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Gene Therapy

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Aims and Scope

Gene Therapy covers both the research and clinical applications of the new genetic therapy techniques currently being developed. Over the last decade, gene therapy protocols have entered clinical trials in increasing

numbers and as they cover a wide spectrum of diseases, these studies promise to unite the diverse organ-based specialities into which modern medicine has become divided. *Gene Therapy* covers all aspects of gene therapy as applied to human disease, including:

- Novel technological developments for gene transfer, control and silencing
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- Cell-based therapies including all aspects of stem cells and genetically modified cellular approaches

Cover Legend: Anti-diabetic effects of lentivirus-mediated Vasoactive Intestinal Peptide (VIP) gene delivery in Diet-Induced Obesity animal model of Type 2 Diabetes. Transient transfection of 293T cells with a transfer vector and three packaging plasmids results in the production of lentiviral vectors carrying a VIP-encoding transgene (LentiVIP). Intraperitoneal injection of LentiVIP into obese mice broke-down insulin resistance, improved glucose tolerance and protected from STZ-induced diabetes. The intracellular signaling pathway triggered by VIP binding to the VIP-shared type 2 receptor (VPAC2) leading to augmentation of glucose-induced insulin secretion is displayed in the figure. See paper by Tasyurek et al. in this issue of Gene Therapy describing this novel gene therapy approach developed against diabetes.

BRIEF COMMUNICATION



HIV-based lentivirus-mediated vasoactive intestinal peptide gene delivery protects against DIO animal model of Type 2 diabetes

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Abstract

Type 2 diabetes mellitus (T2DM) is characterised by insulin resistance, glucose intolerance and beta cell loss leading to hyperglycemia. Vasoactive intestinal peptide (VIP) has been regarded as a novel therapeutic agent for the treatment of T2DM because of its insulinotropic and anti-inflammatory properties. Despite these beneficial properties, VIP is extremely sensitive to peptidases (DPP-4) requiring constant infusion or multiple injections to observe any therapeutic benefit. Thus, we constructed an HIV-based lentiviral vector encoding human VIP (LentiVIP) to test the therapeutic efficacy of VIP peptide in a diet-induced obesity (DIO) animal model of T2DM. *VIP* gene expression was shown by immunocytochemistry (ICC) and VIP peptide secretion was confirmed by ELISA both in HepG2 liver and MIN6 pancreatic beta cell lines. Functional properties of VIP were demonstrated by cAMP production assay and glucose-stimulated insulin secretion test (GSIS). Intraperitoneal (IP) delivery of LentiVIP vectors into mice significantly increased serum VIP concentrations compared to control mice. Most importantly, LentiVIP delivery in DIO animal model of T2DM resulted in improved insulin sensitivity, glucose tolerance and protection against STZ-induced diabetes in addition to reduction in serum triglyceride/cholesterol levels. Collectively, these data suggest LentiVIP delivery should be evaluated as an experimental therapeutic approach for the treatment of T2DM.

Introduction

Blood glucose levels are controlled by the combined and regulated actions of both the insulin released from pancreatic beta cells and glucagon secreted from alpha cells [1]. Glucose toxicity leading to Type 2 diabetes (T2DM) develops when there is an insufficient release of insulin from the pancreatic islets [2]. The amount of insulin released from the pancreas is determined by both intracellular and extracellular signals [3]. Insulin secretion is normally increased following food intake [4]. Postprandial insulin secretion is a complex physiologic process regulated by metabolic products such as glucose, gastrointestinal hormones like glucagon-like peptide-1 (GLP-1), glucosedependent insulinotropic peptide (GIP) and neurotransmitters released from autonomic nerves [5–7]. Interestingly, even before the postprandial increase in blood glucose, blood insulin levels may suddenly rise due to acetylcholine release as a result of vagal nerve activation [8]. However, there are other peptides in the cholinergic neurons with the potential to augment insulin secretion due to vagal nerve stimulation [9, 10]. For example, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclaseactivating polypeptide (PACAP) are two neurotransmitters capable of stimulating pancreatic beta cells to secrete insulin [11, 12] in addition to triggering cellular proliferation [13].

