Molecular Therapy
Original Article

Lentivirus Mediated Pancreatic Beta-Cell-Specific Insulin Gene Therapy for STZ-Induced Diabetes

Fulya Erendor,[1,4] Yunus Emre Eksi,[1,4] Elif Ozgecan Sahin,[1] Mustafa Kemal Balci,[2] Thomas S. Griffith,[3] and Salih Sanlioglu[1]

[1]Department of Gene and Cell Therapy, Faculty of Medicine, Akdeniz University, Antalya 07058, Turkey; [2]Division of Endocrinology and Metabolism, Department of Internal Medicine, Faculty of Medicine, Akdeniz University, Antalya 07058, Turkey; [3]Department of Urology, School of Medicine, University of Minnesota, Minneapolis, MN 55455, USA

Autoimmune destruction of pancreatic beta cells is the characteristic feature of type 1 diabetes mellitus. Consequently, both short- and intermediate-acting insulin analogs are under development to compensate for the lack of endogenous insulin gene expression. Basal insulin is continuously released at low levels in response to hepatic glucose output, while post-prandial insulin is secreted in response to hyperglycemia following a meal. As an alternative to multiple daily injections of insulin, glucose-regulated insulin gene expression by gene therapy is under development to better endure postprandial glucose excursions. Controlled transcription and translation of proinsulin, presence of glucose-sensing machinery, prohormone convertase expression, and a regulated secretory pathway are the key features unique to pancreatic beta cells. To take advantage of these hallmarks, we generated a new lentiviral vector (LentiINS) with an insulin promoter driving expression of the proinsulin encoding cDNA to sustain pancreatic beta-cell-specific insulin gene expression. Intraperitoneal delivery of HIV-based LentiINS resulted in the lowering of fasting plasma glucose, improved glucose tolerance and prevented weight loss in streptozoticin (STZ)-induced diabetic Wistar rats. However, the combinatorial use of LentiINS and anti-inflammatory lentiviral vector (LentiVIP) gene therapy was required to increase serum insulin to a level sufficient to suppress non-fasting plasma glucose and diabetes-related inflammation.

INTRODUCTION

Insulin deficiency manifested following the loss of pancreatic beta cell function is a common feature of both type 1 (T1DM) and type 2 diabetes mellitus (T2DM).[1] T1DM patients require daily insulin injections because of the destruction of pancreatic beta cells by autoreactive T cells.[2] Moreover, glucose intolerance and a decrease in pancreatic islet cell mass also cause hypoinsulinemia as observed in T2DM. For these reasons, both T1DM and T2DM patients eventually require insulin therapy to compensate for the reduced/loss of insulin production.[3] Exogenous insulin therapy postpones the macrovascular (coronary artery disease, peripheral artery disease, and stroke) and microvascular (diabetic nephropathy, neuropathy, and retinopathy) complications of diabetes, but there is a lack in clinical consensus on how to start and continue insulin regimens in diabetic patients.[4,5] The basal insulin secretion profile cannot be preserved using high dose administration of insulin analogs because of the risk of hypoglycemia.[6] Alternatively, hyperglycemia is inevitable when these agents are used at doses inadequate for 24 h insulin coverage.[7] Adding metabolic variability into the equation, it becomes even harder to manage postprandial glucose excursions using commercial insulin preparations.[8] Despite the long-term benefit of intensive insulin therapy, there is still a risk of developing severe hypoglycemia in T1DM patients.[9]

It is noteworthy to mention that exogenous insulin administration cannot mimic the endogenous insulin secretion profile of pancreatic beta-cells,[10] largely because the insulin released from the pancreas travels directly to the liver through the portal vein. By doing so, the liver receives 60% of the pancreatic insulin discharge. Conversely, subcutaneously injected insulin is distributed throughout the body before it reaches the liver. In this case, the liver remains under insulinized. Because physiologic insulin secretion consists of basal insulin production in addition to postprandial bursts in output, a combination of the rapid-acting insulin needed with each meal and basal insulin (basal-bolus therapy) seems to be the preferred choice for the treatment of T1DM patients.[11] Even with numerous fast/long-acting insulin analogs, it remains difficult to achieve optimal glycemic control in T1DM patients which increases the danger of developing diabetes-related complications over time. Furthermore, C-peptide—a by-product of proinsulin processing—has exhibited some beneficial effects in diabetic patients,[12] but regular human insulin and insulin

Received 4 June 2020; accepted 23 October 2020;
https://doi.org/10.1016/j.ymthe.2020.10.025.

[4]These authors contributed equally to this work

Correspondence: Salih Sanlioglu, VMD, PhD, The Department of Gene and Cell Therapy, Faculty of Medicine, Akdeniz University, Antalya 07058, Turkey.
E-mail: ssanlioglu@icloud.com

analogs do not contain the C-peptide. Consequently, there is an ongoing discussion on whether the C-peptide should be included in insulin regimens and the fact that T2DM patients unpredictably exhibited high levels of C-peptide in their blood further heated the debate.[13] The C-peptide is now considered to be more than an insulin indicator and a therapeutic agent with the potential to improve kidney function, nerve function, and blood flow to vital organs.[14] Additionally, C-peptide is involved in the activation of the GLUT1 transporter leading to glucose clearance via ATP release from red blood cells.[15]

Considering all these circumstances, insulin gene therapy is a viable alternative to exogenous insulin administration and has the potential to compensate for insulin deficiency and C-peptide levels by a single injection in diabetic patients.[16,17] In this scenario, recombinant insulin expressed from the gene therapy vector must be released quickly from pancreatic beta cells upon stimulation to manage postprandial glucose levels. Insulin must also be stored in secretory granules until their release since the physiologically controlled insulin production necessitates the regulation of insulin secretion. Insulin is normally released within minutes after beta-cell stimulation through the discharge of secretory granules already present in the cytoplasm. Thus, target cell choice is essential for the success of insulin gene therapy in diabetes.

Pancreatic beta cells possess unique insulin synthesis and secretion features compared to other cell types, including the ability for controlled transcription and translation of proinsulin, presence of a regulated secretory pathway, and inductive secretion.[18] Interestingly, hepatocytes are one of the few cell types with glucose sensors (*Glucokinase* [GK] and GLUT2), but they lack a regulated secretory pathway. Because hepatocytes constitutively secrete proteins, an instant release of insulin from the liver is not achievable to manage meal-related hyperglycemia.[19] Despite having a regulated secretory pathway and insulin secretory granules, neuroendocrine cells lack glucose-sensing machinery.[20] Hence, neither neuroendocrine cells nor hepatocytes can function as beta-cell surrogates. K cells of duodenum and jejunum express GK, GLUT2, and prohormone convertase, just like pancreatic beta cells,[21] suggesting them as ideal targets for glucose-controlled insulin gene expression and secretion.[22,23] Considering the rapid turnover of the intestinal epithelial cells and the scarcity of enteroendocrine cells, the potential of the K cell-targeted gene therapy remains limited.[24] In addition to all the requirements mentioned above, the expression of glucagon-like peptide 1 (GLP-1) receptors essential to control postprandial insulin release is needed in a beta cell surrogate.[25,26] Neuronal inputs from pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) might be required to control glucose metabolism under metabolic stress.[27]

Insulin gene therapy requires a vector for effective gene delivery to the appropriate target cell population. While both viral and non-viral vectors have been tested for the treatment of T1DM and T2DM,[28] viral vectors are undoubtedly superior compared to non-viral vectors in delivering genes to target cells.[29,30] Among the viral vectors used, lentiviral vectors are becoming popular gene delivery vehicles used in clinical trials.[31–34] Currently, lentiviral vector-mediated therapeutic gene delivery is already underway for the treatments of both T1DM[35,36] and T2DM.[37,38] Recent studies concerning liver-directed gene therapy revealed that basal insulin requirements for T1DM could be met using lentivirus-mediated furin-cleavable insulin gene delivery to hepatocytes.[39,40] This approach was aimed at obviating the need for long-acting insulin analogs in diabetic patients. However, the use of constitutive promoters (e.g., CMV) to drive insulin gene expression in targeted tissues would make the construct unresponsive to the fluctuations in blood glucose levels and predispose the host to developing hypoglycemia. Considering the facts listed above, we chose to test the feasibility of targeting pancreatic beta cells via lentiviral vectors carrying an insulin promotor driving the proinsulin gene sequence to manage daily blood glucose levels and decrease the need for exogenously delivered insulin. Interestingly, we found it necessary to include anti-inflammatory and insulinotropic supplementary gene therapy to deal with diabetes-related inflammation. Our data is described herein, and the clinical implications of our approach are discussed.

RESULTS

The Generation of the LentiINS Vector to Drive Insulin Gene Expression Exclusively in Pancreatic Beta Cells

The transfer plasmid carrying insulin promoter connected to the insulin gene (pLentiINS) was generated using Invitrogen's MultiSite Gateway Technology as described in Materials and Methods. The insulin promoter was synthesized as a linear double-stranded DNA (gBlocks) by Integrated DNA Technologies (Iowa City, IA, USA), then cloned into an entry vector (pENTR5′Ip) using pENTR 5′-TOPO-TA Cloning Kit. A MultiSite Gateway LR recombination reaction was carried out using the promoter entry vector (pENTR5′Ip), insulin entry vector (pENTRIns), and a destination vector (pLENTİ6.4/R4R2/V5-DEST) in combination. pLentiINS expression clone was generated using Virapower Hiperform Promoterless Gateway Expression Kit (Figure 1A). pLentiINS plasmid possessed an insulin promoter fused to the insulin gene in addition to the lentiviral vector backbone. *AflII* and *XhoI* restriction enzyme analysis were performed for the diagnostic purposes to pick up the right clones as shown in Figure 1B. Orientation and the sequence identity were confirmed by DNA sequencing (data not shown). Three packaging plasmids (gag/pol [pMDLg/pRRE (HIV-1 pGag-Pol 12251), rev (pRSV-Rev 12253) and vesicular stomatitis virus G glycoprotein (VSV-G; pMD2.G [pVSV-G 12259])]) were obtained from the Addgene plasmid repository (Figures 1C and 1D) and used in transient calcium phosphate transfection with pLentiINS plasmid in 293T cells to produce lentiviral vectors carrying insulin gene (LentiINS) as described previously.[41]

LentiINS Gene Therapy Vector Directed Insulin Gene Expression Exclusively in Pancreatic Beta Cells but not in 293T Cells

One objective of this study was to produce a gene therapy vector in which insulin gene expression could be restricted to pancreatic beta cells. Using the methodology described, we engineered the LentiINS vector, after which the vector was titered by qPCR following CsCl concentration and purification using ion-exchange chromatography.[37,41,42] To confirm insulin gene expression from the newly

