Beta Cell Formation *in vivo* Through Cellular Networking, Integration and Processing (CNIP) in Wild Type Adult Mice

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Abstract: Insulin replacement therapy is essential in type 1 diabetic individuals and is required in ~40-50% of type 2 diabetics during their lifetime. Prior attempts at beta cell regeneration have relied upon pancreatic injury to induce beta cell proliferation, dedifferentiation and activation of the embryonic pathway, or stem cell replacement. We report an alternative method to transform adult non-stem (somatic) cells into pancreatic beta cells. The Cellular Networking, Integration and Processing (CNIP) approach targets cellular mechanisms involved in pancreatic function in the organ's adult state and utilizes a synergistic mechanism that integrates three important levels of cellular regulation to induce beta cell formation: (i) glucose metabolism, (ii) membrane receptor function, and (iii) gene transcription. The aim of the present study was to induce pancreatic beta cell formation *in vivo* in adult animals without stem cells and without dedifferentiating cells to recapitulate the embryonic pathway as previously published [1-3]. Our results employing CNIP demonstrate that: (i) insulin secreting cells can be generated in adult pancreatic tissue *in vivo* and circumvent the problem of generating endocrine (glucagon and somatostatin) cells that exert deleterious effects on glucose homeostasis, and (ii) long-term normalization of glucose tolerance and insulin secretion can be achieved in a wild type diabetic mouse model. The CNIP cocktail has the potential to be used as a preventative or therapeutic treatment or cure for both type 1 and type 2 diabetes.

Keywords: Beta cell formation, insulin secretion, *in vivo*.

INTRODUCTION

Impaired insulin secretion is a characteristic feature of both type 1 and type 2 diabetes mellitus, and a variety of approaches have been employed to generate beta cells *in vivo* and *in vitro* [1-3]. Herein, we describe a completely novel approach to generate beta cells *in vivo* in an adult wild type rodent model of diabetes. This method, which integrates multiple levels of cellular physiology to regulate organ function, results in the long-term normalization of insulin secretion and glycemia and is not associated with pancreatic injury. We labeled our approach "Cellular Networking, Integration and Processing (CNIP)" to distinguish it from the term "system biology" which is used broadly to refer collectively to different levels of cellular, organ and whole body physiology. The fundamental principle underlying CNIP is to target simultaneously and integrate three key levels of cellular physiology that have been implicated in beta cell formation. This is in contrast to the non-integrated model which targets only nuclear reprogramming [1, 2]. Two important concepts characterize the CNIP approach: (i) it is essential to integrate three levels of cellular physiology: carbohydrate metabolism, membrane receptor function, and gene transcription to produce the desired effect, i.e. beta cell

formation, (ii) integration of multiple levels of cellular physiology is essential to produce a synergistic effect on beta cell formation. Synergy is a key factor, in which multiple molecules work together to produce an effect that is greater than the sum of their individual effects. In contrast to previous approaches, CNIP is designed to target cellular mechanisms involved in pancreatic function in the organ's adult state and utilizes a synergistic mechanism that integrates multiple levels of cellular regulation.

Glucose metabolism is the first important element in our CNIP approach to induce beta cell formation in the adult pancreas. Glucose is the major energy source utilized by mammalian cells and provides the energy for cellular function and proliferation [4]. Inhibition of glycolysis stops cell cycle progression, demonstrating the necessity of glucose metabolism to induce cell proliferation [4, 5]. Indeed, the major factors which induce pancreatic beta cell formation *in vivo* require glucose metabolism [6-8]. The first rate limiting step for glucose metabolism in the glycolytic pathway is at the level of glucose phosphorylation by hexokinase. Pancreatic beta cells contain a hexokinase type IV, named glucokinase. We have shown that if glucose is not phosphorylated to G-6-P by glucokinase, it cannot undergo further metabolism and cannot generate a signal to the transcriptional machinery to induce gene expression [9, 10]. Therefore, the first molecule included in our CNIP cocktail to induce pancreatic beta cell formation is glucokinase.

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A second cellular function implicated in beta cell formation is ligand binding to tyrosine kinase receptors [7, 11]. The increase in pancreatic beta cell mass in response to physiological stress, i.e. pregnancy, is mediated by growth hormone, prolactin and placental lactogen working through the prolactin receptor [11, 12]. The prolactin receptor does not have intrinsic tyrosine kinase activity, but it interacts with members of the Janus kinase family of tyrosine kinases [11, 12]. The action of insulin, insulin-like growth factors, hepatocyte growth factor, and epidermal growth factor also increase beta cell mass by activating a membrane bound tyrosine kinase receptor [11]. The protein-tyrosine phosphatase 1B (PTP1B) has been shown to inhibit the ability of insulin, insulin-like growth factor, prolactin and hepatocyte growth factor to activate tyrosine kinase(s) [13-15]. Consequently, the second molecule included in our CNIP cocktail to induce pancreatic beta cell formation by increasing membrane receptor tyrosine kinase activity is a shRNA targeting PTP1B. Suppression of PTP1B protein production by shRNA increases receptor tyrosine kinase activity involved in beta cell formation in the adult pancreas in response to binding of a physiological concentration of the corresponding hormone: insulin, insulin-like growth factor, hepatocyte growth factor, epidermal growth factor, growth hormone, prolactin and placental lactogen [13-15].

