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ARTICLE

Lentiviral gene therapy vectors encoding VIP suppressed diabetes-related inflammation and augmented pancreatic beta-cell proliferation

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Abstract

Type 1 diabetes (T1DM) is an autoimmune condition in which the immune system attacks and destroys insulin-producing beta cells in the pancreas leading to hyperglycemia. Vasoactive intestinal peptide (VIP) manifests insulinotropic and anti-inflammatory properties, which are useful for the treatment of diabetes. Because of its limited half-life due to DPP-4-mediated degradation, constant infusions or multiple injections are needed to observe any therapeutic benefit. Since gene therapy has the potential to treat genetic diseases, an HIV-based lentiviral vector carrying VIP gene (LentiVIP) was generated to provide a stable VIP gene expression in vivo. The therapeutic efficacy of LentiVIP was tested in a multiple low-dose STZ-induced animal model of T1DM. LentiVIP delivery into diabetic animals reduced hyperglycemia, improved glucose tolerance, and prevented weight loss. Also, a decrease in serum CRP levels, and serum oxidant capacity, but an increase in antioxidant capacity were observed in LentiVIP-treated animals. Restoration of islet cell mass was correlated with an increase in pancreatic beta-cell proliferation. These beneficial results suggest the therapeutic effect of LentiVIP is due to the repression of diabetes-induced inflammation, its insulinotropic properties, and VIP-induced beta-cell proliferation.

Introduction

Type 1 diabetes (T1DM)—also known as insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes—is manifested as hyperglycemia due to insufficient production of insulin as a result of autoimmune-mediated destruction of insulin-producing pancreatic beta cells [1]. T1DM is characterized by the production of islet cell-specific autoantibodies, such as those specific for glutamic acid decarboxylase (GAD), the tyrosine phosphatase-related islet antigen 2, and insulin itself, along with mononuclear cell infiltration to pancreatic islets (insulitis) [2]. Although islet-specific antibodies are detectable in the majority (~85%) of patients, genetic (e.g., HLA class II, PTPN22, insulin, IL2RA, and CTLA-4) and environmental factors (e.g., food, stress, chemicals, and microbes) predispose children to disease [3, 4]. In this chronic autoimmune disorder, activated T lymphocytes are the main mediators of autoimmunity by way of recognizing their autoantigens and then attacking pancreatic beta cells [5]. However, the destruction of >70% of the insulin-producing pancreatic beta cells is necessary for clinical manifestation.

In patients with T1D and nonobese diabetic mice (NOD), autoreactive T cells destroy pancreatic beta cells, as transfer or deletion of these cells induces or averts disease, respectively [6]. Pro-inflammatory cytokines (IFN-gamma, TNF-alpha, and IL-1) produced by diabetogenic $CD4^+$ and $CD8^+$ T cells, are indeed generated by reactive oxygen species (ROS) via redox-dependent signaling pathways. Consequently, the most successful immunological therapies for T1DM rely on targeting autoreactive T cells (e.g., cyclosporine A, monoclonal anti-CD3, and thymoglobulin). Moreover, cytokines represent potential immunotherapeutic targets for the treatment of T1DM because they orchestrate

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interactions between immune cells and pancreatic beta cells during diabetes [7]. In this scenario, regulatory cytokines (e.g., IL-10, TGF-β, and IL-33) prevent beta-cell damage by inducing immune tolerance, while pro-inflammatory cytokines such as IL-6, IL-17, IL-21, and TNFα facilitate the development of T1DM [8]. In this regard, TNFα neutralization preserved beta-cell function in young diabetic patients [9], while IL-2 treatment activated regulatory T cells essential for the suppression of autoimmunity in patients with T1DM [10].

To preserve pancreatic beta cells, therapeutic agents are expected to increase glucose-induced insulin secretion, enhance pancreatic beta-cell proliferation, and/or provide protection from inflammatory agents/apoptosis [11]. Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) have been considered for the treatment of diabetic patients due to their potential to induce significant levels of insulin from pancreatic beta cells [12]. Both PACAP and VIP are secreted from the pancreas following parasympathetic nerve activation [13]. Intriguingly, PACAP and VIP expression are also localized to pancreatic islets suggesting these neuropeptides function both as neurotransmitters and endocrine peptides released from pancreatic islets [14]. However, the clinical use of PACAP for antidiabetic purposes is not recommended due to the stimulation of glucagon and epinephrine release. VIP, on the other hand, exhibited significant anti-inflammatory properties, such as suppression of Th1 immune response and activation of regulatory T cells to induce immune tolerance useful for the treatment of patients with various autoimmune diseases including T1DM [15]. Consequently, exogenous administration of VIP displayed therapeutic benefits in models of autoimmune/inflammatory diseases mediated by G-protein-coupled receptors (such as VPAC1 and VPAC2) [16].

Despite having been tested in various clinical trials against diseases such as obstructive pulmonary disease, pulmonary hypertension, sepsis and migraine, the clinical efficacy of VIP was limited due to short half-life requiring multiple high-dose injections. Instead of using peptide forms of drug candidates, some gene therapy vectors can provide long-term gene expression. To better benefit from the therapeutic efficacy of VIP, both viral and nonviral gene delivery methods have been developed [17–19]. Among the viral vectors tested, lentiviral vectors recently prevailed due to their safety and the longevity of transgene expression [20–23]. Lentivirus-mediated VIP gene delivery (LentiVIP) has been tested in inflammatory diseases, but the antidiabetic efficacy of LentiVIP has not yet been revealed in an animal model of T1DM. Thus, the purpose of this study was to test the therapeutic efficacy of a 3rd generation lentivirus encoding VIP in a rat model of T1DM induced by multiple low-dose STZ injections (MLD-STZ).

Results

Generation of an animal model of Type 1 diabetes

To test the therapeutic efficacy of VIP gene transfer in diabetes, we first needed to establish a reliable animal model featuring T1DM. Thus, we used the well-known chemotherapeutic agent, STZ, to induce pancreatic beta-cell damage. Since a single high dose of STZ causes absolute insulin deficiency in a very short time without immune system involvement, multiple low doses of STZ (30 mg/kg IP for 5 consecutive days) were used instead to gradually destroy beta cells in Wistar or Sprague Dawley rats to better conform to T1DM. Blood glucose levels and body weights were recorded following STZ injections. Three days after the last STZ injection, blood glucose rose above 300 mg/dl and remained high during the entire follow-up period indicating successful induction of diabetes in both rat strains (Fig. 1a). Similarly, both rat strains demonstrated a considerable loss in body weight after STZ injection (Fig. 1b). To confirm pancreatic beta-cell loss and its connection to hyperglycemia, animals were sacrificed at different time points after STZ injections and paraffin-embedded pancreatic tissues were analyzed by hematoxylin-eosin staining (Fig. 1c). Histologic analysis of pancreatic tissue sections revealed more than half of the pancreatic islet cell mass was lost during the first 10 days after the STZ injection (Fig. 1d). Also, the majority of pancreatic islet cell loss (above 90%) took place within 20 days of STZ injection indicating that islet cell loss is not a sudden but rather a gradual process in this animal model of T1DM induced by multiple low doses of STZ injections. Since Wistar rats exhibited a more stable blood glucose profile than Sprague Dawley rats, we decided to continue our future assays using Wistar rats exclusively.