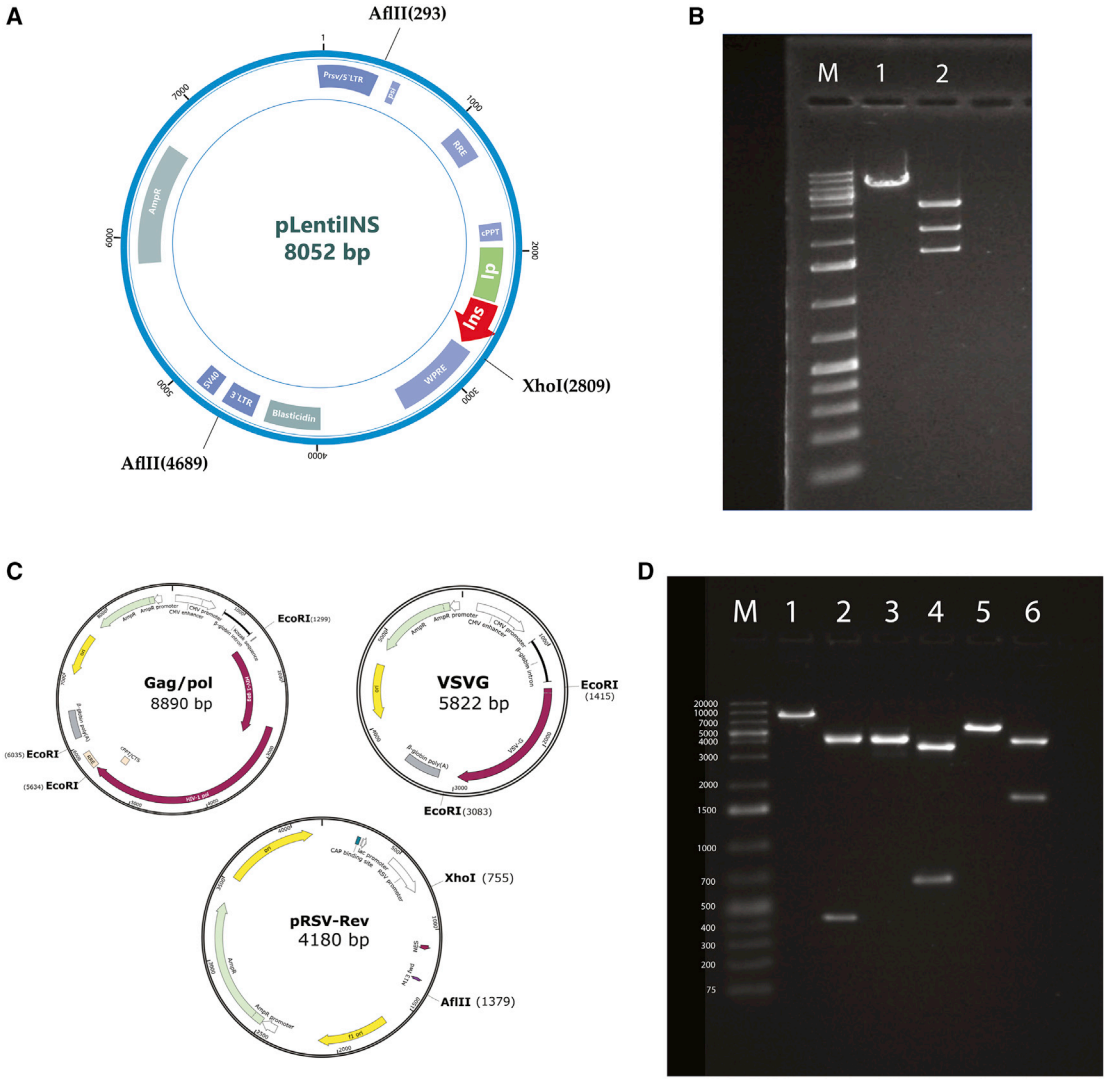


Figure 1. Construction of the pLentiINS Transfer Vector

(A) The insulin promoter was cloned into an entry vector using pENTR 5′-TOPO-TA Cloning Kit generating pENTR5′Ip. Both the insulin gene from pENTRIns plasmid and the insulin promoter from the pENTR5′Ip vector were translocated into the destination vector (pLenti6.4/R4R2/V5-DEST) in a MultiSite Gateway LR recombination reaction to yield pLentiINS. (B) *AflII* and *XhoI* restriction enzyme analysis of pLentiINS vector. Lanes M, MW marker; 1, *XhoI* linearized; 2, pLentiINS vector cut with *XhoI* and *AflII* enzymes. (C) Maps of packaging plasmids (gag/pol [pMDLg/pRRE (HIV-1 pGag-Pol 12251), rev (pRSV-Rev 12253) and VSV-G (pMD2.G [pVSV-G 12259])]) used in the transfection to generate lentivirus vector. (D) Restriction enzyme analysis of packaging constructs for confirmation before the transfection. Lanes M, MW marker; 1, *AflII* Linearized pGag-Pol; 2, pGag-Pol cut with EcoRI; 3, *XhoI* Linearized pRSV-Rev; 4, pRSV-Rev cut with *XhoI* and *AflII*; 5, *NotI* Linearized pVSV-G; 6, pVSV-G cut with EcoRI.

generated LentiINS vector, we transduced NIT-1 and 293T cells with increasing doses of LentiINS (Figure 2). We observed a dose-dependent increase in insulin protein in the culture medium of NIT-1 cells 72 h after LentiINS vector transduction (Figure 2A). In contrast, no insulin protein was detected in the culture medium of 293T cells infected with LentiINS (Figure 2B). The fact that the LentiINS vector provided insulin expression in the NIT-1 pancreatic beta-cell line, but not in the human embryonic kidney 293T cells, revealed cell line-restricted gene expression properties of the LentiINS vector. Furthermore, glucose responsiveness of LentiINS vector was tested in MIN6 pancreatic cell line (Figure 2C). LentiINS-infected pancreatic beta cell line yielded significant levels of insulin secretion only at high glucose concentration compared to LentiLacZ-infected cells.

Testing the *In Vivo* Therapeutic Efficacy of LentiINS Vector in a Rat Model of STZ-Induced Diabetes

Streptozotocin (STZ; 30 mg/kg intraperitoneal [i.p.]) was injected into Wistar rats for 5 consecutive days to destroy pancreatic beta cells and induce hyperglycemia. Blood glucose was measured on days 3 and 5 after the last STZ injection to confirm the establishment of

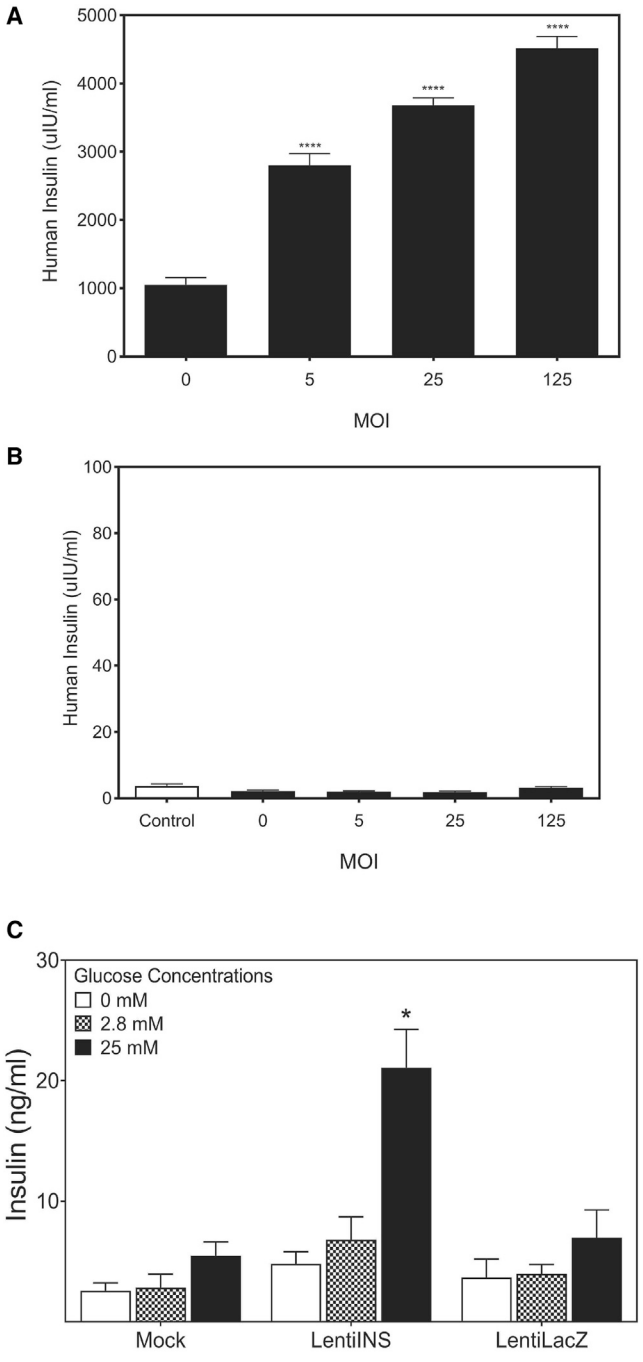


Figure 2. Cell-Type-Restricted Insulin Gene-Expression Profile of the LentiINS Vector

(A and B) Insulin gene expression levels from the LentiINS vector transduced (A) NIT-1 or (B) 293T cell lines as revealed by ELISA (n = 4, one-way ANOVA, $p < 0.0001$). (C) Glucose responsiveness of LentiINS (MOI = 5) or LentiLacZ (MOI = 5) vectors in MIN6 cell line as determined by insulin ELISA (n = 4, two-way ANOVA, $p < 0.0001$). Insulin levels in the culture supernatants were detected using the Insulin Human ELISA kit.

diabetes, and the rats were then divided into two groups to receive either LentiINS or LentiLacZ (10^{10} TU/rat) therapy. Blood glucose levels were then measured periodically. Neither non-fasting or fasting blood glucose levels of LentiLacZ-injected rats returned to normal during the subsequent 5 weeks of monitoring (Figure 3). Experiments on LentiLacZ-injected rats had to be terminated 5 weeks after the gene delivery to prevent animal suffering from prolonged diabetes. In contrast to the LentiLacZ-treated rats, LentiINS injection into diabetic rats lowered fasting blood glucose levels 1 week after gene delivery and remained normal during the entire monitoring period. No sign of hypoglycemia was observed in LentiINS-injected rats; however, the therapeutic efficacy of LentiINS in regard to concerning non-fasting blood glucose levels in diabetic rats appeared to be transient lasting only 3 weeks. Regardless, non-fasting blood glucose levels of LentiINS injected rats were lower than that of LentiLacZ-injected diabetic rats.