The third important step in our CNIP approach is directed at the gene expression level and involves a transcription factor, which is utilized as an attractor to converge and focus the glucose metabolic/molecular effects generated by the tyrosine kinase receptor(s) to form new beta cells in the adult pancreas. An important action of glucose metabolism is to send a signaling to the transcriptional machinery to increase the probability of the transcription factor (TF) binding to the DNA molecule [9, 10]. We have shown that glucose metabolism delivers a signal to the transcriptional machinery to induce TF expression and TF translocation from the cytoplasm to the nucleus to turn on glucose-induced genes [9, 10]. A key transcription factor implicated in beta cell formation is Pdx-1, which has distinct effects before and after birth [16]. At the embryonic stage, Pdx-1 is essential for pancreatic development and it is expressed in endocrine and exocrine tissues. However, after birth, Pdx-1 is expressed only in pancreatic beta cells and somatostatin cells. Pdx-1 has been shown to be involved in beta cell formation in adult animals [17] and has been used in our CNIP approach for its post-embryonic action to enhance beta cell formation. Therefore, overexpression of Pdx-1 plays a central role in converging all of the signaling mechanisms in the CNIP cocktail to stimulate beta cell formation.

We demonstrate in this paper that our CNIP cocktail induces beta cell formation in adult wild type diabetic mice directly from pancreatic somatic cells. This approach to increase the number of insulin secreting pancreatic beta cells in adult subjects can be used as prevention, treatment, or cure of diabetes. We also compared the CNIP approach to a three-pronged cocktail comprised only of transcription factors (1) to demonstrate that integration of multiple levels of cellular physiology is essential to produce a synergistic effect on beta cell formation that cannot be achieved by simply employing

a cocktail of transcription factors that only target nuclear reprogramming *in vivo*.

MATERIALS AND METHODS

Viral Vector Construct

We designed a lentiviral vector construct expressing the glucokinase gene under control of the cytomegalovirus (CMV) promoter. We incorporated into our lentiviral vector construct a posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE) at the 3' untranslated region of coding sequence; this substantially increased the level of expression of the transgene. WPRE functions within the nucleus to stimulate gene expression posttranscriptionally by increasing the level of nuclear transcripts and greatly increasing the RNA half-life. The mouse glucokinase (GCK) gene was subcloned to plasmid CMV-WPRE vector and the insert was verified by DNA sequencing. The plasmid-GCK was treated with LR Clonase II enzyme (Invitrogen) and ligated to a plasmid Lenti vector. The recombination product was transformed into *E. coli* cells. After overnight incubation, the positive clones were selected, and plasmid DNA was purified. The plasmid-GCK and plasmid Lenti-GCK were transfected into 293 cells. 48 hours after transfection, the cells were lysed in SDS-PAGE buffer and subjected to 4-20% SDS-PAGE gel electrophoresis and Western blot analysis. The Western blot was carried out using the anti-GCK antibody at 1:1000 dilution, followed by a HRP conjugated secondary antibody. The Western blot membrane was detected by ECL reagents. The PDX-1 cDNA was introduced into a lentivirus construct using the same method described above for lenti-GCK under the control of the CMV promoter. The coding sequence of Pdx-1 cDNA was ligated downstream of a c-myc tag to allow the protein overexpression to be identified with an antibody against the Myc epitope to differentiate it from the endogenous wild type expression of PDX-1. The plasmid PDX1 and plasmid Lenti-PDX1 were transfected into 293 cells. 48 hours after transfection, the cells were lysed in SDS-PAGE buffer and subjected to 4-20% SDS-PAGE gel electrophoresis and Western blot analysis. The Western blot was carried out using the anti-Myc (for the PDX-1 construct) antibody at 1:1000 dilution, followed by a HRP conjugated secondary antibody. The membrane was detected by ECL reagents. The shRNA PTP1B was introduced into a lentivirus construct using the same method described above for lenti-GCK under the control of the H1 polymerase promoter. The polymerase III promoter H1 is active ubiquitously in all cells, because of the housekeeping function of polymerase III. The lentivirus were generated by Welgen (Worcester, MA, USA). For western blots, equal amounts of total protein were separated on 10 and 15% SDS/PAGE and transferred onto nitrocellulose membranes. Membranes were then blocked with 5% nonfat milk in 0.1% TBS Tween-20 and probed with specific antibodies against PDX-1 (Cell Signaling Technology, Danvers, MA, USA), PTP1B (Abcam Inc., Cambridge, MA, USA), glucokinase (Santa Cruz Inc, Santa Cruz, CA, USA), and GAPDH (G9545, Sigma Aldrich, St Louis, MO, USA). Membranes then were incubated with HRP-conjugated secondary antibody (NA934) and developed with a chemiluminescent reagent (Amersham Bioscience, GE Healthcare,

Pittsburgh, PA, USA). The mouse Ngn3 and MafA genes were subcloned to plasmid CMV vector and inserts were verified by DNA sequencing. The plasmid-Ngn3 and MafA-GFP were treated with LR Clonase II enzyme (Invitrogen) and ligated to a plasmid Lenti vector. The recombination products were transformed into *E. coli* cells. After incubation overnight, the positive clones were selected, and plasmid DNA was purified. The plasmid Lenti-Ngn3 and pLenti-MafA were transfected into 293 cells. 72 hours after transfection, the cells were lysed in SDS-PAGE buffer and subjected to 4-20% SDS-PAGE gel electrophoresis and Western blot analyses. The Western blot was carried out using the anti-Ngn3 and MafA antibody at 1:1000 dilution, followed by a HRP conjugated secondary antibody. The Western blot membrane was detected by ECL reagents. Western blots of pancreatic tissue four-weeks post-injection with cocktail transcription factors (Lenti-MafA + Lenti-PDX-1 and Lenti-Ngn3) and cocktail control with antibodies against MafA, Ngn3, and PDX-1 were stripped and then re-probed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or alpha-tubulin antibodies as loading control. For western blots, equal amounts of total protein were separated on 10 and 15% SDS/PAGE and transferred onto nitrocellulose membranes. Membranes were then blocked with 5% nonfat milk in 0.1% TBS Tween-20 and probed with specific antibodies against PDX-1 (Cell Signaling Technology, Danvers, MA, USA), Ng3 (Santa Cruz Inc, Santa Cruz, CA, USA), MafA (Bethyl Laboratories, Inc. Montgomery, TX, USA), and GAPDH (G9545, Sigma Aldrich, St Louis, MO, USA). Membranes then were incubated with HRP-conjugated secondary antibody (NA934) and developed with a chemiluminescent reagent (Amersham Bioscience, GE Healthcare, Pittsburgh, PA, USA).