Construction of HIV-based lentivirus encoding VIP

Virapower HiPerform Lentiviral Gateway Expression Kit from Invitrogen was used to generate a 3rd generation lentiviral gene therapy vector encoding VIP (LentiVIP). In this procedure, recombination between an entry vector and a destination vector (Fig. 2a) resulted in the generation of the expression clone carrying the VIP transgene on a lentiviral vector backbone (Fig. 2b). Restriction enzyme analysis followed by agarose gel electrophoresis was used to validate both the transfer plasmid (pLentiVIP) and packaging vectors used in the lentiviral vector production (Fig. 2c). Finally, 293 T cells were co-transfected with pLentiVIP and three packaging plasmids to produce virus particles using the transient calcium phosphate transfection method. Virus particles were then concentrated by ultracentrifugation and further purified by anion exchange (AEX)

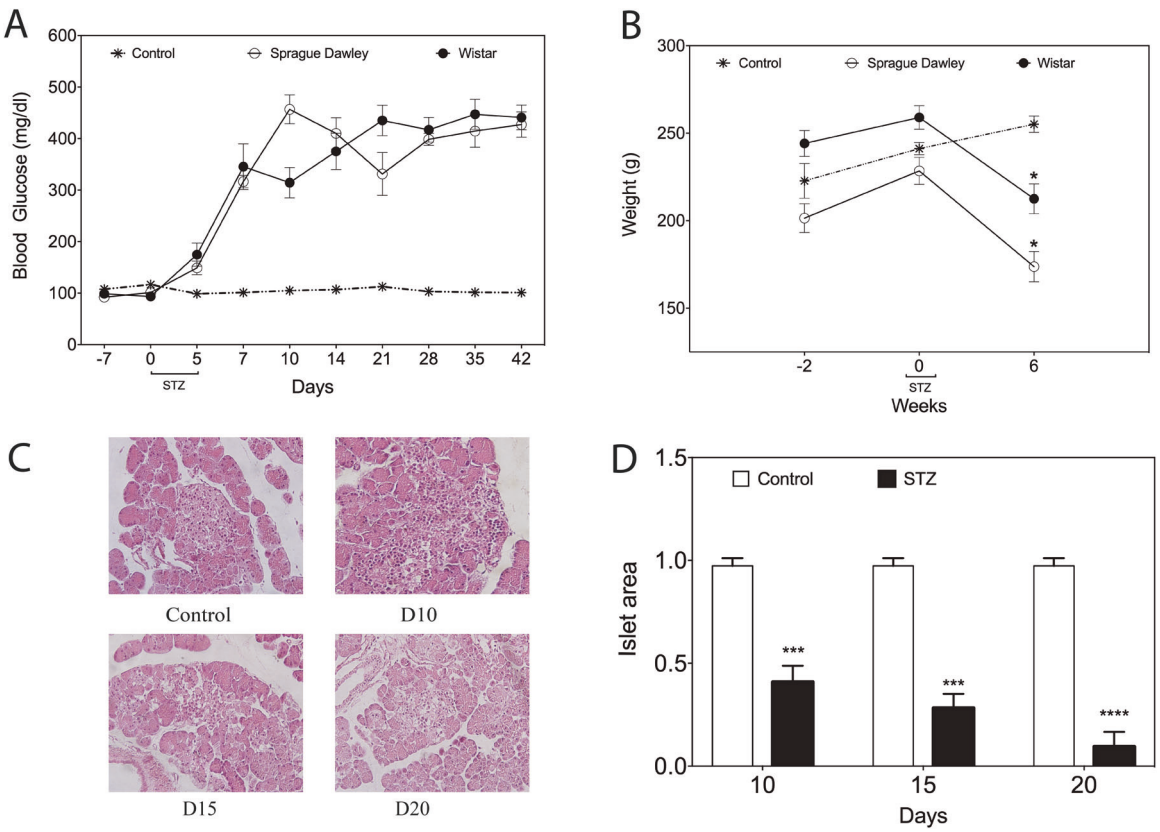

Fig. 1 Experimental animal model of Type 1 diabetes. STZ (30 mg/kg) was injected IP into Wistar or Sprague Dawley rats ($n = 8$) for 5 consecutive days. a Blood glucose measurements were performed periodically (Mann–Whitney U test, $p = 0.01$). **b** Body weight measurements before and after STZ injections. **c** Hematoxylin/Eosin staining of pancreatic sections from Wistar rats ($n = 5$) sacrificed on day 10, 15, and 20 following consecutive 30 mg/kg STZ/day administration. **d** Quantitative analysis of pancreatic islet loss in STZ-injected rats (two-way ANOVA, $p < 0.0001$).

chromatography. Functional viral titers were determined by real-time PCR.

In vitro expression and functional analysis of LentiVIP

To confirm VIP expression from the newly generated gene therapy vector, HepG2 cells, a hepatocellular carcinoma cell line that does not express VIP, were transduced with LentiVIP or LentiLacZ and an ELISA was performed to quantitate the amount of VIP in the culture supernatant. VIP levels increased in a LentiVIP dose-dependent manner (Fig. 3a), and the amount of VIP in the LentiLacZ-infected cells was indistinguishable from uninfected cells. We also used a glucose-stimulated insulin secretion test (GSIS) to analyze the functional properties of LentiVIP in Min6 pancreatic beta-cell line. Increased insulin secretion was detected in the LentiVIP-infected Min6 cell line only when the glucose concentration was high (Fig. 3b). A similar phenotype was not observed in LentiLacZ-infected cells. These results collectively suggest VIP encoded by LentiVIP is functional and displays insulinotropic properties.