Combinatorial Use of LentiINS and LentiVIP Vectors (LentiINSVIP) Was More Effective in Reducing Non-Fasting Blood Glucose than Their Individual Application Alone

We next tested the extent to which antiapoptotic/insulinotropic gene delivery could complement the insulin gene therapy approach to prevent pancreatic beta-cells from degradation and also further boost insulin release from pancreatic beta cells. Antiapoptotic and insulinotropic functional properties of VIP have recently been demonstrated using a lentiviral vector (LentiVIP) in the animal model of T2DM.[38] To test these vectors in combination for STZ-induced diabetes, LentiINS and LentiVIP gene therapy vectors (LentiINSVIP) were delivered to diabetic Wistar rats to assess the efficacy of the combinatorial gene therapy approach in reducing blood glucose. Despite the significant reduction in non-fasting blood glucose of diabetic rats that received LentiVIP alone (compared to LentiLacZ-injected rats), concurrent administration of LentiINS and LentiVIP vectors were more effective than their individual injections (Figure 4A). The difference between LentiINSVIP and LentiVIP gene delivery on blood glucose levels become significant 2 weeks after the injections and continued throughout the entire monitoring period lasting 5 months.

One of the characteristic features of T1DM is the weight loss observed in diabetic patients. Similarly, as LentiLacZ-injected STZ-induced diabetic rats continued to lose weight, LentiINS-injected rats progressively gained body weight (Figure 4B). LentiVIP-injected rats also gradually gained body weight, but the most significant weight gain was observed in LentiINSVIP injected animals. Collectively, the data in Figure 4 demonstrate the additional therapeutic benefit of combining VIP gene transfer (using LentiVIP) with LentiINS to reduce blood glucose levels and promote weight gain in STZ-induced diabetic rats.

Improved Glucose Tolerance Correlates with Higher Levels of Serum Insulin in LentiINSVIP Injected Rats

While it was important to show LentiINS (or LentiINSVIP) could restore normoglycemia in STZ-induced diabetic rats, we also wanted to see how this gene therapy approach affected the ability to respond

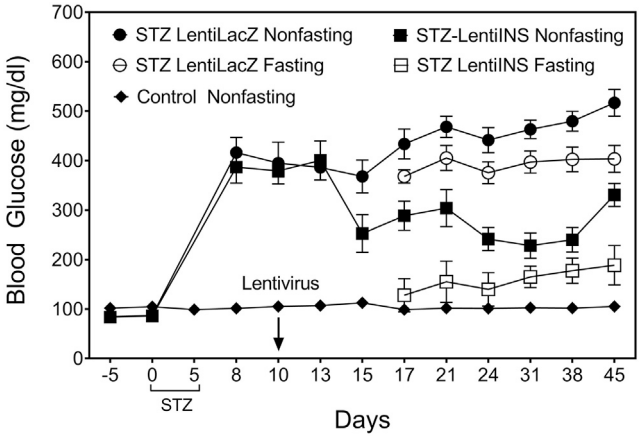


Figure 3. Therapeutic Efficacy of Lentivirus-Mediated Insulin Gene Delivery in Diabetic Rats

After the confirmation of diabetes, Wistar rats were injected either with LentiINS or LentiLacZ vectors (10^{10} TU i.p./rat) 5 days after the last STZ injection. Blood glucose measurements were performed periodically (n = 8/group, two-way ANOVA, LentiLacZ versus LentiINS $p < 0.0001$). Control rats received neither STZ nor gene delivery.

to a sudden rise in glucose. Thus, a glucose tolerance test was performed in diabetic rats 5 weeks after gene delivery (Figure 5A). As LentiLacZ-injected diabetic rats failed to clear blood glucose, LentiINS-injected rats displayed improved glucose tolerance. These data suggest insulin gene delivery facilitate glucose entry into peripheral tissues. Similarly, LentiVIP-injected rats also displayed improved glucose tolerance; however, no significant difference in glucose tolerance was observed between LentiINS- and LentiVIP-treated rats. Furthermore, the efficacy of glucose clearance was much greater in LentiINSVIP injected animals, suggesting LentiINSVIP-injected rats manifested a greater degree of glucose tolerance compared to LentiINS- or LentiVIP-injected rats alone. We also checked serum insulin levels 5 weeks after the gene delivery. As expected, serum insulin levels of LentiLacZ-injected diabetic rats were low correlating with hyperglycemia (Figure 5B). Serum insulin levels of both LentiINS and LentiVIP injected rats were higher compared to the LentiLacZ-injected control rats, but the differences in LentiINS and LentiVIP augmented insulin levels were not statistically significant from each other. Interestingly, LentiINSVIP-injected rats displayed the highest serum insulin levels among the groups tested. Our results suggest serum insulin levels positively correlated with glucose clearance efficiency of lentivirus vectors encoding insulin and VIP in STZ-induced diabetes.

LentiINS Directed Insulin Gene Expression Is Exclusively Limited to Pancreatic Islets

The data showing LentiINSVIP can reduce blood glucose levels, increase glucose tolerance, and generate high level of circulating insulin led us to question role of pancreatic beta cells in this gene therapy approach for treating T1DM. To reveal pancreatic beta-cell status, rats were sacrificed 5 weeks after the gene delivery, and intraabdominal organs were dissected for analysis. After formalin fixation and paraffin embedding, tissue sections were immunostained using insulin antibody (Figure 6). As expected, a faint insulin signal was detected in the pancreatic islets of LentiLacZ-injected rats, suggesting these islets were destroyed following STZ injection and correlating with higher levels of blood glucose. In contrast, pancreatic islets of LentiINS- or LentiVIP-injected rats appeared to be protected from destruction and/or regenerated. Stronger insulin staining and enlarged islets were detected in LentiINSVIP-injected rats, correlating with the higher levels of serum insulin and lower blood glucose. The fact that insulin gene expression was only apparent in pancreatic islets but not in other intraabdominal organs analyzed (e.g., liver, spleen, and kidney) in LentiINS- and LentiINSVIP-injected animals suggested the expression of the LentiINS transgene was restricted to pancreatic islets.

Insulin and VIP Gene Expressions Stimulated Proliferation of Pancreatic Islet Cells in STZ-Induced Diabetic Rats

Our data showing LentiINSVIP restoration of blood glucose levels and glucose tolerance was the expected outcome of this gene therapy approach; however, our histological analysis of pancreatic tissue also revealed some unexpected finding that intrigued us further. Ki67 immunostaining revealed that LentiLacZ-injected diabetic rats displayed a lower proliferation rate in islets compared to control animals (Figure 7A). Conversely, LentiINS- and LentiVIP-injected rats manifested similar cellular proliferation rates in pancreatic islets compared to PBS injected control animals, and the islet cell proliferation rate in LentiINSVIP-injected rats was even higher than those of healthy controls, LentiINS-, or LentiVIP-injected rats. To reveal the intraabdominal tissue distribution profile of our lentiviral vectors, pancreas, liver, spleen, and kidney tissue samples were analyzed to determine the number of integrated lentiviral vector copies. Based on our analysis, a significant number of LentiINSVIP vectors were integrated into the intraabdominal organs analyzed (Figure 7B). Among the tissues analyzed, the highest amount of lentivirus vectors was found in the liver. A similar tissue distribution profile with LentiINS and LentiVIP vectors was detected compared to LentiINSVIP injected rats (data not shown). Together, these data suggest pancreatic islets are maintained/regenerated after LentiINSVIP administration and lentiviral integration occurs in multiple tissues—not just the pancreas.

Insulinotropic and Anti-Inflammatory Gene Delivery Reduces Inflammation during STZ-Induced Diabetes

Serum C-reactive protein (CRP) levels are elevated in patients with impaired glucose tolerance (IGT)[43] or diabetes.[44] To document the influence of insulin and VIP gene delivery on inflammation, we sacrificed STZ-treated rats 5 weeks after gene delivery and blood sera were analyzed for CRP levels (Figure 8A). As expected, LentiLacZ-injected diabetic rats displayed higher levels of serum CRP compared to other groups. LentiINS injection lowered serum CRP levels, but the most significant reduction in serum CRP was obtained after administration of LentiVIP or LentiINSVIP vectors. These data suggest the LentiVIP or LentiINSVIP vectors are effective in suppressing diabetes-induced inflammation. Because of

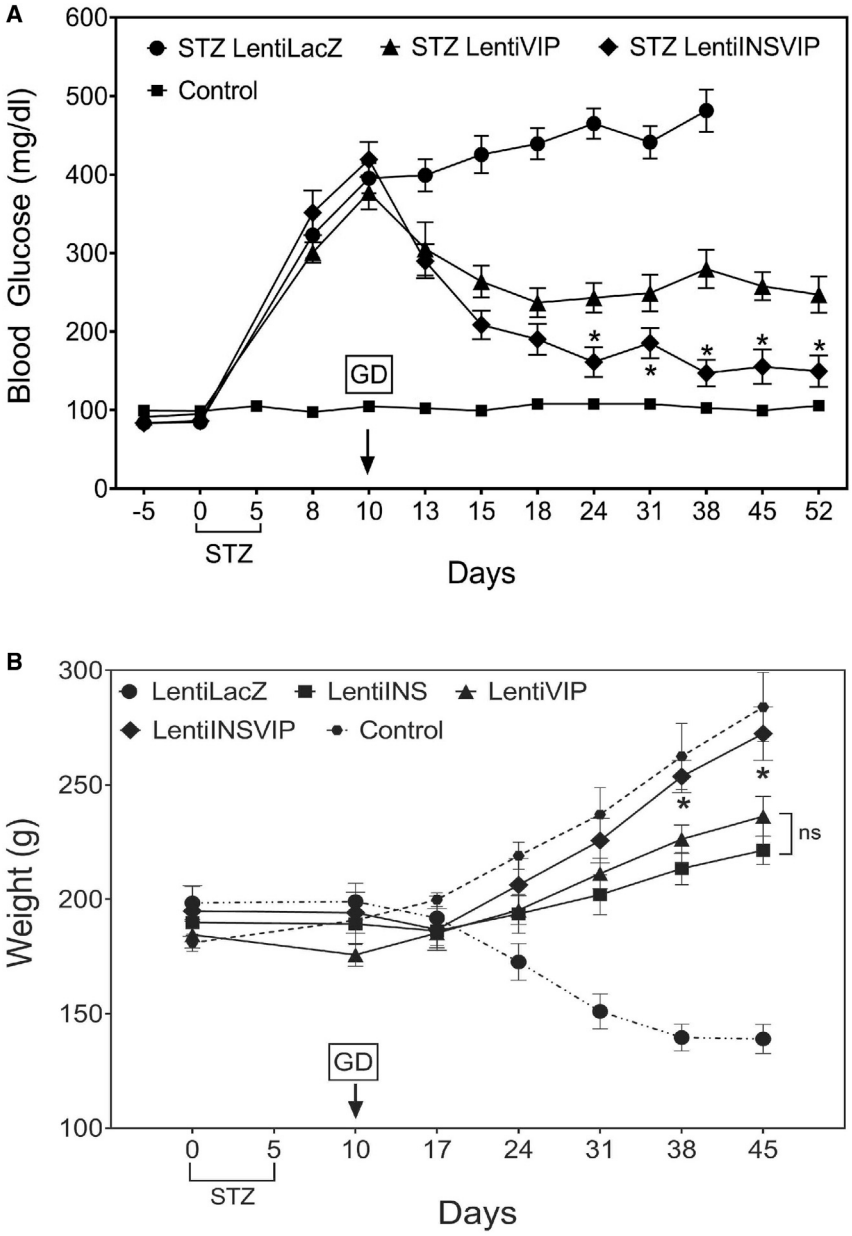


Figure 4. *In Vivo* Efficacy of Insulin and Anti-inflammatory/Insulinotropic Gene Delivery for Diabetes