***In vivo* Method for Targeted Gene Delivery to Adult Pancreas**

To study beta cell formation in the adult animal, we employed a novel *in vivo* approach to target the adult pancreas using a lentiviral vector [18]. We previously validated this technique [18] and employed it in our CNIP approach to generate pancreatic beta cells *in vivo*. The Lentiviral construct (Fig. 1) is introduced into the mouse pancreas via the pancreatic duct as follows: a 32-gauge catheter is inserted into the cystic duct through a small opening in the gallbladder. The catheter is then advanced into the common bile duct and secured in place with a slipknot of 0/0 suture around the bile duct and catheter to prevent vector reflux into the liver. With a micro clamp placed around the sphincter of Oddi to avoid leakage of the vector into the duodenum, 100 μ l of total Lentiviral vector cocktail expressing cDNA glucokinase, Pdx-1, and shRNA PTP1B (Fig. 1) at 10^8 to 10^9 TU/ml is slowly injected into the pancreatic duct through the catheter. The control placebo cocktail was composed of Lentivirus shRNA scramble with Lentivirus expressed GFP at the same concentration and volume as the experimental cocktail. Four weeks post-Lentiviral infection, the entire pancreas is removed for histologic examination. We previously have shown that 48 hours after injection of Lentivirus coding for green fluorescent protein (GFP) under the control of promoter cytomegalovirus (CMV) GFP was detected only in pancreatic tissues [18]. Quantitative morphometric analy-

sis of pancreatic transduction by the Lentivirus vector, based on GFP expression, showed that 60% of the tissue expressed GFP [18]. Importantly, expression was detected in the pancreas even after four weeks (Fig. 1D). The Lentivirus vector expressed green fluorescent protein was not found in any other tissue in the body including heart, lung, liver, brain, leg muscle and kidney by histology and PCR (data not shown). Pancreatic tissue was stained with H & E to look for evidence of inflammation (pancreatitis) at day 2 and day 14 post-injection of the Lentivirus. Consistent with previous studies from our lab [18], no evidence of inflammation was observed in the present study with our method of Lentivirus injection. Male C57BL/6 mice (Charles River, Wilmington, MA, USA) 8 weeks of age were used and maintained on an ad libitum diet of water and normal chow for all experiments. At day 1 post-injection with lentiviral vector, the mice were injected i.p. daily with BrdU (Sigma-Aldrich, St Louis, MO, USA) in PBS at a dose of 50 μ g/g body weight for 12 days to quantitate beta cell proliferation. At 4 weeks post-lentiviral injection, the entire pancreas was removed for histologic examination.

Partially Pancreatectomized Diabetic Mouse

Diabetes was created by removing ~ 80 % of the pancreas as previously described [19]. The partially pancreatectomized diabetes mouse [19] represents an insulinopenic model of diabetes and has the major advantage over other diabetic models that, following resolution of the acute stress of surgery in 5-7 days, the remaining pancreatic tissues are perfectly normal [20]. Of note, we waited two weeks after pancreatectomy before injecting the CNIP cocktail. Two weeks is sufficient for any effect of the surgical pancreatectomy on cell proliferation to have disappeared [21] (see Supplementary Data, Wild-type and partially pancreatectomized diabetic mouse model).

Intraperitoneal Glucose Tolerance Test with Plasma Insulin Response

Before and 4 weeks after partial pancreatectomy, mice (n = 5) received an intraperitoneal glucose tolerance test. Following an overnight fast, mice were weighed and injected i.p. with a glucose bolus (2g/kg). Blood glucose concentrations were determined from tail blood at 0, 10, 15, 30, 60 and 120 min using Ascensia Breeze 2 glucose meter (Bayer HealthCare, Mishawaka, IN). Plasma insulin concentration was measured at 0, 15, 30 and 60 min on 5 μ l (EDTA) samples using the mouse insulin Ultrasensitive ELISA (Alpco Diagnostics, Salem, NH). Fasting blood glucose was measured at weeks -2, -1, 0 and every week post partial pancreatectomy.

Immunofluorescent and Immunohistochemical Analysis

Four weeks post-Lentiviral infection, the entire pancreas was removed for blind histologic examination. Adult mouse pancreatic tissue was fixed by immersion in phosphate buffer 4% paraformaldehyde - 1% glutaraldehyde overnight at 4°C and embedded with Tissues -Tek OCT compound for cryostat sectioning. The following primary antibodies were used: anti-somatostatin (G-10), anti-glucagon (K79bB10) and anti-insulin A (C-12) antibodies and control rabbit IgG (Santa Cruz Inc., Santa Cruz, CA). For proliferation studies,