Therapeutic efficacy of LentiVIP injection in STZ-induced rat model of diabetes

After verification of in vitro expression and functional status of VIP in LentiVIP-infected human cell lines, the in vivo therapeutic efficacy of the LentiVIP gene therapy vector was tested in our STZ-induced T1DM rat model. Wistar rats were injected with 30 mg/kg STZ for 5 consecutive days to induce diabetes as in Fig. 1. Blood glucose levels were measured on day 3 and day 5 after the last STZ injections. After confirmation of hyperglycemia, rats were divided into two groups to receive either LentiVIP or LentiLacZ vectors (10^{10} TU). Blood glucose measurements were taken periodically following the gene transfer. There was a striking normalization in blood glucose levels of LentiVIP-injected rats 5 days after the gene delivery and remained normal during the entire follow-up period (Fig. 4a). In contrast, LentiLacZ-injected rats continued to display hyperglycemia, which necessitated termination of experiments within a month to prevent animal suffering. Furthermore, considerable weight loss was observed in LentiLacZ-injected hyperglycemia rats, while the LentiVIP-injected

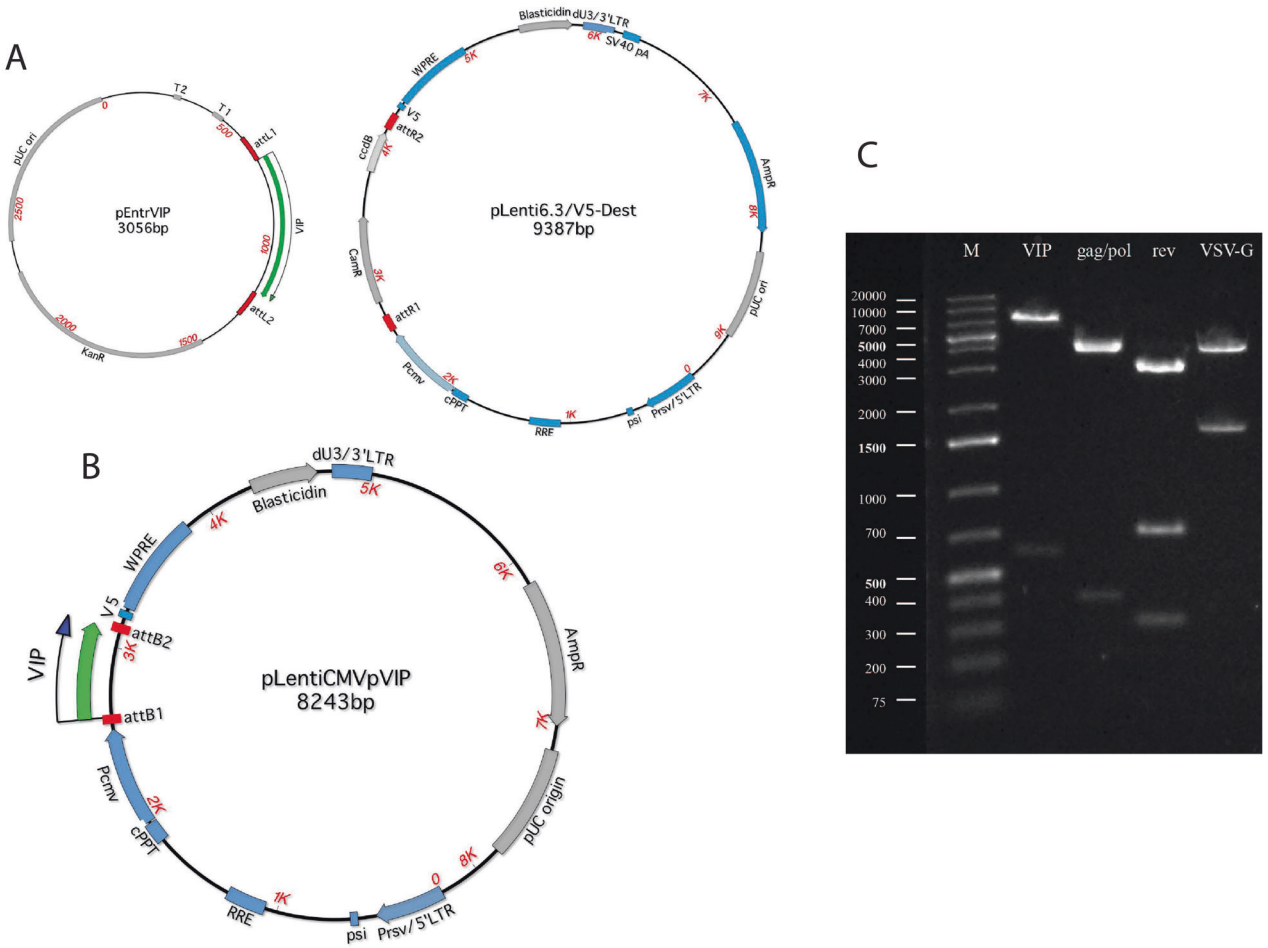

Fig. 2 Construction of pLentiVIP transfer plasmid for lentivirus vector production using Gateway Technology. LR recombination reaction between an entry vector and a destination vector (**a**) resulted in the generation of an expression clone (**b**). **c** Restriction enzyme analysis of pLentiVIP transfer plasmid and packaging plasmids. pLentiVIP (EcoRI and XhoI), gag/pol (pMDLg/pRRE-EcoRI), pRSV/Rev (EcoRI, Afl II), VSV-G (pMD2G-EcoRI). M: Gene ruler 1 kb plus.

normoglycemic rats gained weight over time (Fig. 4b). These results suggest LentiVIP injection into diabetic animals not only reverses hyperglycemia but also further prevents weight loss normally observed in diabetic animals.

Improved glucose tolerance in LentiVIP-injected rats correlated with higher serum VIP levels

To assess the blood glucose clearance profile of LentiVIP- and LentiLacZ-injected rats, a glucose tolerance test was performed 5 weeks after the gene delivery. For this purpose, both groups of rats received 2 g/kg of dextrose IP following 6-h fasting. Blood glucose levels were measured periodically during the 2-h time frame after dextrose injection. LentiVIP-injected rats displayed improved glucose tolerance, but the LentiLacZ-injected diabetic rats failed to clear blood glucose (Fig. 5a). We also measured VIP levels in blood 5 weeks after gene delivery. LentiVIP-injected rats manifested a twofold increase in serum VIP levels compared with LentiLacZ- or PBS-injected healthy controls (Fig. 5b). These results suggest an increase in serum VIP levels sustained from the LentiVIP vector accounts for the improved glucose tolerance observed in LentiVIP-injected normoglycemic rats.