(A) Co-administration of LentiINS and LentiVIP (LentiINS-VIP) is more effective in reducing non-fasting blood glucose of diabetic animals than individual lentiviral vectors (n = 8/group, two-way ANOVA, *p < 0.05 between LentiVIP and LentINSVIP groups; GD, Gene Delivery). LentiINS, LentiVIP, and LentiINSVIP injections but not LentiLacZ injection prevented weight loss caused by STZ injections. (B) Two-way ANOVA, *p < 0.05 between LentINSVIP and LentiINS or LentiVIP injected groups; GD, Gene Delivery; ns, not significant). Control rats received neither STZ nor gene delivery.

chronic hyperglycemia in diabetic patients, the production of oxygen free radicals is increased through glucose autooxidation and nonenzymatic glycation. A previous study conducted in young diabetic patients demonstrated systemic oxidative stress is present upon the early onset of T1DM and is increased by early adulthood.[45] In this particular study, the decreased antioxidant defense was blamed for the increase in the susceptibility of diabetic patients to oxidative injury. To reveal the influence of insulin and VIP gene expression on oxidative stress, we compared serum oxidant and antioxidant capacities. LentiLacZ-injected diabetic rats exhibited higher levels of oxidant capacity while it was normalized in LentiINS- and LentiVIP-injected rats (Figure 8B). Interestingly, the reduction in oxidant levels was greatest in LentiINSVIP-injected rats. Analysis of antioxidant capacities of animals revealed LentiLacZ-injected diabetic rats displayed reduced antioxidant capacity while it was restored to normal in LentiINS-, LentiVIP-, and LentiINSVIP-injected rats (Figure 8C).

DISCUSSION

The main purpose of insulin gene therapy is to transform non-beta cells into a beta-cell-like compartment that can synthesize, store, and secrete insulin just like pancreatic beta cells.[16] In this scenario, the use of non-beta cells is considered to be advantageous since they are expected to be resistant to the beta-cell-specific autoimmune attack present in patients with T1DM.[21] In addition to all these prerequisites, the outcome of insulin gene delivery relies on several additional features necessary for a gene therapy approach to succeed. First, efficient therapeutic protein synthesis requires effective gene delivery to the target organ. Then, the surrogate organ is expected to have enzymes needed for glucose sensing (e.g., GLUT2 and GK) and bioprocessing (e.g., prohormone convertases). More importantly, the presence of a regulated secretory pathway with some degree of storage is essential for the release of therapeutic protein upon stimulation. Finally, insulin transgene expression should be induced by hyperglycemia and repressed by fasting hypoglycemia.

One of the goals of our study was to develop a stable and safe means of expressing an exogenous insulin gene *in vivo*. Expectedly, genome-integrating vectors have a better chance of providing stable gene expression than non-integrating vectors. Transduction of both dividing and non-dividing cells, the lack of immune response and integration-induced mutagenesis upon viral entry/integration made the lentivirus vector of choice for gene delivery.[46,47] Third generation

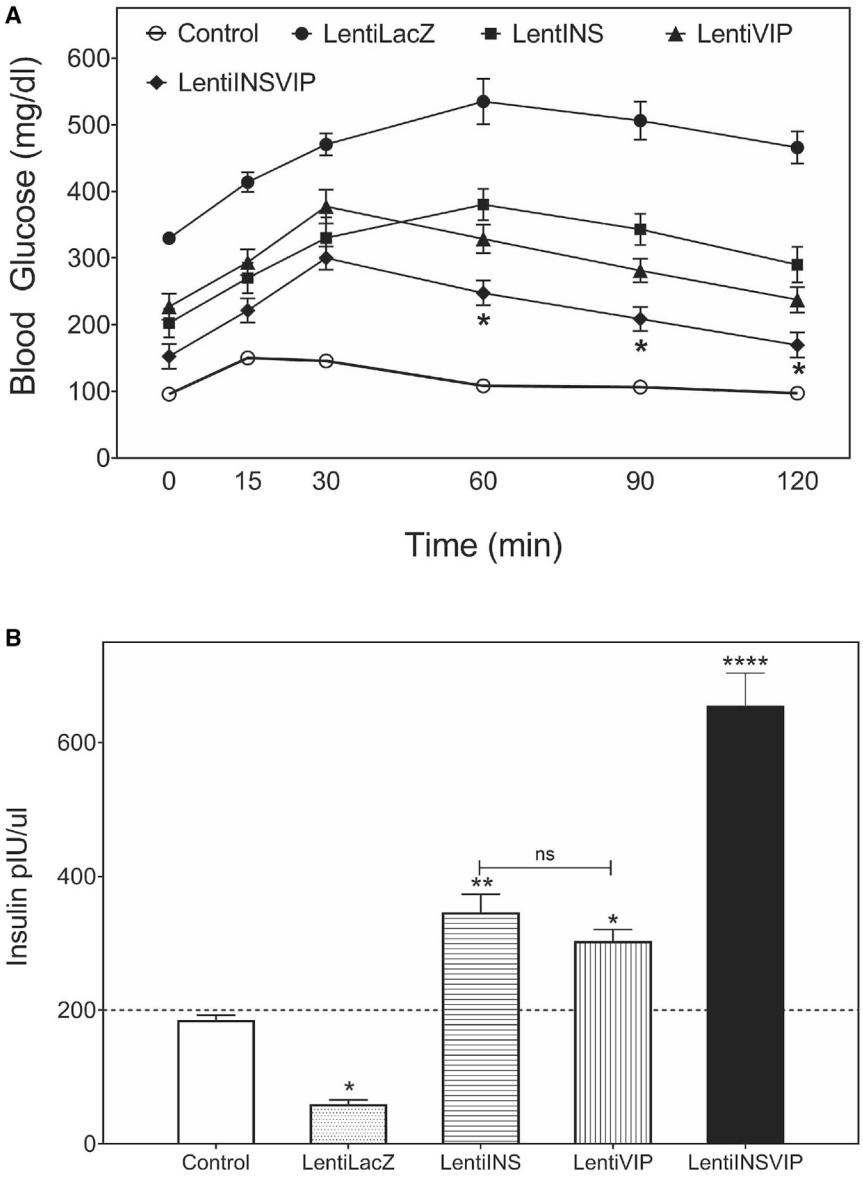


Figure 5. Glucose Clearance Profile and Serum Insulin Levels in Diabetic Rats Treated with Various Gene Therapy Vectors

(A) i.p. glucose tolerance tests following LentiLacZ, LentiINS, LentiVIP, and LentiINSVIP vector delivery conducted 5 weeks after the gene delivery. An asterisk (*) indicates the statistical significance of glucose clearance profile of LentiINSVIP-injected animals compared to LentiINS and LentiVIP injected animals at 60, 90, and 120 min after the glucose administration (n = 8/group, two-way ANOVA; LentiINS versus LentiVIP not significant). (B) Serum insulin profile 5 weeks after the gene delivery (n = 8/group, ordinary one-way ANOVA, Holm-Sidak's multiple comparison test, control versus LentiLacZ, p = 0.034; control versus LentiINS, p = 0.004; control versus LentiVIP p = 0.034; control versus LentiINSVIP $p < 0.0001$; ns = not significant). Control refers to non-diabetic animals.

lentiviral vectors[48] self-inactivate themselves following insertion into the host genome and vector mobilization is not seen even after the infection with wild-type HIV.[32] In this study, a lentiviral vector pseudotyped with VSV-G was employed to increase vector tropism and stability.[49]

For the reasons outlined above, pancreatic beta cells were chosen as target cells. To accomplish this targeting, pancreatic beta-cell specific insulin gene expression was required to prevent insulin gene expression in non-beta cells. Moreover, a glucose-responsive promoter was desired to prevent blood glucose fluctuations leading to hypoglycemia when fasting or hyperglycemia upon feeding. One of the favorite glucose-controlled promoters is undoubtfully the insulin promoter in which the expression is tightly controlled by the amount of glucose present in the blood.[50] For this reason, an insulin-encoding lentiviral vector under the control of an insulin promoter (LentiINS) was generated. LentiINS-directed insulin gene expression was only observable in NIT-1 cells, but not in 293T human embryonic kidney cells. The presence of insulin gene expression in pancreatic beta cells, but absence in non-beta cells, suggested LentiINS vector driven insulin gene expression exhibited some tissue preference. Then, the therapeutic efficacy of LentiINS for T1DM was tested in a rat model of STZ-induced diabetes. Both fasting and non-fasting plasma glucose levels of LentiINS-injected rats were reduced significantly compared to LentiLacZ-injected diabetic rats. However, the efficacy of the LentiINS vector-mediated reduction in non-fasting plasma glucose levels lasted only 3 weeks. These data are in accordance with previous studies in which glucose-responsive promoters were not as effective as desired in responding to blood glucose fluctuation.[51–53] Nevertheless, use of a glucose-responsive promoter prevented the manifestation of insulin-related hypoglycemia in our study.