VIP is a 28 a.a. polypeptide secreted by gastrointestinal cells distributed throughout the intestinal tract. Upon VIP stimulation, intestinal cells secrete electrolytes and water. The fact that VIP expression is also localised to pancreatic islets suggested that VIP could function both as a neurotransmitter released from islet neurons and as an endocrine peptide secreted from pancreatic islets [14]. VIP-induced signalling is mediated by two VIP-shared type 2 receptors: VIP/PACAP

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Fig. 1 Establishment of diet-induced obesity/low-dose STZ animal model of diabetes. **a** Body weight gain in C57BL/6J mice fed HFD (n = 50) or SD (n = 25) (Mann–Whitney *U*-test p < 0.0001). **b** Laparoscopic examination of 8-week SD vs. HFD-fed mice with abdominal fat deposition indicated by black arrows. **c** Insulin sensitivity test conducted in C57BL/6J mice either fed SD (n = 20) or HFD (n = 20). Mice were fed the relevant diet for 8 weeks prior to conducting the

receptor 1 (VPAC1) and VPAC2 [15]. These receptors belong to the G-protein-coupled receptor family and activate adenylate cyclase (AC) through G proteins [16]. As a result of AC stimulation, cyclic adenosine monophosphate (cAMP) is produced from ATP activating protein kinase A (PKA) and/or cAMP exchange factor of EPAC family, closing KATP channels and leading to Ca^{2+} influx into cells, and finally inducing insulin secretion from pancreatic beta cells [17].

An ideal beta cell preserving agent should possess several features, such as stimulating glucose-induced insulin secretion, inducing beta cell replication and islet neogenesis, and protecting islets from apoptosis [18–20]. Since VIP possesses insulinotropic properties, activation of VIP receptors represents a new therapeutic strategy in T2DM. Glucagon secretion can also be induced by VIP, but only at low glucose concentration, which appeared to be beneficial to counteract insulin-induced hypoglycemia [21]. However, VIP is quickly degraded by dipeptidyl peptidase-4 (DPP-4), which is present in many tissues and involved in the functional regulation of several peptides such as GLP-1, GIP, PACAP, and VIP [22]. In this scenario, N-terminal dipeptides (X-Pro or X-Ala) are freed from the regulatory peptides by DPP-4 resulting in their inactivation [23]. Since the N-terminus of VIP is required for

experiment. Seven hours fasted mice were injected IP with 1 U/kg insulin Lispro (HUMALOG[®]). Blood glucose measurements were taken at 0, 15, 30, 60 and 120 min. **d** Glucose tolerance test. C57BL/6J mice fed 8-week SD (n = 20) or 8-week HFD (n = 20) were injected IP with 2 g/kg dextrose to raise blood glucose levels. Glucose levels were measured using blood obtained from the tail vein at 0, 15, 30, 60 and 120 min

the activation of VIP receptors, removal of the N-terminal end of VIP by DPP-4 blocks its agonistic activity [24].

VPAC2 receptors (but not VPAC1) are extensively expressed in pancreatic islets and not involved in the glycogenolitic pathways in liver unlike PACAP, suggesting VPAC2 selective agonists have the potential to stimulate insulin secretion and generate glucose tolerance without the activation of hepatic glucose production or hypoglycemia [25]. Despite limitations concerning stability of VIP peptides, VPAC2 selective agonists represent a good alternative in T2DM [26]. One significant limitation is the need for daily injections to reveal any therapeutic benefit from VPAC2 selective agonists in clinical settings. One way to relinquish the necessity of repeated administration of VPAC2 selective agonists and to relieve concerns over stability problems of VIP peptide is to develop VIP gene transfer applications to provide stable and long-term VIP gene expression in vivo. Gene transfer studies have been under development for the treatment of autoimmune diseases, including Type 1 diabetes [14]. Considering the variety of gene therapy vectors available, lentiviral vectors are the vector of choice when considering long-term gene expression, transduction efficacy and safety. However, the