VIP restores beta-cell loss in STZ-induced diabetes

Based on the data in Fig. 5 showing improved glucose tolerance following LentiVIP injection, we were interested to see the effect of LentiVIP on beta-cell mass. Rats were sacrificed 5 weeks after the gene delivery to observe any alterations in pancreatic beta-cell mass as a result of STZ injections. Pancreatic tissues were dissected and embedded in paraffin and later immunostained with an anti-insulin antibody to reveal the beta-cell content of the pancreatic islets. Interestingly, while there was a significant pancreatic beta-cell loss in LentiLacZ-injected animals correlating with hyperglycemia (Fig. 6a), strong insulin-specific

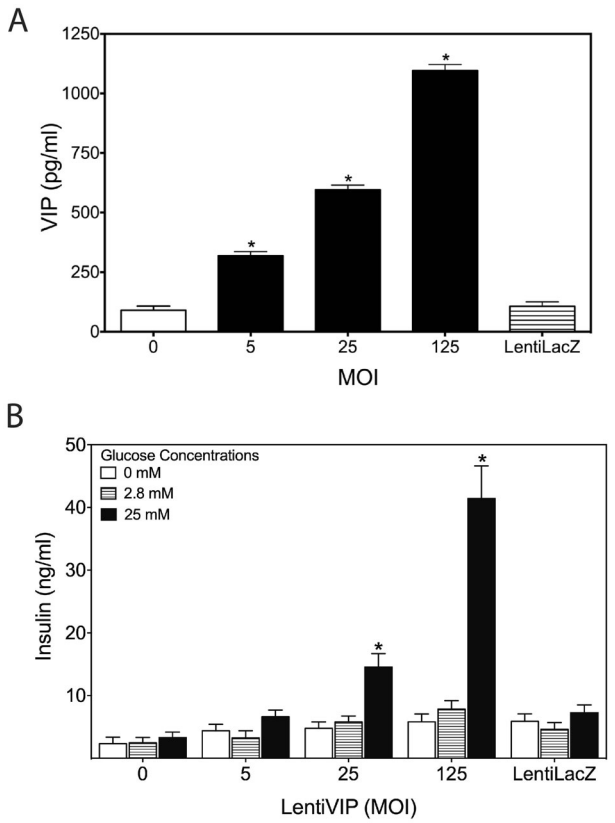

Fig. 3 In vitro expression and functional analysis of the LentiVIP vector. a LentiVIP transduction of HepG2 cells at increased MOI resulted in augmented VIP expression ($n = 6$, one-way ANOVA, Dunnett Multiple Comparison Test $p = 0.0001$). **b** Increased insulin secretion is observed in the LentiVIP-transduced pancreatic beta-cell line only at high glucose concentrations ($n = 6$, two-way ANOVA, $p < 0.001$). LentiLacZ vector is used at an MOI of 100.

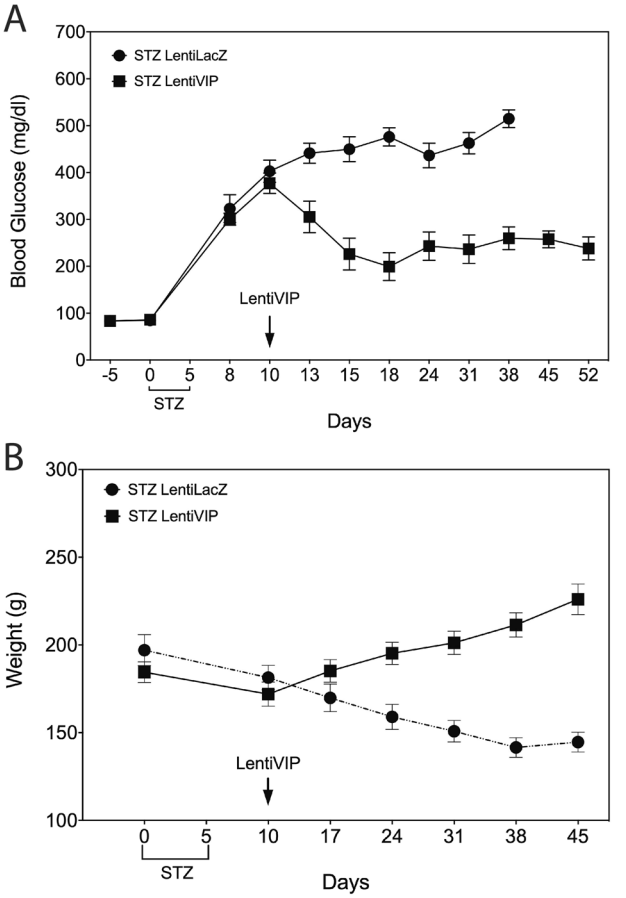

Fig. 4 Therapeutic efficacy of LentiVIP injection in STZ-induced rat model of diabetes. a LentiVIP injection decreased blood glucose levels in diabetic animals ($n = 8$/group, Mann–Whitney U Test, $p = 0.0138$). **b** While LentiLacZ-injected diabetic animals continued to lose weight, LentiVIP-injected rats progressively gained body weight ($n = 8$/group, two-way ANOVA, Sidak Multiple Comparison Test, $p < 0.0054$).

immunostaining of pancreatic islets was detected in normoglycemic LentiVIP-injected animals. These results suggest LentiVIP gene delivery somewhat reverses the STZ-induced pancreatic beta-cell damage. To reveal the mechanism of the VIP-induced restoration of pancreatic beta-cell mass, Ki-67 immunostaining was performed on pancreatic tissue sections. Scoring of Ki-67 positive beta cells revealed a significant increase in beta-cell proliferation rate in LentiVIP-injected normoglycemic animals compared with the LentiLacZ-injected diabetic animals (Fig. 6b). These data suggest the restoration of pancreatic beta-cell mass is due to VIP-enhanced beta-cell proliferation in the pancreas.

LentiVIP injection suppressed diabetes-induced inflammation

Glucotoxicity, inflammation, and oxidative stress induced by autoimmunity against islet beta cells increase in T1DM (Pietropaolo ve ark., 2007). Accordingly, serum CRP, oxidant, and antioxidant levels were measured in LentiVIP/LentiLacZ-injected animals to reveal the outcome of gene transfer on diabetes-related inflammation. Based on the statistical analysis, normoglycemic LentiVIP-injected animals displayed significantly lower levels of serum CRP and total oxidants compared with LentiLacZ-injected diabetic animals (Fig. 7a, b). As total antioxidant capacity was decreased in diabetic LentiLacZ-injected animals, it was increased back to normal levels in animals injected with LentiVIP (Fig. 7c).