Insulin analogs cannot cure diabetes; instead, they delay the appearance of diabetic complications.[10] This limitation is mainly due to the nature of insulin analogs and route of insulin delivery. Thus, a glucose-regulated insulin gene therapy approach targeting pancreatic beta cells is expected to better mimic endogenous insulin synthesis and secretion profile compared to exogenously injected insulin. In reality, a complete cure requires not only the compensation of insulin deficiency, but also the suppression of the ongoing autoimmunity observed in T1DM patients. Unfortunately, due to its cost and side-effects, immunosuppressive drugs are not recommended to diabetic

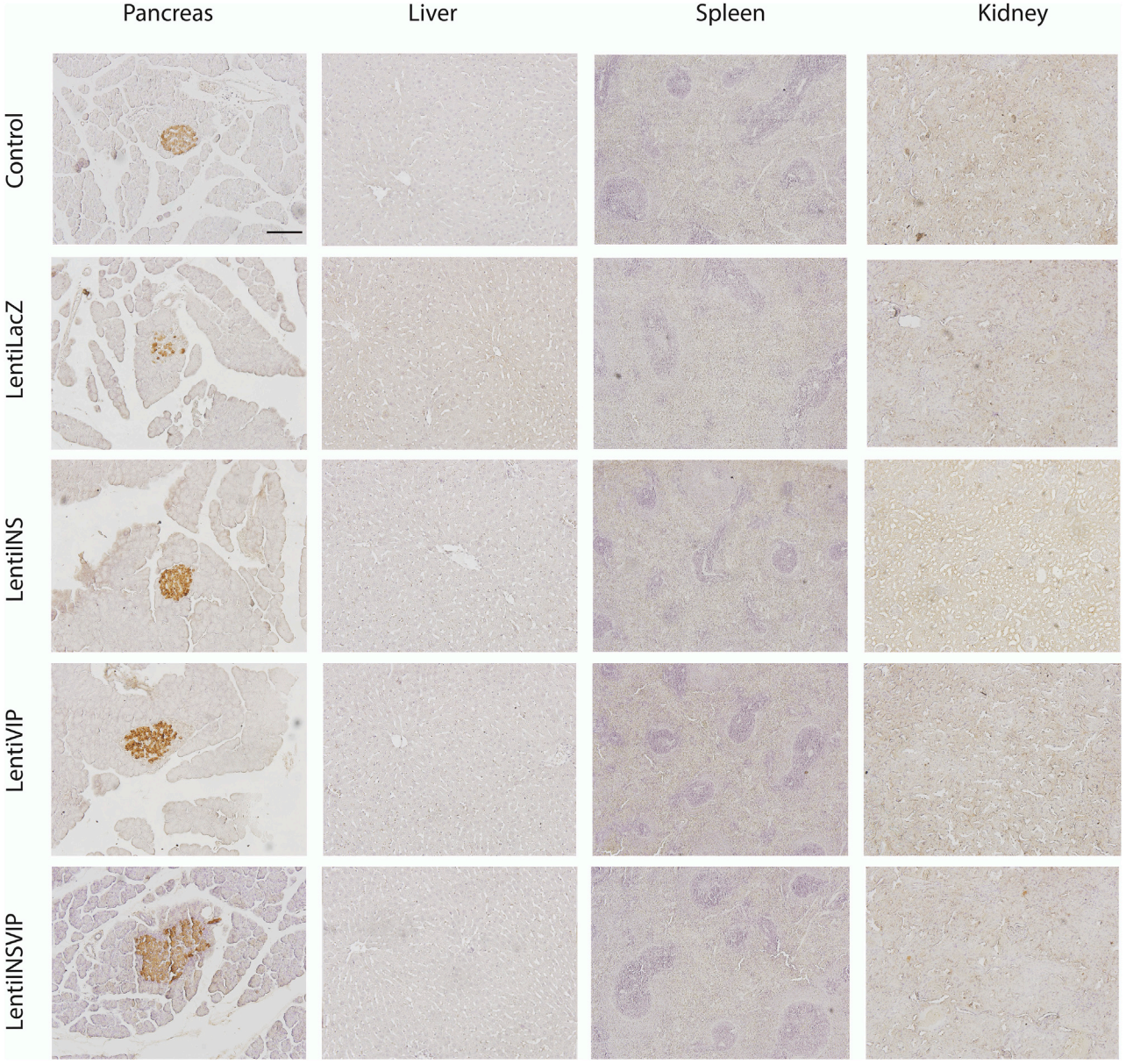


Figure 6. Insulin Immunostaining of Tissue Sections Obtained from Various Intraabdominal Organs following Gene Delivery (n = 6)

Gene therapy vector types are given on the left side of each panel. Intraabdominal tissues used in the analysis are listed above the panels. Immunohistochemistry analysis was performed on paraffin-embedded tissues using an anti-insulin antibody. Scale bar, 100 μm.

patients. Therefore, we hypothesized that if we could suppress diabetes-related inflammation and further boosted insulin gene expression by complementary gene therapy, we would be able to increase the therapeutic efficacy of LentiINS in a rat model of diabetes. VIP exhibits anti-inflammatory and insulinotropic properties.[54] However, it was unknown whether VIP could induce pancreatic beta cell proliferation following STZ destruction of pancreatic islets.[27] The therapeutic effect of VIP either in peptide form or a plasmid has already been tested in non-obese diabetic (NOD) mice in two similar studies. For example, plasmid delivery of VIP encoding gene decreased the incidence of cyclophosphamide (CP)-accelerated diabetes (CAD) in NOD mice by way of shifting proinflammatory cytokine profile (T helper 1 [Th1]) to an anti-inflammatory type (Th2).[55] In another study, i.p. injection of 2.5 nmoles VIP/animal every other day reduced the severity of insulitis and prevented development of diabetes in NOD mice.[56] The suppression of Th1 cytokines, activation of regulatory T cells, and increased interleukin-10 (IL-10) synthesis were all blamed for the observed phenotype. In this study, VIP injection drastically decreased the expression of T-bet (Th1 lineage-specific transcription factor) and increased the expression of the GATA-3 (Th2

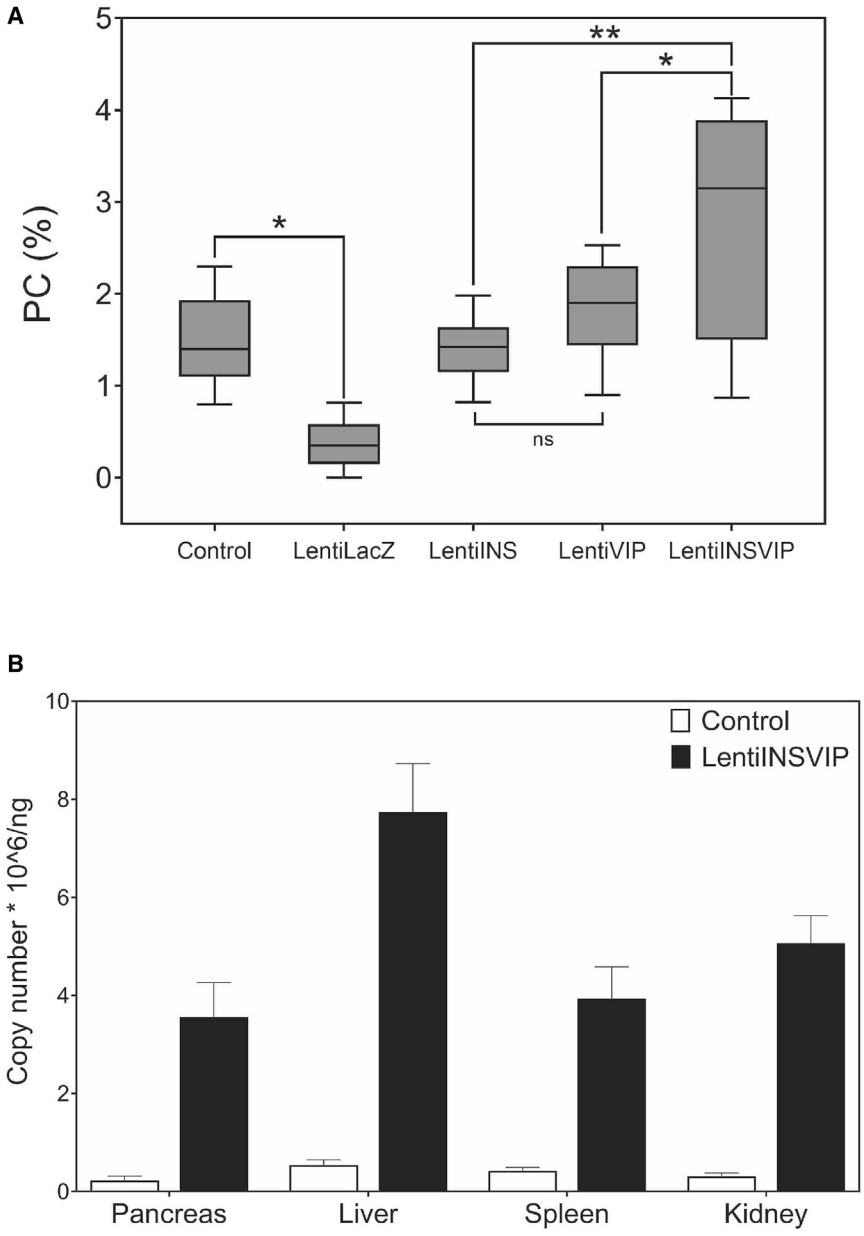


Figure 7. Induction of Pancreatic Islet Cell Proliferation and *In Vivo* Tissue Distribution of LentiINSVIP Vector

(A) Pancreatic islet cell proliferation rate in lentivirus injected animals. Values are expressed as the median (the interquartile range). Statistical analysis was performed using one-way ANOVA followed by Holm-Sidak's multiple comparisons test (Control versus LentiLacZ *p = 0.0407, LentiINS versus LentiINSVIP **p = 0.0012, LentiVIP versus LentiINSVIP *p = 0.0406, ns, not significant). (B) Genomic integrated copy number in intraabdominal organs following LentiINSVIP vector delivery (copy number $\times 10^6$/ng). qPCR analysis was performed using genomic DNA isolated from selected intraabdominal organs of sacrificed animals (n = 6). Control represents PBS injected animals.

lineage-specific transcription factor), and the markers for regulatory T cell function (FoxP3 and transforming growth factor β [TGF-β]) in pancreatic tissue of NOD mice. In the current study, injection of LentiVIP had the ability to significantly reduce non-fasting blood glucose levels in diabetic rats compared to LentiLacZ-injected diabetic rats. However, concurrent use of LentiINS and LentiVIP vectors were even more effective than their individual administration. Despite a significant decrease in blood glucose, no hypoglycemia was detected in LentiINSVIP injected animals. One of the characteristic features of T1DM patients is the loss of body weight. Interestingly, individual LentiINS and LentiVIP administration led to increased body weight, which was even more pronounced in rats receiving both vectors.

We also noted that glucose tolerance was improved after LentiINS or LentiVIP injection compared to diabetic LentiLacZ-injected rats, but once again LentiINSVIP-injected rats displayed the greatest improvement in beta-cell tolerance to glucose. Consistent with these data, LentiLacZ-injected diabetic rats displayed lower levels of insulin in blood compared to untreated controls, while LentiINS- and LentiVIP-injected animals manifested higher levels of insulin. The highest level of circulating insulin was achieved with LentiINSVIP injection. Higher levels of serum insulin translated into better reduction in hyperglycemia, glucose tolerance, and increase in body weight. Immunohistochemical analysis of insulin expression revealed pancreatic beta-cells were destroyed in LentiLacZ-injected diabetic rats, while they were protected and/or regenerated in LentiINS- and LentiVIP-injected rats. LentiINSVIP-injected rats manifested stronger insulin staining and larger islets compared to the other groups. Ki67 immunostaining revealed islet cell proliferation in LentiLaZ-injected diabetic rats was significantly reduced compared to control rats, suggesting these animals could not recover from STZ-induced diabetes without intervention. In comparison, the increased islet cell proliferation rate observed in LentiINS- and LentiVIP-injected rats suggested insulin and VIP gene expression had the ability to restore islet cell mass through an enhanced proliferation of pancreatic islet cells. It is noteworthy to mention that LentiINSVIP-injected rats displayed the highest islet cell proliferation rate. Moreover, lentiviral integration studies revealed the LentiINSVIP vectors were integrated into various intraabdominal organs—particularly the liver. However, LentiINS-directed insulin gene expression was only observable in pancreatic beta cells but not in other tissues analyzed.

