed longer time euglycemia in NOD mice without immune cells attacked in seven months. This observation may let us think about the promoter regions may also be a factor to induce immune cells sensing beta cells causing it to be attacked.

In summary, we report here that the human short promoter-driven reporter GFP gene could target pancreatic alpha cells specifically in the mouse pancreas. The short human GCG promoter is enough to drive two transcription factors, Pdx1 and MafA, and combination with AAV serotype 8 is capable of normalizing blood glucose either in chemically induced or autoimmune diabetes in animals. We reported that normal blood glucose restoration and beta cell survival in NOD/ShiLt mice by AAV8-CMV drive Pdx1, and MafA appears to last 4 months prior to re-establishment of autoimmunity, but in our GCG promoter driven Pdx1 and MafA, euglycemia of NOD mice lasts 7 months and without immune cells found in the neogenic islets. This may be the motivation for us to better use the GCG promoter driving Pdx1 and MafA gene reprogramming alpha cells into insulin-producing beta cells for T1D.

Four weeks after infusion, we observed blood glucose normalization in ALX-induced diabetic mice, and the new insulin-positive cells were found to be derived exclusively from alpha cells. Similarly, we observed that the blood glucose of hyperglycemic NOD/ShiLt mice became normal at approximately 4 weeks and lasted more than 7 months to the end of the experimental design date without immune cell infiltration in islets. Therefore, we conclude that this alpha cell-specific promoter in combination with AAV 8 serotype viral reprogramming provides the vision to develop a novel therapy that can potentially be translated to the clinic for the treatment of T1D.

Declarations

AUTHOR CONTRIBUTIONS

P.G. designed experiments, constructed viral vectors, and wrote and edited the manuscript. T. Z. and A. L. generated reagents and performed experiments. C. S. contributed to the discussion on the characterization of the GCG promoter. M. H. taken care the animals. K. W. contributed to manuscript editing and proofreading. J. H. contributed to the discussion and reviewed the manuscript.

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Figures

Figure 1

T1D mouse alpha cell number increased following disease advancement. The beta-cells losing the remaining alpha-cells occupied the islet center, and more alpha cells in the NOD mouse islets were observed as the blood glucose increased. A: normal islet of C57BL/6J (WT). B: islet of NOD/ShiLt mouse at normal glycemia (NG). C: Alpha cells tend to center in the islet at the early onset of diabetes (250 mg/dl). D and E: Alpha cell distribution in the islets at the disease development stage. F: Alpha cells occupied the center area of the islet at a blood glucose level increase near the limit (600 mg/dl) measurement of the glucometer. H to J: alpha cell proliferation rate was reached high level around 300mg/dl hyperglycemia of NOD compared to WT mice (G). K: quantification of Ki67 positive alpha cells in NOD mice islets. * $p < 0.05$. Immunofluorescence staining, x200, scale bar = 25 μ M.

Figure 2

Construction for AAV vector of GCG promoter drive gene. A schematic showing the construction of the AAV vector and GCG promoter driving GFP, Cre. For AAV-GCG-Pdx1-MafA, the GCG promoter drives Pdx1 and MafA open reading frames (ORFs), and the GFP tag was connected to the 2A peptide sequence, pAAV-GCG-Pdx1-P2A-MafA-T2A-GFP (Fig. 2A). The Pdx1 and MafA expression of plasmids was determined by Western blot (Fig. 2C) and GFP cell transduction efficiency in alpha-TC1 clone 9 cells (Fig.

2B). Min6: Min-6 cell line as a positive control. Schematics showing the therapeutic strategy for using AAV8-GCG-Pdx1-MafA-specific reprogramming of pancreatic alpha cells into insulin-producing cells in diabetic mice (Fig. 2D). Bright field and fluorescence images, x100.

Figure 3

AAV8 GCG promoter drives Pdx-MafA-GFP-specific targeting alpha cells Specific α cell expression of GFP in vivo (Fig. 3A) indicated that Pdx1 and MafA expression was specific to α cells compared to AAV8-CMV-GFP virus-infused pancreas. Quantification of GFP-positive cells (more than 96%) is shown in Fig. 3B. RT-PCR images of Pdx1 and MafA after FACS sorting of GFP-positive cells from AAV8-GCG-PM-infused islets (Fig. 3C). Immunofluorescence staining, x200, scale bar = 25 μ M.