Intraabdominal tissue distribution profile of lentiviral vectors following IP injection

To quantify the amount of virus integrated into the intraabdominal organs, qPCR was performed using genomic DNA isolated from various tissues of LentiVIP-injected animals. Table 1 indicates the liver contained the highest

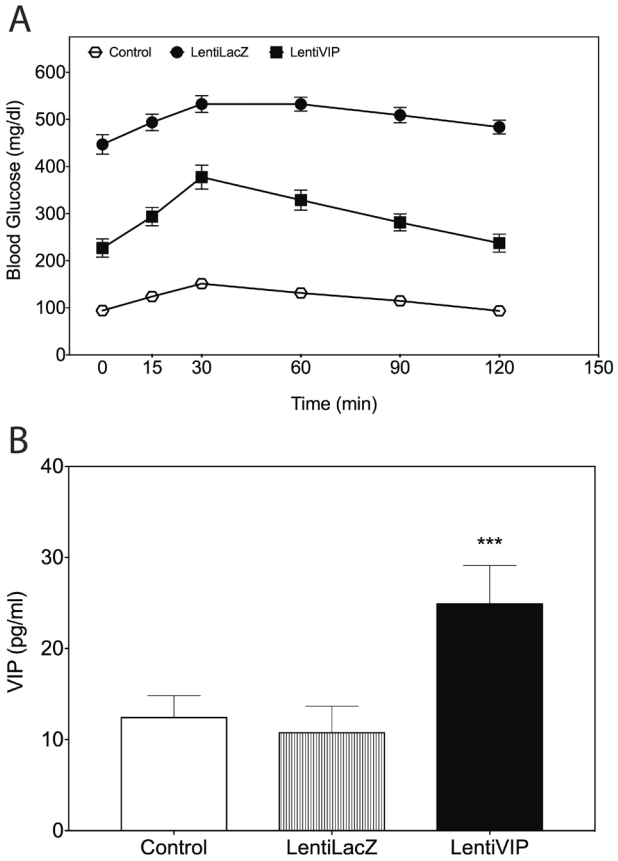

Fig. 5 Improved glucose tolerance in LentiVIP-injected rats correlated with higher serum VIP levels. a. Glucose tolerance test of rats injected with LentiVIP or LentiLacZ (**a**, $n = 8$/group, Tukey's Multiple Comparison Test, $p < 0.0001$). **b** Serum VIP levels following gene delivery ($n = 8$/group, one-way ANOVA, $p < 0.0001$).

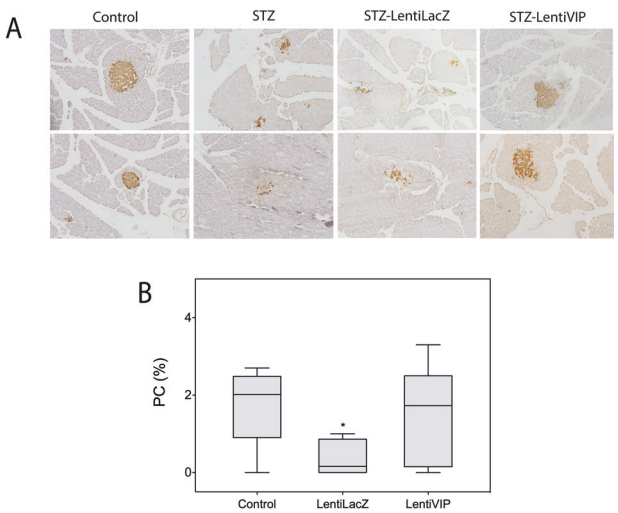

Fig. 6 VIP restores beta-cell loss in STZ-induced diabetes. a Insulin immunostaining of pancreatic sections following gene delivery ($n = 6$). **b** The proliferation rate of islet cells in all groups. Values are expressed as the median (the interquartile range). Statistical analysis was performed using the Kruskal–Wallis H test ($p < 0.05$).

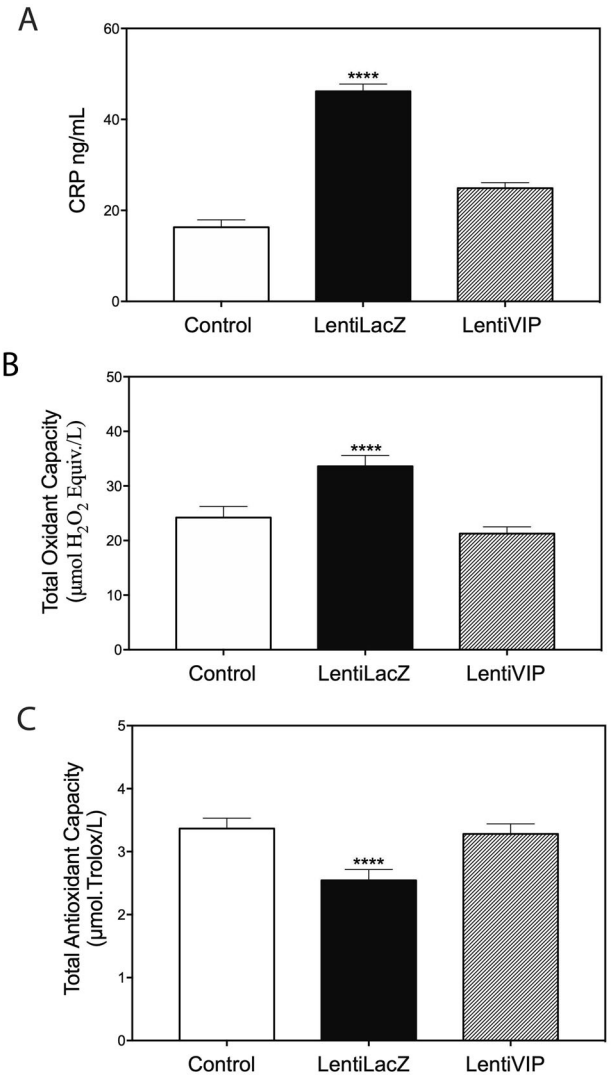

Fig. 7 LentiVIP suppresses diabetes-induced inflammation. a LentiVIP delivery decreased serum CRP levels in diabetic animals ($n = 8$/group, one-way ANOVA, $p < 0.0001$). Total oxidant (**b**) and antioxidant capacity (**c**) following gene delivery ($n = 8$/group, one-way ANOVA, $p < 0.0001$).

copy number of integrated lentivirus particles compared with other intraabdominal organs tested. Immunohistochemical staining in intraabdominal tissue sections from LentiVIP-delivered animals revealed the highest levels of VIP in liver sections, but VIP expression was also detectable in other intraabdominal tissues analyzed (data not shown).

Discussion

Type 1 Diabetes (T1DM) is a metabolic disease caused by the destruction of insulin-secreting pancreatic beta cells by autoreactive T cells [24]. All T1DM patients eventually

Table 1 Integrated genomic copy number of different tissues after intraperitoneal gene delivery using the LentiVIP vector (copy number[a] 10^5/ng).

	Control	LentiVIP
Pancreas	0.21 ± 0.08	5.45 ± 0.5
Liver	0.27 ± 0.06	8.38 ± 1.14
Spleen	0.24 ± 0.1	4.35 ± 0.64
Kidney	0.47 ± 0.22	5.16 ± 0.82

[a]Data are reported as means ± SEM.

become insulin dependent due to pancreatic beta-cell loss [25]. Despite the beneficiary effects provided by insulin replacement therapy, these regimens do not cure the disease but rather just delay the appearance of diabetic complications [26]. Thus, the autoreactive immune system has to be properly dealt with to successfully manage T1DM. Unfortunately, the use of immunosuppressive drugs for patients with T1DM is currently not recommended due to side effects and cost. Consequently, novel approaches with the potential to suppress islet-related inflammation and/or stimulate pancreatic beta-cell proliferation to compensate islet cell loss actively being explored to effectively treat patients with T1DM [27].