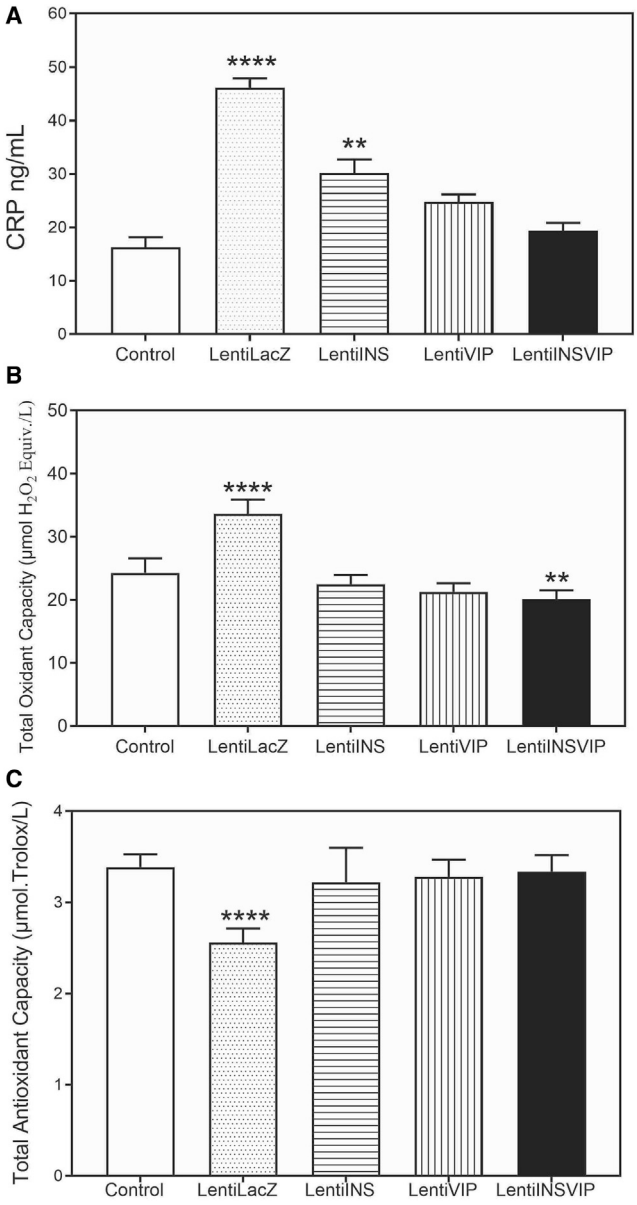


Figure 8. LentiINS, LentiVIP, and LentiINSVIP Administrations Reduced Serum CRP Levels in Diabetic Animals

(A) n = 8/group, ordinary one-way ANOVA, Kruskal-Wallis Test, **p = 0.0031, ****p < 0.0001. (B and C) Alterations in total (B) oxidant and (C) antioxidant capacity following gene delivery (n = 8/group, ordinary one-way ANOVA, Tukey's multiple comparison test, ****p < 0.0001, **p = 0.0042). Control represents PBS injected animals.

Prolonged hyperglycemia correlating with elevated CRP values has been blamed for the chronic inflammatory changes seen in both T1DM and T2DM patients.[57] Furthermore, insulin has anti-inflammatory properties and can lower serum CRP levels.[58] In our study, LentiLacZ-treated diabetic rats displayed higher levels of serum CRP compared to control animals, but serum CRP levels were reduced in LentiINS vector injected rats. The reduction in serum CRP levels were more prominent, and almost indistinguishable from the controls, in LentiVIP- or LentiINSVIP-injected rats. Moreover, the production of reactive oxygen species (ROS) and subsequent oxidative stress has been proposed as the root cause underlying the progression of insulin resistance, glucose intolerance, and beta-cell loss in T2DM.[59] Similarly, the sensitivity of pancreatic beta-cells to hyperglycemia-induced oxidative stress is a major role in the pathogenesis of T1DM.[60] In this scenario, the ROS generated as a result of oxidative stress contributed to pancreatic beta-cell death during the development of T1DM. The fact that T1DM patients manifest higher serum oxidant enzyme levels but lower antioxidant enzyme repertoire further support this hypothesis.[61,62] As shown in our study, LentiLacZ-injected STZ-induced diabetic rats displayed higher serum oxidant but lower antioxidant capacities. Both the insulin and VIP expressions were equally effective in suppressing increased oxidant capacity due to hyperglycemia, and the lower antioxidant capacity observed in diabetic rats was normalized by LentiINS and LentiVIP injections.

In conclusion, pancreatic beta-cell specific insulin gene expression combined with the suppression of diabetes-related inflammation manifested beneficial outcomes in multiple low dose STZ-induced animal model of T1DM. By deploying a complementary gene therapy approach involving LentiVIP, we were able to rescue and induce proliferation of pancreatic beta-cells and simultaneously increase insulin gene expression via LentiINS transduced pancreatic beta-cells. However, late-stage T1DM patients may not have sufficient numbers of pancreatic beta cells for the insulin gene therapy to be successful. In this scenario, complementary gene therapies to induce pancreatic beta cell regeneration like the one employed in this study (LentiVIP) might be necessary prior to insulin gene delivery. Similar to our studies, the lentivirus vector was used as a transgene carrier for FDA-approved Kymriah and EMA-approved Zynteglo. Lentiviral vectors are considered to be one of the safest gene therapy vectors and no integration induced mutagenesis was observed in the clinical trials performed to date.[32] Although, we observed no increase in serum alanine aminotransferase (ALT) levels (an indication of liver damage) in rats injected with individual lentiviral vectors (LentiINS, LentiVIP, or LentiLacZ; data not shown), further research is warranted to reveal any other possible side effects of the treatment. Additionally, future studies are needed in non-obese diabetic (NOD) mice or BioBreeding (BB) Wistar rats to better assess the efficacy of lentivirus-mediated pancreatic beta-cell specific insulin gene therapy before planning large animal models (dogs, pigs, and nonhuman primates) or human clinical trials.

MATERIALS AND METHODS

Construction of Lentiviral-Based Expression Plasmid Encoding the Minimal Insulin Promoter and Insulin Gene

The human insulin promoter (−364/+31) was obtained from Integrated DNA Technologies (IDT, Iowa City, IA, USA) as synthetic double-stranded DNA. After resuspending DNA fragments in Tris-EDTA buffer, an adenylation reaction was set up using Taq polymerase in the presence of dATP and $MgCl_2$ by incubating at 70°C in 9800 Fast Thermal Cycler. The insulin promoter was then cloned into an entry vector

(pENTR5′Ip) using pENTR 5′-TOPO-TA Cloning Kit (İnvitrogen K591-20). The pENTR5′-TOPO TA Cloning Kit and MultiSite Gateway Technologies are linked to simplify one-step cloning of Taq polymerase-processed DNA fragments encoding a eukaryotic promoter of interest into a MultiSite Gateway entry vector. In this protocol, the cloned eukaryotic promoter is transferred from the pENTR5′-TOPO vector to a suitable MultiSite Gateway destination vector in a MultiSite Gateway LR recombination reaction with the other entry clone to generate an expression construct. The entry vector carrying the gene encoding for the human insulin (pENTRIns; Invitrogen, Ultimate ORF Clone ID: IOH7334) was obtained as a bacterial stab culture. QIAGEN Plasmid Mega Kit (QIAGEN, Cat. No: 12183) was used to obtain purified pENTRIns and pENTR5′Ip plasmids. Virapower Hiperform Promoterless Gateway Expression Kit (İnvitrogen, A11145) was used to generate the expression clone (pLentiIINS). To do this, the MultiSite Gateway reaction was set up using both entry vectors (pENTR5′Ip and pENTRIns) and a destination vector (pLENTİ6.4/R4R2/V5-DEST) in the presence of LR Clonase II Plus enzyme by incubating at room temperature overnight. After proteinase K digestion, the reaction was transformed into *E. coli* and spread on LB agar plates with ampicillin. Plasmid DNA was isolated using QIAGEN Plasmid Mini Kit (QIAGEN, Cat. No. 12125) and cut with *AflII* versus *XhoI* restriction enzymes to idenfity the right bacterial colonies. Plasmid sequence identity was confirmed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Cat. No. 4337455). QIAGEN Plasmid Mega Kit (QIAGEN, Cat. No. 12183) was used to obtain a large quantity of plasmid DNA needed for transfection. Construction of LentiLacZ and LentiVIP vectors was performed as described previously.[37,38] Lentivirus vectors were produced by the transient transfection of 293T cells in roller bottles and purified by anion-exchange chromatography as described previously.[41,42]

Functional Titration of Lentiviral Vectors by qPCR

Concentrated lentivirus vectors were stored at −80°C for at least 24 h before the titration. HT1080 cells (ATCC CCL-121) were added to 24-well plates at 50,000 cells/well the day before the transduction. After thawing of lentivirus stocks, HT1080 cells were transduced with increasing doses of the vector (10^{-4}, 10^{-2}, 1, 2, 4, 8 μL) in the presence of polybrene (6 μg/mL). The cell culture medium was replaced with 500 μL of fresh DMEM without polybrene the day after transduction. Lentivirus-transduced HT1080 cells were kept at in an incubator (37°C and 5% CO_2) for 2 additional days. Lysis buffer was added at 100 μL/well after removing the cell culture media. Samples were kept at 96°C then centrifuged at 14,000 rpm for 2 min. Cell lysates were held at −20°C before use. WPRE primers (Fwd: 5′-CCGTTGTCAGGCAACGTG-3′; Rev: 5′-AGCTGACAGGTGGTG GCAAT-3′) were used to detect integrated lentiviral vector copies while albumin primers (Fwd: 5′-GCTGTCATCTCTTGTG GGCTGT-3′; Rev: 5′-ACTCATGGGAGCTGCTGGTTC-3′) were used as internal qPCR controls. Albumin plasmids obtained from Addgene were used to establish the standard curve. Real-time PCR reactions were carried out using the Quantitect SYBR Green PCR Kit (QIAGEN, Cat. No. 204143). *In vivo* administration of LentiIINS, LentiVIP, and LentiLacZ used a single dose of 10^{10} TU/rat. Combined LentiIINS and LentiVIP delivery included 5×10^9 TU of each vector (totaling 10^{10} TU/rat).