Fig. (1). (A) We designed a lentiviral vector construct expressing the glucokinase gene under control of the cytomegalovirus (CMV) promoter. (B) The PDX-1 cDNA was introduced into a lentivirus construct using the same method described in the methodology section in supplementary data for lenti-GCK under the control of the CMV promoter. (C) The shRNA PTP1B was introduced into a lentivirus construct under the control of the H1 polymerase promoter. The internal ribosome entry site (IRES) element in the Lenti-vector co-expressing GFP protein was used to identify the co-localization of the shRNA in histologic sections. The IRES element enables the coordinated co-expression of two genes with the same vector. Co-transfection experiment was set up using the target gene expression plasmid PTP1B and one plasmid PTP1B-shRNA vector. The Western blot was carried out using the anti-PTP1B antibody at 1:1000 dilution. The membrane was detected by ECL reagents. (D and E) Expression of CNIP cocktail molecules in pancreatic tissue. Four-weeks post-injection we demonstrated in the pancreas *in vivo* over-expression of PDX-1, glucokinase, and suppression of PTP1B expression by the CNIP cocktail (Lenti-GCK + Lenti-Pdx-1 and Lenti-shRNA PTP1B) (Fig. 1D). Glucokinase over expression was detected in the islets and in exocrine tissues (Fig. 1D). Expression of glucokinase in exocrine tissues confirms the over expression of glucokinase by the experimental cocktail (Lenti-GCK + Lenti-Pdx-1 and Lenti-shRNA PTP1B) since pancreatic tissues only express glucokinase in endocrine cells. In Fig. 1D histology in experimental mice is paired with the corresponding control below. Pdx-1 over expression is detected by a tag c-Myc incorporated into the cDNA of Pdx-1 to differentiate the exogenous versus wild type endogenous expression (Fig. 1D). shRNA PTP1B co-expressing GFP is shown in green (Fig. 1D). (E) Western blots of pancreatic tissue four-weeks post-injection with CNIP cocktail (Lenti-GCK + Lenti-Pdx-1 and Lenti-shRNA PTP1B) and control cocktail with antibodies against glucokinase (GK), protein-tyrosine phosphatase 1B, (PTP1B), and PDX-1 were stripped and then re-probed with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibodies as loading control. * = $p < 0.001$, CNIP cocktail ($n=3$) vs Control cocktail ($n=4$). Data are present as mean $\pm$ SE. Statistical comparisons were performed with Student's *t* test.

pancreatic tissues were stained with rat monoclonal BrdU antibody (Abcam Inc., Cambridge, MA). Antigen retrieval was performed for BrdU antibodies by boiling sections for 10 min in 10 mM citrate buffer followed by cooling for 30 min to room temperature. Nuclei were counterstained with DAPI (Vector Laboratories, Inc., Burlingame, CA). Fluorescent secondary antibodies included donkey anti-goat-fluorescein, goat anti-mouse-fluorescein, goat anti-rabbit Texas red, and

donkey anti-goat Texas red (Santa Cruz Inc., Santa Cruz, CA). Beta cell areas represent the surface area of cells staining positively for insulin immunostaining divided by the total pancreatic surface scanned with Olympus FV-1000 laser scanning confocal microscope. Insulin positive and total pancreatic areas were quantified with Image J (National Institutes of Health, Bethesda, MD). Beta cell mass was calculated as beta-cell area multiplied by pancreatic wet weight.

Fig. (2). (A, B, D and E) One or two insulin-positive cells in acinar tissues 4 weeks post-injection of cocktail (Lenti-GCK + Lenti-Pdx-1 + Lenti-shRNA PTP1B). (C and F) Control cocktail pancreatic tissues 4 weeks post-injection show no insulin-positive cells in acinar tissues. Insulin positive cells only were demonstrated in islets. Insulin is shown in green in fig A, B and C. Insulin is shown in red in figs D, E and F. DAPI in blue is all figures. The figure is representative of different areas of the pancreas examined in ten sections per animal, separated by 200 μ m. Confocal laser microscopy was used for analysis. * $p < 0.001$, CNIP cocktail ($n=4$) vs Cocktail control ($n=6$). ** $p < 0.001$, CNIP cocktail ($n=5$) vs Cocktail control ($n=5$). The histologic analysis was performed in a blinded fashion to the examiner. Data are presented as mean $\pm$ SE. Statistical comparisons were performed with Student's t test.

BrdU Marker of Proliferation

Fig. (3). BrdU marker of proliferation in islets (A and D) and acinar tissues (B and E) 4 weeks post-injection of cocktail (Lenti-GCK + Lenti-Pdx-1 + Lenti-shRNA PTP1B). (C and F) Control cocktail pancreatic tissues 4 weeks post-injection show no BrdU marker staining. BrdU is shown in green and DAPI is shown in blue. The figure is representative of different areas of the pancreas examined in ten sections per animal, separated by 200 μ m. The results are expressed as the fold-increase in number of BrdU-labeled cells compared with controls. Confocal laser microscopy was used for analysis. * $p < 0.001$, CNIP cocktail ($n=4$) vs Control cocktail ($n=6$). ** $p < 0.001$, CNIP cocktail ($n=5$) vs Control cocktail ($n=5$). The histologic analysis was performed in a blinded fashion to the examiner. Data are presented as mean $\pm$ SE. Statistical comparisons were performed with Student's t test.

At least four mice were analyzed per condition. Pancreatic tissue was stained with H & E to look for evidence of inflammation (pancreatitis) at days 2 and 14 post-injection of Lentivirus.