VIP is a neuroendocrine peptide with antidiabetic features including insulinotropic and anti-inflammatory properties [16]. However, the extent to which VIP overexpression stimulates pancreatic beta-cell proliferation in a diabetic setting remains unknown [11]. The NOD mouse has been the primary animal model for studying autoimmune diabetes, because of its striking similarity to the human disease [28]. At the same time, the progression of diabetes in NOD mice can be accelerated using cyclophosphamide (CP), an immunosuppressive drug modulating Th1/Th2 response [29]. Accordingly, plasmid-mediated VIP gene delivery reduced the incidence of CP-accelerated diabetes in NOD mice via shifting pro-inflammatory cytokine profile (Th1) to an anti-inflammatory type (Th2) [19]. The purpose of using plasmid-mediated gene delivery was to avoid the conventional problems of neuropeptide administration such as unstable biological activity, purification and treatment costs, and the need for frequent administration. Knowing that VIP can skew the pro-inflammatory cytokine profile to an anti-inflammatory type, antidiabetic properties of VIP was also tested in an animal model of autoimmune diabetes [30]. VIP-treated NOD mice did not develop diabetes and exhibited milder insulitis compared with non-treated controls. Beneficiary effects of VIP included (but were not limited to) the suppression of Th1 cytokines, activation of regulatory T cells, and increased IL-10 synthesis. The spontaneously diabetic NOD mouse is one of the most extensively studied animal models for T1DM used for more than 35 years [28]. However, spontaneous diabetes in NOD mice is quite variable and nearly takes 30 weeks to develop without intervention under pathogen-free conditions. To alleviate this problem, diabetes in NOD mice is often accelerated using agents like streptozotocin or CP [31]. Even though the NOD mouse is a useful preclinical model for studying T1DM, there are differences in many key aspects questioning whether data obtained from such models will apply to human patients [32]. Hence, investigators are strongly advised not to assume that the available spontaneous diabetic mouse and rat models represent complete surrogates for humans [33]. Since few patients with T1D exhibit the same clinical features as NOD mice, research in other animal models of diabetes are also required. Because the therapeutic effect of VIP has already been tested to some degree either in peptide form or in a plasmid in NOD mice in two similar studies [19, 30], we were motivated to use a different animal model of diabetes to assess VIP influence on beta-cell proliferation than the one used before. Besides, VIP exhibited some antidiabetic properties in animal models of Type 2 diabetes (T2DM). Our group has recently shown that lentivirus-mediated VIP gene delivery protected obese mice from developing diabetes [23]. Besides altered blood lipid profile and improved glucose tolerance, obese mice became resistant to STZ-induced diabetes upon therapeutic gene delivery. However, it remains undetermined whether LentiVIP administration would reverse STZ-induced hyperglycemia in obese-diabetic animals.

Gene therapy has the potential to treat human diseases with a single injection. Among the viral vectors tested, 3rd generation lentiviral vectors are safe and provide long-term gene expression suitable for treating complex genetic diseases like diabetes [34]. These attributes are what drove us to generate the lentiviral vector carrying the VIP transgene used in this study. To test the antidiabetic properties of lentivirus-mediated VIP gene delivery, we used an STZ-induced model of T1DM. MLD-STZ injections has also been used to induce IDDM in mice [35]. In this model, diabetes is induced by STZ mediated beta-cell toxicity as a result of T-cell-dependent immune reactions manifested by the presence of mononuclear cell infiltrates [36]. Consequently, IP injections of low-dose STZ (40 mg/kg) for 5 consecutive days into male C57Bl/6 or C57Bl/KsJ mice resulted in the induction of diabetes exhibiting insulitis [37, 38]. Th_1 pro-inflammatory cytokines (IFN-γ and TNF-α) produced by the autoreactive T cells were responsible for the beta-cell death observed in animal models of spontaneous diabetes, such as the NOD mouse and the Bio-Breeding (BB) Wistar rat. Considering cyclophosphamide accelerated diabetes in NOD mice, cyclophosphamide exerts its effects by shifting cytokine profile from Th_2 (IL-4 and IL-10) to Th_1 (IFN- γ and TNF-α). Similarly,

MLD-STZ administration into male C57BL/6 mice stimulated the production of IFN-γ and TNF-α, but significantly reduced IL-4 and IL-10 levels [36]. In terms of the activation of the cellular immune system, both models (spontaneously diabetic vs. MLD-STZ) share some similarities in the mechanisms of pancreatic beta-cell destruction. A single dose STZ injection rat model of T1DM [39, 40] and multiple low-dose STZ injection in a mouse model of T1DM [36–38] have been reported, but we found no reference of a multiple low-dose STZ injection rat model of T1DM. In this context, it is noteworthy to mention that BB rats are the most extensively studied rat model of T1DM [33]. They were originally derived from a Canadian colony of outbred Wistar rats. When we administered MLD-STZ into Wistar rats, we expected to simulate IDDM as observed in BB rats. Thus, we first optimized MLD-STZ injections to gradually induce diabetes on two different rat strains. In our hands, five daily injections of 30 mg/kg STZ successfully and reproducibly induced diabetes in both Wistar and Sprague Dawley rats. Hyperglycemia correlated with the loss in body weight and reduction in pancreatic islets cell mass, all of which are characteristic features of T1DM. Surprisingly, we could not find evidence of insulitis in our multiple low-dose STZ-induced rat model. We observed a few scattered mononuclear cells in the islets of STZ-treated rats, in contrast to the robust inflammatory cell infiltration observed in NOD mice. We can think of two reasons why we did not detect insulitis in our diabetic rats. First, it is possible that insulitis may have resolved by Day 10 (the earliest time point we analyzed islet morphology) after the last STZ administration. If this is the case, we could simply be missing the insulitis window. A second possibility is that the mononuclear cell infiltration in MLD-STZ-injected rats might be less apparent, as generally reported in diabetic patients. While insulitis is the characteristic lesion in young patients with the acute disease [41], it is often absent in young adults and older patients [42]. Historically, leukocytic infiltration was associated with islets in only 10% of patients with diabetes, but often under conditions in which more generalized pancreatitis was also present [43]. In accordance with this observation, studying young patients with a disease duration of <1 year revealed no sign of insulitis, suggesting lymphocytic insulitis was less common than suggested previously [44]. Considering the variability and intensity of insulitis between mice vs. humans, the relevance of the NOD mouse model for human T1DM has recently been challenged due to past disappointments when translating therapies from NOD mice to humans [28]. Based on the comprehensive research recently conducted using pancreata data from JDRF Network for Pancreas Organ Donors with Diabetes (nPOD) bio-repository, insulitis seen in humans appeared to be less severe and less frequent than what was observed in NOD mice [45]. In this particular study, insulitis was detected only in 23% of T1DM donors (18 of 80 donors) and 11% of islet autoantibody-positive donors without diabetes (2 of 18 AAb^{+} donors). Intriguingly, insulitis frequency was inversely correlated with the duration of diabetes. In another study, insulitis prevalence has been reported in 51% of patients (41 of 81 patients) with disease duration of <1 year, compared with only 3% of patients (4 of 132 patients) with duration >1 year [42]. But only certain lobes and a limited number of islets in the pancreas manifested lymphocytic infiltration. Based on this information, to observe the influence of VIP overexpression on insulitis, it might be helpful to use the NOD mouse, the BB Wistar rat, or MLD-STZ applied C57Bl/6 mice [32, 33, 36].