In Vitro Gene Expression Analysis of LentiIINS Vector

To confirm transgene expression from the LentiIINS vector, we used 293T (ATCC CRL-3216) and NIT-1 pancreatic beta-cell lines (ATCC CRL-2055). Briefly, cells were cultured in 24-well plates (50,000 cells/well) and then transduced with LentiIINS at increasing MOIs (0, 5, 25, 125) in the presence of polybrene. Supernatants were collected 72 h later to detect insulin levels using the Insulin Human ELISA kit (Abcam, ab100578). MIN6 pancreatic beta cell line was kindly provided by Jun-Ichi Miyazaki (Osaka University). To assess glucose responsiveness of lentiviral vectors, we washed LentiIINS or LentiLacZ-infected MIN6 cells with PBS and then incubated in Krebs Ringer Buffer (KRB) with no glucose. All cells were exposed to KRB containing 2.8 mM glucose for 30 min at 37°C. After washing cells with glucose-free KRB, cells were treated with 250 μL KRB supplemented with 0, 2.8, or 25 mM glucose for 1 h at 37°C. Cell culture supernatants were collected from wells and stored at −20°C prior to detection of secreted insulin. Serum insulin ELISA was performed similarly using blood collected from animals sacrificed 5 weeks after the gene delivery using Insulin ELISA Kit (Abcam, ab100578).

Development of STZ-Induced Diabetes

4-week-old Wistar rats were attained from the Animal Care Unit of Akdeniz University Hospitals. Rats were housed in a humidity and temperature-controlled environment under a 12-h light/dark cycle and received standard chow and water *ad libitum*. STZ was used to induce diabetes in 6-week-old Wistar rats. STZ was dissolved in 0.01 M citrate buffer (pH 4.5) just before use and injected i.p. to establish diabetes. After testing various doses and repeated administrations, injection of 30 mg/kg STZ (Sigma-Aldrich; St. Louis, MO, USA) for 5 consecutive days gave the optimum dose to induce hyperglycemia. Blood glucose levels and body weight of diabetic rats were logged intermittently. Blood samples from sacrificed rats were taken by intracardiac puncture when necessary. Serum samples were obtained from the blood via centrifugation and stored at −20°C for further analyses. The animal study was conducted under the supervision of the Institutional Animal Care and Use Committee of Akdeniz University School of Medicine.

Blood Glucose Measurements and i.p. Glucose Tolerance Test

Glucose levels were measured using blood taken from the tail vein via Accu-Check Compact Glucometer (Roche Diagnostics, Indianapolis, IN, USA). The sensitivity of the Accu-Check Compact Glucometer is between 10–600 mg/dL, and glucose values >600 mg/dL are displayed as "HI." Thus, "HI" displayed values on glucometer were recorded as 600 mg/dL. Blood glucose values >250 mg/dL on two consecutive readings were considered diabetic. Blood glucose measurements were taken in the early afternoon to prevent alterations due to differences in animal feeding behavior. Animals were refrained from food for 8 h to obtain fasting blood glucose levels. i.p. glucose tolerance test was performed in rats after 6 hr of fasting. To do this, gene-delivered

rats received i.p. 2 g/kg of glucose. Blood glucose measurements were taken at 0, 15, 30, 60, and 120 min using blood obtained from the tail vein.

Immunohistochemical Studies

To demonstrate that pancreatic islets were destroyed/restored following STZ injection and gene delivery, we sacrificed rats 5 weeks after i.p. injections. After intracardiac perfusion with 4% paraformaldehyde, intraabdominal organs (pancreas, spleen, liver, and kidney) were dissected, fixed in formalin, and embedded in paraffin. Hematoxylin and eosin staining were performed to reveal general cytoarchitecture of tissues, while immune staining was used to reveal insulin gene expression using polyclonal rabbit anti-insulin antibody (Abcam, Cat. No. ab63820). Ki-67 immunostaining (Biorbyt, St. Louis, MO, USA, Cat. No. orb389335) was conducted to determine proliferating islet cells, and the proliferation rate in each islet (%) was estimated as the number of proliferating cells/the total number of cells $\times$ 100%.[63] Sections were analyzed under an Olympus IX81 Inverted Fluorescein Microscope and the images were processed using the ImageJ program (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

Detection of Serum CRP, Total Oxidant, and Total Antioxidant Capacities

CUSABIO ELISA kit (CSB-E07922r, Wuhan, Hubei, China) was used for the quantitative determination of rat C-reactive protein (CRP) concentrations in serum. Total oxidant and total antioxidant capacities were measurements as described previously.[64,65] Briefly, total oxidant capacity analysis is a colorimetric method based on the oxidation of ferrous iron (Fe^{+2}) to the ferric iron complex (Fe^{+3}). The intensity of this colored compound is measured at 600 nm using a spectrophotometer. Total antioxidant capacity relies on the antioxidant-induced color change in ABTS measured at 660 nm using a spectrophotometer.

AUTHOR CONTRIBUTIONS

F.E. and Y.E.E. performed the experiments. E.O.S. helped with the assays. T.S.G. and M.K.B. acted as consultants and edited the manuscript. S.S. designed and supervised the study.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

This work is supported by the Akdeniz University Scientific Research Administration Division and The Scientific and Technological Research Council of Turkey (TUBITAK) under grant number 215S820. Special thanks to Dr. Hamit Yasar Ellidag for his technical assistance.

REFERENCES

1. Pietropaolo, M., Barinas-Mitchell, E., and Kuller, L.H. (2007). The heterogeneity of diabetes: unraveling a dispute: is systemic inflammation related to islet autoimmunity? Diabetes *56*, 1189–1197.
2. Ramos-Rodríguez, M., Raurell-Vila, H., Colli, M.L., Alvelos, M.I., Subirana-Granés, M., Juan-Mateu, J., Norris, R., Turatsinze, J.V., Nakayasu, E.S., Webb-Robertson, B.M., et al. (2019). The impact of proinflammatory cytokines on the β-cell regulatory landscape provides insights into the genetics of type 1 diabetes. Nat. Genet. *51*, 1588–1595.
3. Peters, A., and Laffel, L.; American Diabetes Association Transitions Working Group (2011). Diabetes care for emerging adults. Diabetes Care *34*, 2477–2485.
4. Atkinson, M.A., Eisenbarth, G.S., and Michels, A.W. (2014). Type 1 diabetes. Lancet *383*, 69–82.
5. Barbetti, F., and Taylor, S.I. (2019). Insulin: still a miracle after all these years. J. Clin. Invest. *129*, 3045–3047.
6. Heise, T., and Pieber, T.R. (2007). Towards peakless, reproducible and long-acting insulins. An assessment of the basal analogues based on isoglycaemic clamp studies. Diabetes Obes. Metab. *9*, 648–659.
7. Ashwell, S.G., Gebbie, J., and Home, P.D. (2006). Twice-daily compared with once-daily insulin glargine in people with Type 1 diabetes using meal-time insulin aspart. Diabet. Med. *23*, 879–886.
8. Mohn, A., Marcovecchio, M., and Chiarelli, F. (2005). Insulin analogues. N. Engl. J. Med. *352*, 1822–1824, author reply 1822–1824.
9. Crasto, W., Jarvis, J., Khunti, K., and Davies, M.J. (2009). New insulins and new insulin regimens: a review of their role in improving glycaemic control in patients with diabetes. Postgrad. Med. J. *85*, 257–267.
10. Sanlioglu, A.D., Altunbas, H.A., Balci, M.K., Griffith, T.S., and Sanlioglu, S. (2013). Clinical utility of insulin and insulin analogs. Islets *5*, 67–78.
11. Mathieu, C., Weisnagel, S.J., Stella, P., Bruhwyler, J., and Alexandre, K. (2020). Impact of Switching from Twice-Daily Basal Insulin to Once-Daily Insulin Glargine 300 U/mL in People with Type 1 Diabetes on Basal-Bolus Insulin: Phase 4 OPTIMIZE Study. Diabetes Ther. *11*, 495–507.
12. Wahren, J., Kallas, A., and Sima, A.A. (2012). The clinical potential of C-peptide replacement in type 1 diabetes. Diabetes *61*, 761–772.
13. Marx, N., Silbernagel, G., Brandenburg, V., Burgmaier, M., Kleber, M.E., Grammer, T.B., Winkelmann, B.R., Boehm, B.O., and März, W. (2013). C-peptide levels are associated with mortality and cardiovascular mortality in patients undergoing angiography: the LURIC study. Diabetes Care *36*, 708–714.
14. Kamiya, H., Zhang, W., and Sima, A.A. (2009). The beneficial effects of C-Peptide on diabetic polyneuropathy. Rev. Diabet. Stud. *6*, 187–202.
15. Meyer, J.A., Froelich, J.M., Reid, G.E., Karunarathne, W.K., and Spence, D.M. (2008). Metal-activated C-peptide facilitates glucose clearance and the release of a nitric oxide stimulus via the GLUT1 transporter. Diabetologia *51*, 175–182.
16. Sanlioglu, A.D., Altunbas, H.A., Balci, M.K., Griffith, T.S., and Sanlioglu, S. (2012). Insulin gene therapy from design to beta cell generation. Expert Rev. Mol. Med. *14*, e18.
17. Chan, L., Fujimiya, M., and Kojima, H. (2003). In vivo gene therapy for diabetes mellitus. Trends Mol. Med. *9*, 430–435.
18. Tiedge, M., and Lenzen, S. (1991). Regulation of glucokinase and GLUT-2 glucose-transporter gene expression in pancreatic B-cells. Biochem. J. *279*, 899–901.
19. Dong, H., Morral, N., McEvoy, R., Meseck, M., Thung, S.N., and Woo, S.L. (2001). Hepatic insulin expression improves glycemic control in type 1 diabetic rats. Diabetes Res. Clin. Pract. *52*, 153–163.
20. Moore, H.P., Walker, M.D., Lee, F., and Kelly, R.B. (1983). Expressing a human proinsulin cDNA in a mouse ACTH-secreting cell. Intracellular storage, proteolytic processing, and secretion on stimulation. Cell *35*, 531–538.
21. Dong, H., and Woo, S.L. (2001). Hepatic insulin production for type 1 diabetes. Trends Endocrinol. Metab. *12*, 441–446.
22. Niu, L., Xu, Y.C., Dai, Z., and Tang, H.Q. (2008). Gene therapy for type 1 diabetes mellitus in rats by gastrointestinal administration of chitosan nanoparticles containing human insulin gene. World J. Gastroenterol. *14*, 4209–4215.
23. Cheung, A.T., Dayanandan, B., Lewis, J.T., Korbutt, G.S., Rajotte, R.V., Bryer-Ash, M., Boylan, M.O., Wolfe, M.M., and Kieffer, T.J. (2000). Glucose-dependent insulin release from genetically engineered K cells. Science *290*, 1959–1962.