RESULTS

Following the Lentiviral vector injection containing the experimental cocktail or control placebo cocktail, daily food intake was monitored over the four weeks post-injection (CNIP cocktail mice, 5.4 ± 0.3 grams/day [$n=4$] versus control placebo cocktail mice, 5.9 ± 0.5 grams/day [$n=6$]). Weight gain was similar in the CNIP and control groups. No diarrhea was observed in either the cocktail placebo control or experimental cocktail groups after Lentivirus injection. Pancreatic (lipase) and hepatic (AST, ALT) enzymes measured on days 7 and 14 post-Lentiviral injection were not elevated, consistent with previous observations [18]. These results demonstrate that the Lentivirus injection technique does not cause adverse gastrointestinal, pancreatic, or hepatic effects. Four-weeks post-injection, we demonstrated in the pancreas over-expression of PDX-1 and GK and suppression

of PTP1B expression by the CNIP cocktail (Fig. 1D and 1E). We quantified the number of single or double insulin-positive cells in exocrine tissues as an indication of beta cell formation by comparing the CNIP cocktail group to cocktail control group. We demonstrated a significant increase in single or double insulin-positive cells in exocrine tissues (Fig. 2). The increase in single or double insulin positive cells in the CNIP cocktail group correlated with the increase in beta cell proliferation, quantitated by BrdU (Fig. 3). The BrdU marker demonstrated beta cell proliferation in islets and exocrine tissues. Co-localization of the proliferation marker BrdU with insulin positive cells documents the formation of newly formed pancreatic beta cells in mice injected with CNIP cocktail (Fig. 4A). Only pancreatic beta (insulin) cell proliferation was observed. No alpha (glucagon) or delta (somatostatin) cell proliferation was demonstrated (data not shown), consistent with our aim to only induce the post-embryonic formation of pancreatic beta cells.

We quantified beta cell mass in CNIP cocktail and control groups. Pancreatic beta cell mass was significantly increased in adult mice injected with CNIP cocktail versus

Fig. (4). CNIP cocktail induced pancreatic beta cell proliferation and increased beta cell mass in adult mice. (A1) Beta cell proliferation in islets. (A3) Beta cell proliferation inside the beta cell cluster. (A2 and A4) represent control cocktail without beta cells proliferation. Insulin is shown in red and DAPI is shown in blue. BrdU marker is shown in green. (B) The beta cell mass 4-weeks post-injection with cocktail CNIP (Lenti-GCK + Lenti-Pdx-1 + Lenti-shRNA PTP1B) and cocktail control is shown. Total pancreatic and insulin positive staining areas of each section were measured using Image J (NIH, Bethesda, USA). Beta cell mass was calculated as the ratio of total insulin positive area to total pancreatic area of all sections, multiplied by the pancreatic tissue wet weight. The figure is representative of an area of the pancreas examined in ten sections per animal, separated by 200 μ m. Confocal laser microscopy was used for analysis. $*=p < 0.001$, CNIP cocktail ($n=3$) vs Control cocktail ($n=4$). Data are presented as mean $\pm$ SE. (C) Beta cell cluster density in adult mice injected with CNIP cocktail compared with the adult mice injected with the control cocktail 4 weeks post-injection. Cluster density was determined as the number of beta cell clusters (3 to 5) divided by the total area of the pancreas. The figure is representative of an area of the pancreas performed in ten sections per animal, separated by 200 μ m. $*=p < 0.001$, CNIP cocktail ($n=4$) vs Control cocktail ($n=6$). The histologic analysis was examined in a blinded fashion to the examiner. Data are presented as mean $\pm$ SE. Statistical comparisons were carried out with Student's *t* test.

mice injected with the control placebo (Fig. 4B). We also quantified the number of beta cell clusters (represent 3 to 5 cells connected together and excludes islets) in the pancreas and documented a significant increase in the CNIP group (Fig. 4C). The increase in pancreatic beta cell proliferation and mass was associated with 2.5-fold increase in fasting (overnight) plasma insulin concentration in mice injected with CNIP cocktail compared to the control group (Fig. 5A; $P < 0.01$). HOMA-beta, an index of insulin secretion (22), increased 3.9-fold in CNIP versus control group ($P < 0.001$) (Fig. 5B). Fasting plasma glucose (FPG) concentration was reduced to normal in CNIP versus control group ($P < 0.01$) (Fig. 5A); hypoglycemia (FPG < 70 mg/dl) was not observed in any CNIP treated mouse, indicating that the newly formed beta cells were physiologically responsive to the ambient glucose concentration (Fig. 5A). It is important to emphasize that we validated the CNIP approach in a partially pancreatectomized diabetic wild-type mouse model. This eliminates the confounding variables associated with the use of other mouse models (see Supplementary Data, Wild-type and partially pancreatectomized diabetic mouse model) previously employed to examine beta cell formation *in vivo*. Four-weeks post-injection of CNIP cocktail in partially pancreatectomized diabetic mice, the glucose tolerance test was completely normalized (Fig. 6A) and insulin secretion was restored to normal (Fig. 6B). Most importantly, the fasting plasma glucose concentration remained normal after one year without any FPG measurement less than 70 mg/dl (Fig. 7A). The synergistic effect of the CNIP cocktail to induce pancreatic beta cell formation was documented by the failure of each individual component of the CNIP cocktail to induce beta cell proliferation, as demonstrated with BrdU quantification and failure to increase beta cell mass (Fig. 5C and Fig. 5D). Two molecules in the CNIP cocktail (glucokinase plus shRNA PTP1B) induced some beta cell proliferation and increase in beta cell mass compared to the control group (Fig. 5C and 5D), but the increase was much less than in the complete CNIP cocktail group. Only the CNIP cocktail had an effect to increase fasting plasma insulin and decrease fasting plasma glucose concentrations (data not shown).

We also examined whether a cocktail of transcription factors (1) that target only one level of cellular function, the transcriptional machinery, could induce beta cell formation *in vivo* in the partially pancreatectomized diabetic mouse model. Unlike our CNIP approach, the cocktail containing three transcription factors (PDX-1, Ngn3, MafA) (Fig. 8) (1) failed to induce beta cell formation whatsoever and had no effect on either fasting or postprandial plasma glucose or insulin levels in diabetic mice (Fig. 9).