Fig. 2 a Blood glucose levels of C57BL/6J mice fed SD (n = 15) or HFD (n = 15) (Mann–Whitney *U*-test, p < 0.0001). **b** Development of STZ-induced diabetes in obese vs. non-obese C57BL/6J mice. After feeding C57BL/6J mice with relevant diet for 8 weeks, STZ (150 mg/ kg IP) was injected to induce diabetes and blood glucose levels were recorded over a 28-day-period. Control mice (n = 15) were injected with PBS (One-way ANOVA with Bonferroni's multiple comparison test, p = 0.0002). **c** Insulin immunostaining (upper panel) and staining scores (lower panel) of pancreatic beta cells (n = 5/group). Treatment

efficacy of lentivirus-mediated *VIP* gene delivery remains to be tested in experimental animal model of T2DM. Since insulin resistance, glucose intolerance and hyperglycemia due to beta cell loss are the prominent features of T2DM, the high-fat diet/low-dose STZ diabetic animal model has been suggested for testing of the therapeutic efficacy of newly developed therapeutic agents for T2DM [27]. In this animal model, insulin resistance is generated via feeding animals with diets enriched in fat, and hyperglycemia is induced by STZ injection. Thus, an HIV-based lentivirusmediated *VIP* gene delivery was tested as a new experimental gene therapy strategy to provide sustained VIP peptide synthesis and secretion in vivo in a diet-induced obesity (DIO)/low-dose STZ animal model of T2DM.

Results

Diet-induced obesity (DIO)/low-dose STZ-induced animal model of diabetes

To establish obese mice with T2DM, 8-week-old C57BL/6J mice were fed high-fat diet (HFD) for 36 weeks. Measuring body weight over this timeframe revealed significant differences between HFD-fed mice and mice fed a standard diet (SD; Fig. 1a). There was an obvious increase in the amount of visceral fat in the HFD mice after 8 weeks on the HFD (Fig. 1b). Since insulin resistance and glucose intolerance are prominent findings in obese patients, insulin sensitivity

conditions are provided above each panel. Duplicate samples are provided for comparison. **d** Insulin scores of STZ-injected C57BL/6J mice are much lower compared to STZ-uninjected C57BL/6J mice regardless of diet type (One-way ANOVA with Tukey's Multiple Comparison Test, p = 0.001). **e** Glucagon immunostaining (upper panel) and **f** staining scores (lower panel) of pancreatic beta cells (n = 5/group). Treatment groups are provided above each photomicrograph and shown on *x*-axis in panel **b** (One-way ANOVA with Tukey's Multiple Comparison Test, p = 0.4390)

and glucose tolerance tests were conducted on mice fed HFD or SD, with only the HFD mice displaying insulin resistance (Fig. 1c) and glucose intolerance (Fig. 1d).

Even though the blood glucose levels of HFD-fed mice were consistently higher than SD-fed mice, all values were <180 mg/dl during the entire follow-up period (Fig. 2a). Since we considered mice diabetic when their blood glucose levels were > 250 mg/dl, HFD feeding alone was not sufficient to induce diabetes in obese C57BL/6J mice. To induce diabetes, Streptozotocin (STZ; 150 mg/kg) was injected intraperitoneally into the mice and blood glucose levels were recorded for the following 30 days. As expected, STZ induced diabetes in both SD- and HFD-fed mice (Fig. 2b). While blood glucose levels were > 250 mg/dl as early as 3 days following STZ administration in HFD-fed mice, it took longer (14 days) to establish diabetes in SD-fed mice. Furthermore, insulin immunohistochemical staining of pancreatic islets suggested more severe destruction of pancreatic islets in STZ-treated HFD-fed mice compared to SD-fed mice (Fig. 2c, d). The fact that neither HFD feeding nor STZ injections altered glucagon expression in alpha cells supported beta cell-specific action of STZ in pancreas (Fig. 2e).