24. Corbett, J.A. (2001). K cells: a novel target for insulin gene therapy for the prevention of diabetes. Trends Endocrinol. Metab. *12*, 140–142.

25. Tasyurek, H.M., Altunbas, H.A., Balci, M.K., and Sanlioglu, S. (2014). Incretins: their physiology and application in the treatment of diabetes mellitus. Diabetes Metab. Res. Rev. *30*, 354–371.

26. Tasyurek, M.H., Altunbas, H.A., Canatan, H., Griffith, T.S., and Sanlioglu, S. (2014). GLP-1-mediated gene therapy approaches for diabetes treatment. Expert Rev. Mol. Med. *16*, e7.

27. Sanlioglu, A.D., Karacay, B., Balci, M.K., Griffith, T.S., and Sanlioglu, S. (2012). Therapeutic potential of VIP vs PACAP in diabetes. J. Mol. Endocrinol. *49*, R157–R167.

28. Chellappan, D.K., Sivam, N.S., Teoh, K.X., Leong, W.P., Fui, T.Z., Chooi, K., Khoo, N., Yi, F.J., Chellian, J., Cheng, L.L., et al. (2018). Gene therapy and type 1 diabetes mellitus. Biomed. Pharmacother. *108*, 1188–1200.

29. Wong, M.S., Hawthorne, W.J., and Manolios, N. (2010). Gene therapy in diabetes. Self Nonself *1*, 165–175.

30. Lundstrom, K. (2018). Viral Vectors in Gene Therapy. Diseases *6*, 42.

31. Shirley, J.L., de Jong, Y.P., Terhorst, C., and Herzog, R.W. (2020). Immune Responses to Viral Gene Therapy Vectors. Mol. Ther. *28*, 709–722.

32. Milone, M.C., and O'Doherty, U. (2018). Clinical use of lentiviral vectors. Leukemia *32*, 1529–1541.

33. White, M., Whittaker, R., Gándara, C., and Stoll, E.A. (2017). A Guide to Approaching Regulatory Considerations for Lentiviral-Mediated Gene Therapies. Hum. Gene Ther. Methods *28*, 163–176.

34. Carbonaro-Sarracino, D.A., Tarantal, A.F., Lee, C.C.I., Kaufman, M.L., Wandro, S., Jin, X., Martinez, M., Clark, D.N., Chun, K., Koziol, C., et al. (2019). Dosing and Re-Administration of Lentiviral Vector for *In Vivo* Gene Therapy in Rhesus Monkeys and ADA-Deficient Mice. Mol. Ther. Methods Clin. Dev. *16*, 78–93.

35. Ren, B., O'Brien, B.A., Swan, M.A., Koina, M.E., Nassif, N., Wei, M.Q., and Simpson, A.M. (2007). Long-term correction of diabetes in rats after lentiviral hepatic insulin gene therapy. Diabetologia *50*, 1910–1920.

36. Doiron, B., Hu, W., and DeFronzo, R.A. (2016). Beta Cell Formation in vivo Through Cellular Networking, Integration and Processing (CNIP) in Wild Type Adult Mice. Curr. Pharm. Biotechnol. *17*, 376–388.

37. Tasyurek, H.M., Altunbas, H.A., Balci, M.K., Griffith, T.S., and Sanlioglu, S. (2018). Therapeutic Potential of Lentivirus-Mediated Glucagon-Like Peptide-1 Gene Therapy for Diabetes. Hum. Gene Ther. *29*, 802–815.

38. Tasyurek, H.M., Eksi, Y.E., Sanlioglu, A.D., Altunbas, H.A., Balci, M.K., Griffith, T.S., and Sanlioglu, S. (2018). HIV-based lentivirus-mediated vasoactive intestinal peptide gene delivery protects against DIO animal model of Type 2 diabetes. Gene Ther. *25*, 269–283.

39. Elsner, M., Jörns, A., and Lenzen, S. (2008). Diabetes therapy by lentiviral hepatic insulin gene expression without transformation of liver. Diabetologia *51*, 694–695, author reply 696.

40. Elsner, M., Terbish, T., Jörns, A., Naujok, O., Wedekind, D., Hedrich, H.J., and Lenzen, S. (2012). Reversal of diabetes through gene therapy of diabetic rats by hepatic insulin expression via lentiviral transduction. Mol. Ther. *20*, 918–926.

41. Olgun, H.B., Tasyurek, H.M., Sanlioglu, A.D., and Sanlioglu, S. (2019). High-Titer Production of HIV-Based Lentiviral Vectors in Roller Bottles for Gene and Cell Therapy. Methods Mol. Biol. *1879*, 323–345.

42. Olgun, H.B., Tasyurek, H.M., Sanlioglu, A.D., and Sanlioglu, S. (2019). High-Grade Purification of Third-Generation HIV-Based Lentiviral Vectors by Anion Exchange Chromatography for Experimental Gene and Stem Cell Therapy Applications. Methods Mol. Biol. *1879*, 347–365.

43. Temelkova-Kurktschiev, T., Henkel, E., Koehler, C., Karrei, K., and Hanefeld, M. (2002). Subclinical inflammation in newly detected Type II diabetes and impaired glucose tolerance. Diabetologia *45*, 151.

44. Ford, E.S. (1999). Body mass index, diabetes, and C-reactive protein among U.S. adults. Diabetes Care *22*, 1971–1977.

45. Domínguez, C., Ruiz, E., Gussinye, M., and Carrascosa, A. (1998). Oxidative stress at onset and in early stages of type 1 diabetes in children and adolescents. Diabetes Care *21*, 1736–1742.

46. Naldini, L., Trono, D., and Verma, I.M. (2016). Lentiviral vectors, two decades later. Science *353*, 1101–1102.

47. Kobinger, G.P., Deng, S., Louboutin, J.P., Vatamaniuk, M., Matschinsky, F., Markmann, J.F., Raper, S.E., and Wilson, J.M. (2004). Transduction of human islets with pseudotyped lentiviral vectors. Hum. Gene Ther. *15*, 211–219.

48. Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. J. Virol. *72*, 8463–8471.

49. Zhang, B., Xia, H.Q., Cleghorn, G., Gobe, G., West, M., and Wei, M.Q. (2001). A highly efficient and consistent method for harvesting large volumes of high-titre lentiviral vectors. Gene Ther. *8*, 1745–1751.

50. Kato, I., Suzuki, Y., Akabane, A., Yonekura, H., Tanaka, O., Kondo, H., Takasawa, S., Yoshimoto, T., and Okamoto, H. (1994). Transgenic mice overexpressing human vasoactive intestinal peptide (VIP) gene in pancreatic beta cells. Evidence for improved glucose tolerance and enhanced insulin secretion by VIP and PHM-27 in vivo. J. Biol. Chem. *269*, 21223–21228.

51. Alam, T., and Sollinger, H.W. (2002). Glucose-regulated insulin production in hepatocytes. Transplantation *74*, 1781–1787.

52. Han, J., McLane, B., Kim, E.H., Yoon, J.W., and Jun, H.S. (2011). Remission of diabetes by insulin gene therapy using a hepatocyte-specific and glucose-responsive synthetic promoter. Mol. Ther. *19*, 470–478.

53. Lu, D., Tamemoto, H., Shibata, H., Saito, I., and Takeuchi, T. (1998). Regulatable production of insulin from primary-cultured hepatocytes: insulin production is up-regulated by glucagon and cAMP and down-regulated by insulin. Gene Ther. *5*, 888–895.

54. Martínez, C., Juarranz, Y., Gutiérrez-Cañas, I., Carrión, M., Pérez-García, S., Villanueva-Romero, R., Castro, D., Lamana, A., Mellado, M., González-Álvaro, I., and Gomariz, R.P. (2019). A Clinical Approach for the Use of VIP Axis in Inflammatory and Autoimmune Diseases. Int. J. Mol. Sci. *21*, 65.

55. Herrera, J.L., Fernández-Montesinos, R., González-Rey, E., Delgado, M., and Pozo, D. (2006). Protective role for plasmid DNA-mediated VIP gene transfer in non-obese diabetic mice. Ann. N Y Acad. Sci. *1070*, 337–341.

56. Rosignoli, F., Torroba, M., Juarranz, Y., García-Gómez, M., Martinez, C., Gomariz, R.P., Pérez-Leirós, C., and Leceta, J. (2006). VIP and tolerance induction in autoimmunity. Ann. N Y Acad. Sci. *1070*, 525–530.

57. Treszl, A., Szereday, L., Doria, A., King, G.L., and Orban, T. (2004). Elevated C-reactive protein levels do not correspond to autoimmunity in type 1 diabetes. Diabetes Care *27*, 2769–2770.

58. Dandona, P., Aljada, A., and Mohanty, P. (2002). The anti-inflammatory and potential anti-atherogenic effect of insulin: a new paradigm. Diabetologia *45*, 924–930.

59. Dos Santos, J.M., Tewari, S., and Mendes, R.H. (2019). The Role of Oxidative Stress in the Development of Diabetes Mellitus and Its Complications. J. Diabetes Res. *2019*, 4189813.

60. Varvarovská, J., Racek, J., Stetina, R., Sýkora, J., Pomahacová, R., Rusavý, Z., Lacigová, S., Trefil, L., Siala, K., and Stozický, F. (2004). Aspects of oxidative stress in children with type 1 diabetes mellitus. Biomed. Pharmacother. *58*, 539–545.

61. Fatima, N., Faisal, S.M., Zubair, S., Ajmal, M., Siddiqui, S.S., Moin, S., and Owais, M. (2016). Role of Pro-Inflammatory Cytokines and Biochemical Markers in the Pathogenesis of Type 1 Diabetes: Correlation with Age and Glycemic Condition in Diabetic Human Subjects. PLoS ONE *11*, e0161548.

62. Menon, V., Ram, M., Dorn, J., Armstrong, D., Muti, P., Freudenheim, J.L., Browne, R., Schunemann, H., and Trevisan, M. (2004). Oxidative stress and glucose levels in a population-based sample. Diabet. Med. *21*, 1346–1352.

63. He, Y., Shi, B., Zhao, X., and Sui, J. (2019). Sphingosine-1-phosphate induces islet β-cell proliferation and decreases cell apoptosis in high-fat diet/streptozotocin diabetic mice. Exp. Ther. Med. *18*, 3415–3424.

64. Malik, A., Morya, R.K., Bhadada, S.K., and Rana, S. (2018). Type 1 diabetes mellitus: Complex interplay of oxidative stress, cytokines, gastrointestinal motility and small intestinal bacterial overgrowth. Eur. J. Clin. Invest. *48*, e13021.

65. Yaribeygi, H., Atkin, S.L., and Sahebkar, A. (2019). A review of the molecular mechanisms of hyperglycemia-induced free radical generation leading to oxidative stress. J. Cell. Physiol. *234*, 1300–1312.