DISCUSSION AND CONCLUSION

CNIP was designed to induce pancreatic beta cell formation in the adult mouse pancreas and effectively achieved its goal (see Figures 1-7). Insulin secretion by the newly formed beta cells was completely normalized and this resulted in normalization of both the fasting and postprandial blood glucose concentrations (Fig. 6A and 6B) without hypoglycemia for more than one-year post-injection of the CNIP cocktail (Fig. 7A). The CNIP cocktail induced beta cell formation in the absence of injury to the pancreas (Fig. 2, Fig. 3, Fig. 4 and Fig. 5C and 5D), as occurs with streptozotocin or by

surgical manipulations of pancreatic tissue, both of which have been shown to induce beta cell proliferation (2, 23). We compared our CNIP approach with an alternative method which used a cocktail of three transcription factors (TFs) to induce beta cell formation (1). This cocktail of TFs (Ngn3, MafA and PDX-1) (1), when injected *in situ* into the pancreas of an immunodeficient transgenic STZ-diabetic mouse with an adenovirus vector, had a very modest effect to improve glycemia (1). We injected the same cocktail of TFs (Ngn3, MafA and PDX-1) into wild-type, partially pancreatectomized diabetic mice *in vivo* using our pancreatic intra-ductal lentivirus injection method (18) and found no effect on beta cell proliferation or on plasma glucose or insulin levels four-weeks post-injection (Fig. 8 and 9). Our CNIP cocktail and wild-type diabetic mouse model differed significantly from the cocktail of TFs and immunodeficient mouse model used by the previous investigators (1). We used lentivirus instead of adenovirus to express the cocktail of transcription factors. Lentivirus integrates into the genome, whereas adenovirus does not. Lentivirus also has stable, long term gene expression, whereas the adenovirus has transient gene expression. The adenovirus elicits a strong immune response to the viral protein, while the lentivirus does not. By using a lentivirus vector, we were able to utilize a wild-type mouse instead of an immunodeficient mouse, since the adenovirus elicits a strong immune reaction. Further, use of immunodeficient mice introduces confounding effects (inflammation, cell division, spontaneous neoplasia) that enhance cell proliferation and can influence the mechanism of action of the cocktail of TFs (see Supplementary Data, diabetic mouse model). The partially pancreatectomized diabetic wild-type mouse model avoids the confounding effects of the immunodeficient mouse model and the confounding effect of streptozotocin (STZ) to stimulate beta cell proliferation. Importantly, STZ has the potential to induce beta cell proliferation by itself and to amplify any effect of the TFs on beta cell proliferation [23, 24]. STZ also induces expression of the transcription factor Ngn3 in pancreatic tissues [24]. Using a wild-type partially pancreatectomized diabetic mouse with lentivirus gene transfer vector, which more closely approximates the real life situation, we have been unable to demonstrate any effect on beta cell proliferation when only targeting gene expression level with transcription factors (Fig. 8 and 9). Further, four-weeks post-injection of the TFs cocktail, we failed to observe any beneficial effect on fasting or postprandial plasma glucose or insulin levels during an intraperitoneal glucose tolerance test. Collectively, these results demonstrate the advantage of the CNIP approach which integrates three key levels of cellular function: (i) glucose metabolism, (ii) membrane receptor function, (iii) gene expression, implicated in beta cell formation *in vivo*. By targeting all three levels simultaneously, one can generate a synergistic effect that cannot be achieved with any single component of the CNIP cocktail and that results in the desired cellular effect, i.e. beta cell formation (Fig. 5C and 5D).

Interestingly, overexpression of glucokinase with shRNA PTP1B had a modest effect to augment beta cell mass and increase beta cell proliferation compared to the control group (Fig. 5C and 5D). However, only the CNIP cocktail had an effect on fasting plasma insulin and glucose concentrations

Fig. (5). (A) Fasting plasma insulin and fasting plasma glucose concentrations in the adult mouse group injected with CNIP cocktail (Lenti-GCK + Lenti-Pdx-1 + Lenti-shRNA PTP1B) compared to the group injected with the control cocktail (placebo). $\ast = p < 0.01$, CNIP cocktail (n=4) vs Control (n=6). Data are presented as mean $\pm$ SE. (B) Effect of CNIP cocktail treatment on HOMA- β compared to the group injected with the control cocktail. $\ast = p < 0.01$, CNIP cocktail (n=4) vs Control (n=6). (C) BrdU marker of proliferation in islets and exocrine tissue 4 weeks post-injection of the full cocktail (Lenti-GCK + Lenti-Pdx-1 + Lenti-shRNA PTP1B) or by two molecules or each molecule individually. The figure is representative of an area of the pancreas examined in ten sections per animal (control [n=4] and experimental cocktail [n=3]; for all other experimental conditions), separated by 200 μ m. The results are expressed as the fold-increase in number of BrdU-labeled cells compared with controls. Confocal laser microscopy was used for analysis. $\ast = p < 0.001$, CNIP cocktail vs control; $\ast = p < 0.05$, CNIP cocktail vs [GK + PTP1B]; $\# = p < 0.01$ [GK + PTP1B] vs control. The histologic analysis was performed in a blinded fashion to the examiner. Data are presented as mean $\pm$ SE. Statistical comparisons were performed with one-way ANOVA for multiple comparisons. (D) Beta cell mass 4-weeks post-injection with the full CNIP cocktail or by two molecules or each molecule individually compared to the control cocktail and each other (control [n=4] and experimental cocktail [n=3] for all other experimental conditions). Total pancreatic and insulin positive staining areas of each section were measured using Image J (NIH, Bethesda, USA). Beta cell mass was calculated as the ratio of total insulin positive area to total pancreatic area of all sections, multiplied by the pancreatic tissue wet weight. The figure is representative of an area of the pancreas examined in ten sections per animal, separated by 200 μ m. Confocal laser microscopy was used for analysis. $\ast = p < 0.001$, CNIP cocktail vs control; $p < 0.001$, CNIP cocktail vs [GK + Pdx-1]; $p < 0.001$, CNIP cocktail vs [PTP1B + Pdx-1]; $\blacksquare = p < 0.05$, CNIP cocktail vs [GK + PTP1B]. $\# = p < 0.05$ [GK + PTP1B] vs [PTP1B + Pdx-1]; $p < 0.05$ [GK + PTP1B] vs control. The histologic analysis was performed in a blinded fashion to the examiner. Data are presented as mean $\pm$ SE. Statistical comparisons were performed with one-way ANOVA for multiple comparisons.