VIP and VIP receptor expression profile in pancreatic islets of diabetic vs. non-diabetic C57BL/6J mice

To reveal any potential alterations in VIP and VIP receptor expression in obese, and obese diabetic mice compared to controls, 8-week-old C57BL/6J mice were fed HFD or SD Fig. 3 a VIP and VIP receptor expression on pancreatic islets of T2DM animal model (n = 5/group). Obese (HFD-fed) and non-obese (SD-fed) mice received IP injection of 150 mg/ kg STZ to induce diabetes. STZ uninjected SD-fed or HFD-fed animals were used as controls. **b** VIP expression scores. STZinjected obese diabetic and nonobese diabetic animals displayed lower levels of VIP expression compared to STZ uninjected counterparts (p = 0.0098)



and then sacrificed 28 days after STZ administration. Pancreata were dissected and prepared for immunohistochemical analysis to detect VIP, and VIP receptor (VPAC1 and VPAC2) expression (Fig. 3a). Low VIP expression was detected on pancreatic islets of SD- and HFD-fed mice (p = 0.22). In contrast, STZ-treated SD-fed and HFD-fed mice displayed a significant reduction in VIP expression due to destruction of pancreatic beta cells compared to untreated mice (Fig. 3b). When we analysed VIP receptor expression, no detectable VPAC1 expression on pancreatic islets was observed in any of the groups (Fig. 3a). Pancreatic islets of SD- and HFD-fed mice, however, displayed significant VPAC2 expression. Despite the smaller islet size after STZ treatment VPAC2 staining was still detectable in both the SD- and HFD-fed diabetic mice.

Construction of lentivirus vectors encoding VIP

After successfully establishing a T2DM mouse model, we next generated VIP-encoding lentiviral vectors and tested their therapeutic efficacy. Accordingly, the Gateway Hiperform Lentiviral Expression System was used to generate VIP-encoding lentivirus vectors (LentiVIP; Fig. 4). Site-specific recombination properties of bacteriophage lambda were used in the generation of the expression plasmid. Diagnostic restriction enzyme analysis was performed using *Eco*RI and *XhoI* enzymes to select correct clones carrying VIP transgene (Fig. 4a). Packaging plasmids were also cut with restriction enzymes to validate their identity (Fig. 4b). Orientation and sequence identity were confirmed by DNA sequencing (Fig. 4c). Calcium phosphate-mediated transient transfection method involving the use of three packaging plasmids and a transfer vector was deployed in 293T cells to produce LentiVIP vectors.

LentiVIP encodes functional VIP peptide

After constructing a lentiviral vector encoding VIP (Lenti-VIP), we infected HepG2 cells to test the extent to which VIP was expressed from the gene transfer vector. Immunohistochemical and enzyme-linked immunosorbent assay (ELISA) analysis of infected cells revealed VIP expression increased in a dose-dependent manner (Fig. 5).



Fig. 4 Construction of LentiVIP vector. *Eco*RI and *XhoI* restriction enzyme analysis of pLentiVIP expression clone is given in **a**. **b** Shows restriction enzyme confirmation of packaging clones, gag/pol (pMDLg/pRRE-Not I, Afl II), pRSV/Rev (Eco RI, Afl II), vsv-g (pMD2G-Eco RI, Not I), used in the lentiviral vector production. MW: Gene Ruler 1 kb Plus (Fermentas). **c** Physical map of LentiVIP showing the orientation of *VIP* gene in the destination vector. VIP:

vasoactive intestinal peptide sequence, pRSV: Rous Sarcoma Virus promoter; 5' LTR: modified HIV-1 5'- and 3'-long terminal repeats, Pcmv: Human Cytomegalovirus promoter, V5: C-terminal V5 epitope, attB: recombined attachment sites in expression vector, dU3/3'LTR: deletion of U3 region of the 3'-long terminal repeats, Blasticidin: Blasticidin resistance gene, AmpR: Ampicillin resistance gene, pUC ori: origin of replication site derived from pUC plasmid