To test the in vivo therapeutic efficacy of LentiVIP in T1DM, diabetic rats were injected with LentiVIP or LentiLacZ and blood glucose levels were followed periodically. Blood glucose levels of diabetic animals were normalized within 5 days after LentiVIP gene delivery and normoglycemia persisted during the entire follow-up period. LentiVIP-injected rats exhibited improved glucose tolerance and body weight gain, and these features correlated with increased levels of VIP in the blood compared with LentiLacZ-injected diabetic animals. Similarly, transgenic mice overexpressing VIP in pancreatic beta cells displayed decreased plasma glucose and improved glucose tolerance in 70% depancreatized mice [46]. The fact that VPAC2 knockout mice displayed reduced glucose-induced insulin secretion further supports the importance of VIP on the maintenance of glucose homeostasis [47].

Immunohistochemical analysis of pancreatic sections using anti-insulin antibodies revealed pancreatic islet destruction in LentiLacZ-injected diabetic animals, while a considerable degree of islet cell restoration was observed in LentiVIP-injected normoglycemic animals. Moreover, Ki-67 staining suggested the restoration of islet cell mass was due to VIP-augmented pancreatic beta-cell proliferation, a novel finding in our study. The effect of VIP on cell proliferation appears to be dependent on the cell type. For example, VIP exhibits antiproliferative effects on vascular [48] and airway smooth muscle cells [49]. Intriguingly, islet cell mass was not altered in VIP knockout mice [50]. The fact that PACAP transgenic mice displayed increased beta-cell mass suggested PACAP might play essential roles in islet cell proliferation and differentiation [51]. Therefore, the lack of alteration in islet cell mass in VIP knockout mice could be attributed to the presence of compensatory mechanisms involving PACAP-induced activation of VPAC2 receptors present on pancreatic beta cells. Interestingly, the stimulatory effect of VIP on islet cell proliferation was not observed when it was administered into young normoglycemic mice [52]. This could be due to the instability of the VIP peptide in the blood or the necessity

for additional signals like cellular damage to induce proliferation. Carbachol plus PACAP/VIP can increase pancreatic beta cells through the FoxM1 pathway [53]. In this scenario, carbachol combined with either PACAP or VIP displayed a synergistic effect on the induction of cell cycle-related genes stimulating proliferation of pancreatic beta cells. The VIP/PACAP shared receptor, VPAC2, is the predominant receptor type in pancreatic beta cells [23] and its coupled to the stimulatory G protein (Gs). The fact that insulinotropic glucagon-like peptide 1 stimulated pancreatic beta-cell proliferation through the same Gs proteins suggests these insulinotropic peptides might enhance beta-cell proliferation using similar mechanisms [54]. However, the mechanism of VIP-induced beta-cell proliferation following STZ damage remains to be better clarified.

ROS and free radicals are produced during the progression of diabetes interfering with molecular functions of DNA, RNA, lipids, and proteins important for cellular activity. While these free radicals can enter the body from outside, they can also be generated during normal metabolic processes. Organisms possess antioxidant defense mechanisms to protect themselves from the detrimental effects of oxidants [55]. Under physiological conditions, there is a balance between oxidants and antioxidants which can be disturbed by acute or chronic inflammation resulting in tissue damage [56]. Inflammation is generally characterized by an increase in pro-inflammatory cytokine production such as C-reactive protein (CRP), TNF-α, and IL-6. Because CRP is very stable and has a longer half-life (20 h) compared with IL-6 and TNF-α, it has been considered to be a better marker to monitor systemic inflammation [57, 58]. Both glucotoxicity and autoimmune reaction against islet cells instigated inflammation and oxidative stress in T1DM [59, 60]. Expectedly, LentiLacZ-injected diabetic animals manifested higher serum CRP and oxidant levels compared with controls. Conversely, LentiVIP-injected rats displayed a decrease in serum CRP levels and oxidant capacity, but an increase in antioxidant capacity. These results suggest VIP gene delivery suppressed diabetes-related inflammation in our model. Tissue distribution profile of IP injected LentiVIP vectors were determined by qPCR. These results indicated that most intraabdominal organs analyzed were effectively transduced by lentiviral vectors. Immunohistochemical staining using VIP antibodies revealed the strongest VIP expression in the liver following lentiviral gene delivery.

3rd generation SIN lentiviral vectors like the one used in our study are considered to be one of the safest gene delivery vehicles among the other viral vectors used in the clinical trials [61]. To date, clinical trials using the 3rd generation lentiviral vectors did not reveal any concerns regarding integration-induced mutagenesis. FDA-approved Kymriah and EMA-approved Zynteglo are the two representative drugs in which the lentivirus vector was used as a transgene carrier. Despite this fact, the use of integrase-defective lentivirus vectors could be considered in future similar studies to formally investigate the potential for integration-induced mutagenesis. In conclusion, LentiVIP delivery reduced blood glucose, improved glucose tolerance, prevented body weight loss in multiple low-dose STZ-induced diabetes. Suppression of diabetes-induced inflammation resulted in the protection of pancreatic beta cells from apoptosis while VIP-induced beta-cell proliferation restored pancreatic beta-cell loss. Collectively, these data highlight the potential for LentiVIP as a novel gene therapy treatment agent for patients with T1DM.