(Fig. 5). Further, the effect of CNIP cocktail on cell proliferation and beta cell mass was significantly greater compared to that observed with overexpression of GK with shRNA PTP1B. These data suggest that there is a critical threshold that must be exceeded to increase beta cell proliferation and mass in order to enhance insulin secretion and improve glycemic control. This effect on the critical threshold only was reached with the synergistic effect of the full CNIP cocktail. The modest effect of Lentivirus-CMV-GK

combined with Lentivirus -H1-shRNA PTP1B on cell proliferation and beta cell mass could be explained by the effect of the two molecules to increase the cellular glucose metabolic rate [9], which has been shown to induce chromatin remodeling at the binding site of the transcription factor(s) implicated in gene-transcription induced by glucose [25-27]. On the other hand, glucose metabolism has no effect on the activity of membrane tyrosine kinase. This would explain why it is essential to combine the effect of shRNA PTP1B with

Fig. (6). (A) Effect of CNIP cocktail treatment on glucose tolerance test in partially pancreatectomized mice. Two weeks following partial pancreatectomy, the CNIP cocktail was injected as described in methodology section. Before CNIP treatment represents the GTT performed two weeks after partial pancreatectomy. (B) Plasma insulin concentration during GTT in partially pancreatectomized diabetic mice. After CNIP treatment represents mice studied four weeks post CNIP treatment and six weeks after partial pancreatectomy. GTT = Glucose Tolerance Test; * $p < 0.01$ and # $p < 0.05$ Before CNIP treatment vs after CNIP treatment ($n=5$). Data are presented as mean $\pm$ SE. Statistical comparisons were performed with Student's t test.

the increase of glucose signaling to the transcriptional machinery [9, 10] to synergistically induce pancreatic beta cell formation. However, the effect of membrane tyrosine kinase activity on beta cell mass also relies on glucose metabolism. In the absence of glucose metabolism, the ability of hormones acting through the tyrosine kinase family to increase pancreatic beta cell mass is lost [28]. Ultimately, the effect of any approach to induce beta cell formation must be judged by its impact to augment insulin secretion and normalize glucose metabolism. With respect to this, the CNIP approach increased insulin secretion and completely normalized glucose tolerance, whereas the transcription factor cocktail [1] had no effect on either plasma insulin or glucose levels.

As demonstrated with BrdU, cell proliferation observed with CNIP occurred in both acinar and endocrine (islets), but not in ductal, cells. Ductal cells are associated with embryonic development of the pancreas, including endocrine and exocrine tissues [29-31]. This provides further support that

the CNIP cocktail induces post-embryonic developmental formation of beta cells. Consistent with this, we failed to detect expression of any gene involved in pancreatic embryonic development in mice injected with CNIP (Gene name and GeneBank: Ptf1a, NM_018809; Sox9, NM_011448; Hex, NM_008245 and Nr5a2, NM_030676). Our CNIP approach provides a novel approach that overcomes many of the hurdles involved with islet cell transplantation and the stem cell approach to obtain fully differentiated beta cells *in vivo* [32-33], including limited donor islet/stem cell availability and immune/inflammatory reaction to the transplanted islets/stem cells [34-35]. Moreover, it is difficult to target islets/stem cells to the pancreas [36-37] where the secretion of insulin directly into the portal circulation exerts a major regulatory effect to suppress hepatic glucose production [38] without causing peripheral hyperinsulinemia, which causes insulin resistance [39]. Most previous attempts to induce beta cell proliferation have involved injury to the

Fig. (7). (A) Effect of CNIP treatment on fasting plasma glucose in partially pancreatectomized mice. Mice were fasted overnight and the fasting blood glucose concentration was measured. One year post-injection of CNIP cocktail the fasting blood glucose concentration remained normal in partially pancreatectomized mice without hypoglycemia ($n=5$). Data are presented as mean $\pm$ SE. (B) Weight gain is normal in mice following partial pancreatectomy ($n=5$). Data are presented as mean $\pm$ SE. (C) CNIP concept to induce *de novo* beta cell formation in adult mouse pancreas.

Fig. (8). (A and B) We incorporated into our lentiviral vector construct a posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE) at the 3' untranslated region of the coding sequence; this substantially increased the level of expression of the transgene. (C) Western blots of pancreatic tissue four-weeks post-injection with cocktail transcription factors (Lenti-MafA + Lenti-Pdx-1 and Lenti-Ngn3) and cocktail control with antibodies against MafA, Ngn3, and PDX-1. The figure is representative of three to four individual samples in each group.