To test to what extent pancreatic beta cells were transducable with LentiVIP, the pancreatic beta cell-line Min6 was infected with increasing LentiVIP doses. Similar to the HepG2 cells, increased VIP synthesis and secretion were detected with the Min6 cells (Fig. 6a). As all the in vitro assays conducted so far only validated VIP synthesis and secretion, we next evaluated the functional status of VIP encoded by LentiVIP. cAMP production assays demonstrated LentiVIP infection (but not LentiLacZ) resulted in increased cAMP production in Min6 cells (Fig. 6b). To define the insulinotropic potential of the LentiVIP-encoded VIP, a glucose-stimulated insulin secretion test (GSIS) was performed. LentiVIP transduction, but not LentiLacZ, increased insulin secretion only at the high-glucose concentration, suggesting LentiVIP indeed possesses insulinotropic activity (Fig. 6c).

LentiVIP injection reverses insulin resistance and improves glucose tolerance in obese C57BL/6J mice

After testing the in vitro functionality of LentiVIP, HFD-fed C57BL/6J mice were IP injected with LentiVIP to determine VIP production in vivo. We first determined the

distribution profile of lentivirus within various internal organs after IP injection. While the pancreas, liver and spleen were efficiently transduced with lentiviral vectors, gene delivery to kidney and heart was low (Fig. 7a). To correlate gene expression to lentivirus integration, immunohistochemistry analyses of abdominal organs (pancreas, liver and spleen) highly transduced with lentivirus vectors (based on quantitative PCR data) were performed following gene delivery. High levels of VIP were detected in pancreas, liver and spleen of LentiVIP-injected mice compared to those receiving LentiLacZ (Fig. 7b). We next determined VIP production in LentiVIP-injected mice. We detected ~2fold increase in serum VIP levels (1.7-fold with 10⁸ TU and 2.2-fold with 3×10^8 TU of LentiVIP) compared to PBSinjected mice (Fig 8a). We then assessed the antidiabetic efficacy of LentiVIP. Consequently, insulin-resistant and glucose intolerant HFD-fed obese mice received either LentiVIP or LentiLacZ, and insulin sensitivity and glucose tolerance tests were conducted 28 days later. While LentiLacZ-injected obese mice were completely resistant to insulin injection, LentiVIP delivery restored insulin sensitivity (Fig. 8b). Furthermore, glucose tolerance was only restored in LentiVIP-injected obese mice (Fig. 8c).

Fig. 5 a Immunohistochemical analysis of HepG2 liver carcinoma cell-line infected with increasing doses of LentiVIP (n = 6). "C" represents uninfected controls. b VIP secretion from LentiVIP-transduced HepG2 liver carcinoma cell-line detected by ELISA (n = 6, p < 0.0001, One-way ANOVA)



LentiVIP protects obese C57BL/6J mice from developing STZ-induced diabetes

After defining VIP expression in LentiVIP-treated obese mice, additional studies were conducted to determine the extent to which LentiVIP injection would prevent the development of T2DM in C57BL/6J mice. Specifically, C57BL/6J mice were fed HFD for 2 months. Insulin-resistant and glucose intolerant obese mice received an IP injection of either LentiVIP or LentiLacZ vector. One month after gene delivery, 150 mg/kg STZ was IP injected into both LentiVIP- or LentiLacZ-treated mice. Blood glucose levels were measured periodically over the next 28 days (Table 1). While T2DM developed in the LentiLacZ-injected, STZ-treated DIO mice, LentiVIP injection prevented development of disease (Fig. 9a), as blood glucose levels in the LentiVIP-injected mice remained normoglycemic throughout the entire follow-up period.