Materials and methods

Animal studies

Sprague Dawley and Wistar rats (4-week-old) were obtained from the Animal Care Unit of Akdeniz University and housed in a humidity and temperature-controlled environment under a 12-h light/dark cycle and fed standard chow and water ad libitum. Animal handling conformed to the regulations of the Institutional Animal Care and Use Committee of Akdeniz University School of Medicine. The T1DM animal model was established via IP injection of multiple low-dose Streptozotocin (STZ; 30 mg/kg for 5 consecutive days—Sigma-Aldrich; St. Louis MO, USA). STZ was dissolved in 0.01 M citrate buffer (pH 4.5) just before use. Body weight changes and blood glucose levels were recorded periodically. Blood glucose was measured with Abbott FreeStyle glucose strips using Abbott glucometer. Rats were sacrificed as required and blood samples were taken by intracardiac puncture under anesthesia. After centrifugation of blood samples, serum was separated and stored at −20°C for further analyses. Abdominal tissues (liver, pancreas, kidney, and spleen) were fixed in formalin, dehydrated, and embedded in paraffin for immunohistochemical analysis.

Construction of HIV-based lentivirus encoding VIP

The open reading frame of the human VIP gene was obtained from the Invitrogen ORF Clone library (Invitrogen, IOH80326). The VIP coding sequence, 513 bp in length, was cloned into a pENTR221 entry vector containing the Kanamycin resistance gene. Plasmid purification was performed using Invitrogen Purelink HiPure Plasmid Midiprep Kit (Cat No. K2100-05). Taking advantage of the site recombination properties of Gateway technology, recombination between an entry vector and the pLenti6.3/

V5-DES destination vector generated the desired pLentiVIP expression clone. This transfer plasmid carrying the CMV-driven VIP encoding sequence, in addition to the lentiviral vector backbone, was generated using a Virapower HiPerform Lentiviral Gateway Expression Kit (Invitrogen, K5330-00). The transfer plasmid also included a Woodchuck posttranscriptional regulatory element (WPRE; needed for the increased transgene expression) and polypurine tract (cPPT) from the HIV-1 integrase gene (to increase transduction efficiency by facilitating the nuclear import of HIV-1 cDNA). Restriction enzyme analysis using EcoRI and XhoI followed by agarose gel electrophoresis was carried out to identify positive bacterial colonies. DNA sequence identity and the orientation were confirmed by sequencing using the Big DyeTerminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Cat No. 4336917) in ABI Prism 310 Genetic Analyzer. pLentiLacZ control vector encoding β-galactosidase was produced similarly using the expression vector (Plasmid 12108) obtained from the Addgene plasmid repository.

Production of HIV-based lentivirus encoding VIP

Lentivirus vectors were produced by mixing the transfer vector (pLentiVIP or pLentiLacZ) with three packaging plasmids [gag/pol (pMDLg/pRRE (HIV-1 pGag-Pol 12251)), rev (pRSV-Rev 12253), and VSV-G (pMD2.G (pVSV-G 12259))] obtained from the Addgene plasmid repository, using transient calcium phosphate transfection methodology in 293 T cells as described previously [62]. After harvesting and centrifugation of viral supernatant at $2000 \times g$ for 15 min to remove the cell debris, the supernatant was passed through a 0.45-μm filter before aliquoting into ultracentrifuge tubes (polyallomer ultracentrifuge tubes, Beckman Coulter, Cat No: 326823) for concentration. A sucrose cushion (10%) was included during ultracentrifugation to reduce vector toxicity. Ultracentrifugation was carried out at 25,000 rpm, 4 °C for 2.5 h using Beckman SW28 rotors in a Beckman Coulter Optima L-90K ultracentrifuge. The virus pellet was resuspended in HBSS and stored at −80 °C in aliquots. Concentrated viral samples were then purified by AEX chromatography after benzonase treatment to remove the residual cellular DNA [63]. The functional titer of lentiviral vector stocks was determined by qPCR by transducing HT1080 cells (ATCC®CCL-121™) with a serial dilution of lentivirus in the presence of polybrene (6 μg/ml). To do this, WPRE primers were used for the quantification of the viral genome, while albumin primers were used for the internal control. QuantiTect SYBR Green PCR Kit (Qiagen, Cat No: 204143) was used to perform the qPCR reaction in an ABI 7500 Fast Real-Time PCR System.

In vitro expression and functional analysis of lentiviral vectors

HepG2 cells grown in a 24-well cell culture plate were transduced at increasing doses of LentiVIP or LentiLacZ vector in the presence of polybrene (6 μg/ml). VIP levels in culture supernatants were determined by ELISA, with DPP-4 inhibitor added according to the manufacturer's instructions (Peninsula Laboratories, LLC, Bachem; Cat. No. S-1183, San Carlos, CA 94070, USA). The insulinotropic property of VIP expressed from the LentiVIP vector was confirmed by the GSIS. For this, the pancreatic beta-cell line Min6 (the gift of Prof. Dr. Jun-ichi Miyazaki, Japan) was infected with LentiVIP or LentiLacZ vector at increasing doses. Following the transduction, beta cells were exposed to low/high concentrations of glucose. The amount of insulin released from the transduced beta cells was determined by using an Ultra-Sensitive Mouse Insulin ELISA kit (CrystalChem, Cat No: 90080, Downers Grove, IL USA).

Histopathological studies

To evaluate STZ-induced pancreatic beta-cell damage and the outcome of gene transfer experiments for pathological analysis, animals were sacrificed 5 weeks after gene delivery. All dissected tissues, including pancreas, liver, spleen, and kidney, were fixed in formalin then embedded in paraffin. Tissue sections were analyzed initially by hematoxylin-eosin staining. Immunohistochemical analysis was performed using anti-insulin antibodies (Abcam, Cat No. ab63820) to visualize pancreatic beta cells or using anti-VIP antibodies to detect VIP expression in situ (Abcam, Cat No. ab8556). Ki-67 staining (Biorbyt LLC, St. Louis, US, Cat No. orb389335) [64] was performed to identify proliferating islet cells and the proliferation rate in each islet (%) was calculated as the number of proliferating cells/the total number of cells ×100%. Image J software (http://rsb.info.nih.gov/ij/) was used for imaging analysis.

Assessment of serum CRP, total oxidant capacity (TOC), and total antioxidant capacity (TAC)

CRP level was determined by using the CUSABIO ELISA kit (CSB-E07922r, Wuhan, Hubei, China). The TOC, and TAC of the subjects were measured as previously described [65, 66]. TOC analysis is a colorimetric method based on the oxidation of ferrous iron (Fe^{+2}) to the ferric iron complex (Fe^{+3}). In an acidic medium, ferric iron forms a colored compound. The intensity of this colored compound is then measured at 600 nm in a spectrophotometer. Hydrogen peroxide is used for the calibration and the result is given in

μmol H_2O_2 Equiv./L. The expected coefficient of variation of the method is <5%. TAC is determined by the antioxidant-induced color change in ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) measured at 660 nm using a spectrophotometer. Vitamin E is used for the calibration and the result is expressed in μmol H_2O_2 Equiv./L. The expected coefficient of variation of the method is <3%.

Statistical analysis

Statistical analyzes were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Data were presented as ±SEM and *P* value was determined as 0.05.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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