Fig. (9). (A) Effect of transcription factors (TFs) cocktail (Pdx-1, Ngn3 and MafA) on glucose tolerance test (GTT) in partially pancreatectomized mice. Two weeks following partial pancreatectomy the TFs cocktail was injected as described in the methodology section. Before TFs cocktail injection represents the GTT performed two weeks after partial pancreatectomy. After TFs cocktail injection represents mice studied four-weeks post-injection and six weeks after partial pancreatectomy ($n=5$). (B) Plasma insulin concentration during GTT in partially pancreatectomized mice before and after TFs cocktail injection ($n=5$). (C) Effect of TFs cocktail treatment on fasting plasma glucose in partially pancreatectomized mice. Mice were fasted overnight and the fasting plasma glucose concentration was measured. Mice were tested weekly for 4-weeks post-injection of TFs cocktail, and no decrease in the elevated fasting plasma glucose concentration was observed in the partially pancreatectomized diabetic mice ($n=5$). (D) BrdU marker of proliferation in islets and exocrine tissue 4 weeks post-injection of the cocktail (Lenti-Pdx-1 + Lenti-ng3 + Lenti-MafA). The figure D is representative of an area of the pancreas examined in ten sections per animal ($n=5$ per group), separated by 200 μm . The results are expressed as the fold-increase in number of BrdU-labeled cells compared with controls. Confocal laser microscopy was used for analysis. The histologic analysis was examined in a blinded fashion to the examiner. Before injection of the transcription factors cocktail vs after transcription factors injection ($n=5$). Data are presented as mean $\pm$ SE. Statistical comparisons were performed with Student's *t* test.

pancreas with a chemical agent or ligature of the pancreatic duct, both of which induce a regenerative pathway that recapitulates embryonic development of pancreatic tissues [1, 2, 31, 40]. The pancreatic injury induces expression of genes involved in embryonic development of the pancreas [2, 3]. Thus, these studies do not confirm the presence of stem cells in the pancreas but rather a regenerative pathway similar to the one involved in wound healing [41].

The induction of pancreatic beta cell formation with the CNIP cocktail occurred without injury to pancreatic tissues. Plasma lipase and amylase levels were normal and there was no histologic evidence of inflammation in the pancreas. Most published studies involving pancreatic beta cell formation have employed a chemical agent or surgical manipulation of pancreatic tissues immediately prior to injection of the cocktail of transcription factors [1, 42]. In this case, the synergistic effect of the natural embryonic regenerative pathway and the wound healing pathway following pancreatic injury cannot be excluded as the major mechanism of action of the injected transcription cocktail [41]. For example, there was

no evidence of beta cell proliferation when the transcription factor NeuroD1 was injected 7 days (as opposed to 1 day) after streptozotocin [42], when the inflammatory process had dissipated. From a clinical standpoint, the CNIP cocktail approach has the major advantage of not destroying functioning pancreatic beta cells to induce new beta cell formation, [1, 42]. Further, there is no guarantee that the beta cell destructive approach will work and further loss of beta cells will eventuate in worsening of glycemic control.

The CNIP cocktail did not induce pancreatic ductal cell proliferation, which has been associated with pancreatitis [43]. Since ductal cell proliferation is not observed during the induction of beta cell formation, the CNIP cocktail avoids the risk of pancreatitis. The CNIP cocktail is designed to mimic a natural phenomenon, as occurs in pregnancy, where beta cell mass is increased as a result of placental/fetal development which triggers a response leading to a compensatory change in the mother's physiology [44]. A major advantage of the CNIP cocktail is that only a 20-25% increase in beta cell mass is required as treatment, prevention or cure

for type 1 diabetes, since destruction of >80% of the pancreatic beta cells is required to produce type 1 diabetes [45]. The CNIP approach has the advantage of its simplicity of application to induce pancreatic beta cell formation *in vivo* at the site, i.e. portal circulation, which is essential to restore the normal physiologic regulation of glucose homeostasis. Our results demonstrate that, utilizing the Cellular Networking Integration and Processing (CNIP) approach, we can stimulate pancreatic beta cell formation *in vivo* in adult animals, restore insulin secretion and normalize fasting and postprandial glycemia without hypoglycemia in partially pancreatectomized diabetic mice. In summary, the present results provide a novel approach to the generation of beta cells using the natural physiologic cellular capacity of adult cells in the pancreas to form beta cell *in vivo*. The CNIP cocktail has the potential to be used as a preventative or therapeutic treatment or cure for both type 1 and type 2 diabetes. The lentivirus injection method employed can be used to target beta cell proliferation within pancreatic tissues *in vivo* in man or can be modified by using small molecules or nonviral vectors in the future.

LIST OF ABBREVIATIONS

CNIP	= Cellular Networking, Integration and Processing
CMV	= Cytomegalovirus
FPG	= Fasting plasma glucose
GFP	= Green fluorescent protein
PTP1B	= Protein-tyrosine phosphatase 1B
STZ	= Streptozotocin
TF	= Transcription factor

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Images were generated in the Core Optimal Imaging Facility which is supported by the University of Texas Health Science Center at San Antonio (UTHSCSA), National Institutes of Health (NIH)-National Cancer Institute (NCI) P30 CA54174 (Cancer Therapy & Research Center at UTHSCSA) and NIH-National Institute on Aging (NIA) P01AG19316. We thank Nikhil K. Acharya from the Diabetes Division of UTHSCSA for performing RT-PCR and animal experiments. Dr. Bruno Doiron is the guarantor of this work, and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Part of RAD's salary is supported by the Veterans Administration Health Care System. B.D. initiated and directed the project, developed the CNIP concept, designed and performed the experiments (histology; animal research; molecular analyses), analyzed the data and wrote the manuscript; WH performed the histologic analyses, western blots, animal research, and reviewed the manuscript; R.A.D. was involved in the analysis and interpretation of all physiologic data and writing of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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