Figure 4

AAV8 GCG promoter drives Cre-specific targeting alpha cells in the pancreas of tomato reporter mice. The specific alpha cell expression of tomato red in the nuclei of alpha cells by Cre cleaved stop codons in the tomato reporter mouse pancreas via AAV8-GCG-Cre virus infusion (Fig. 4). More than 94% alpha cells and few beta-cells are tomato red in nuclei. These glucagon α -positive cells are Cre cleaved which showed red fluorescence. They are not beta-cells via confocal microscope scan. Few nonnuclear red fluorescent cells

were found outside of islets, and they were not glucagon positive (Fig. 4A). Alpha cells transduced with AAV8-GCG-Cre virus proliferated, and some insulin-positive cells were red and glucagon positive, indicating that the cells transdifferentiated to insulin-producing cells 10 days post viral infusion in the ALX-induced diabetic pancreas (Fig. 4B). Immunofluorescence staining, x200, scale bar = 25 μ M. These results are confirmed by confocal microscope scan and some cells are insulin and glucagon double positive (Supplementary Figure 1).

Figure 5

AAV8 GCG-driven Pdx1 and MafA normalize blood glucose in ALX-induced diabetic mice Blood glucose was normalized 3 weeks post AAV8-GCG-PM viral infusion (Fig. 5A). Compared to AAV8-GCG-GFP viral infused islets (Fig. 5 C e to h) in ALX induced diabetes mice, ALX Islets was normalized by reprogrammed alpha cells into insulin producing cells in ALX induced C57/6 mice pancreas (Fig. 5 i to l). IPGTT showed that glucose tolerance was better than that of the pancreas infused with AAV GCG GFP virus (Fig. 5B). Beta cell mass was significantly increased compared to AAV8-GCG-GFP-infused pancreas ($p<0.01$) and up to 65% of WT mice 4 weeks after AAV8-GCG-PM viral infusion (Fig. 5D). Immunofluorescence staining, x200, scale bar = 25 μ m

Figure 6

AA8 GCG-driven Pdx1 and MafA normalize blood glucose in autoimmune diabetes mouse NOD/ShiLt mouse blood glucose levels to normal 4 weeks after AAV8-GCG-PM viral infusion (Fig. 6A). Immunohistochemistry of glucagon and insulin in AAV8-GCG-GFP-infused NOD/ShiLt autoimmune diabetes mouse pancreas (Fig. 6B a to d). Islets were normalized by reprogramming alpha cells into insulin-producing cells in AAV8-GCG-PM-infused NOD/ShiLt mouse pancreases (Fig. 6B e to l shows two representative mouse pancreases). Immunofluorescence staining, x200, scale bar = 25 μ m. IPGTT

showed that glucose tolerance was better than that of the pancreas infused with AAV GCG GFP virus (Fig. 6C). Beta cell mass was increased at 4 weeks (*p<0.01) (Fig. 6D) and maintained until the end of the 30-week experiment. There were no surviving AAV8-GCG-GFP-infused NOD/ShiLt mice after 6 weeks (Fig. 6D). Concentrations of triglycerides, free fatty acids and β -hydroxybutyrate in the serum of AAV8-GCG-PM virus-infused mice had normal insulinemia (Fig. 6E). NG: NOD/ShiLt normoglycemic, HG: hyperglycemic NOD/ShiLt mice. NOD+GCG-PM: AAV8-GCG-PM infused NOD/ShiLt mice. *:p<0.01, #: p<0.05

Figure 7

The GCG promoter drives Pdx1- and MafA-reprogrammed insulin-producing beta cells to reduce immune cell homing to the islets of the pancreas in NOD/ShiLt mice. CD45-positive immune cells infiltrated the islets of NOD mice (Fig. 7) but not near the islets in the pancreas of AAV8-GCG-PM virus-infused NOD/ShiLt mice (Fig. 7). Immunofluorescence staining, x200, scale bar = 25 μ M.

Supplementary Files

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