Pancreata of LentiVIP- or LentiLacZ-injected animals were dissected and prepared for histological analysis. Pancreatic beta cells were revealed by insulin immunostaining (Fig. 9b). LentiLacZ-injected DIO mice displayed significant reduction in beta cell mass, but pancreatic beta cells of LentiVIP-injected mice were protected from STZ- induced destruction (Fig. 9c). As LentiVIP-mediated protection was correlated with normoglycemia, destruction of pancreatic islets resulted in induction of T2DM in LentiLacZ-injected animals. While DIO mice displayed increased serum insulin levels correlating with insulin resistance, a drastic reduction in serum insulin levels was observed in LentiLacZ-injected mice due to the destruction of pancreatic beta cells by STZ leading to hyperglycemia (Fig. 9d). In contrast, serum insulin levels were normalised in LentiVIP-injected mice restoring insulin sensitivity similar to that seen in PBS-injected SD-fed control mice. We next analysed the potential alteration of serum lipid profile by gene delivery in DIO mice. LentiVIP injection reduced serum triglyceride levels of obese mice (Fig. 10a). In addition, serum cholesterol levels were also reduced in LentiVIP-injected DIO mice compared to DIO controls or LentiLacZ-injected DIO mice (Fig. 10b). Because LentiVIP delivery altered blood lipid profile of diabetic DIO mice, we decided to analyse endogenous VIP ligand and receptor expression in intra-abdominal tissues (liver and spleen) highly transduced with lentiviral vectors. Both VPAC2 (Fig. 11a) and VPAC1 (Fig. 11b) receptors were detectable in liver and spleen with VPAC2 being the predominant receptor type just like pancreas.

Fig. 6 a VIP secretion from LentiVIP-infected Min6 pancreatic beta cell line (n = 6,p < 0.0001, One-way ANOVA; C = Control). **b** LentiVIPmediated cAMP production in Min6 pancreatic beta cell line (n= 6, p < 0.0001, One-wayANOVA). Mock = uninfected cells. c LentiVIP transduction augments glucose-induced insulin secretion from pancreatic beta cell line. (n = 6, p < 0.0001,Two-way ANOVA) LentiLacZ vector was used as a control vector at an MOI of 100 both in cAMP production and GSIS tests



Discussion

Much like other members of the secretin protein family (e.g., GLP-1 and PACAP), VIP is a neuropeptide involved in the stimulation of insulin secretion from pancreatic islets [9, 28]. VIP is essential for the development of endocrine pancreas, as well as physiologic insulin secretion after meals and possess a broad spectrum of cellular activity [29-31]. For example, VIP functions as an anti-inflammatory agent involved in the suppression of Th1 responses and regulatory T cell-mediated induction of immune tolerance [32, 33]. Moreover, the therapeutic activity of VIP has been tested in several auto inflammatory diseases such as rheumatoid arthritis, ulcerative colitis and multiple sclerosis [34-37]. Clinical trials conducted to test the therapeutic efficacy of VIP to reduce pulmonary hypertension, obstructive pulmonary disease, migraine and sepsis (ClinicalTrials.gov identifiers: NCT00272896, NCT00464932, NCT00004494 and NCT00255320) revealed VIP was well tolerated and exhibited only minimal side effects [14].

Despite these positive aspects of VIP there are several obstacles that must be overcome for VIP-based treatment strategies to be effective in clinical settings. In particular, there is the necessity for multiple injections at high doses of VIP to observe any therapeutic benefit. Stable and longterm gene expression of VIP can be obtained by gene delivery, and viral and non-viral methods of introducing the VIP coding sequence have been under development [38, 39]. For example, plasmid-mediated VIP gene transfer protected NOD mice from cyclophosphamide-induced diabetes [36]. Similarly, recombinant adenoviruses encoding functional VIP inhibited proliferation of smooth muscle cells to relieve pulmonary arterial hypertension [40, 41]. Injection of a rAAV-2 vector encoding the human VIP gene into submandibular glands of NOD mice resulted in immunosuppression of Sjogren's syndrome [39]. Lastly, lentiviral vectors encoding VIP successfully blocked both autoimmune and inflammatory response in an experimental animal model of collagen-induced arthritis [35]. These gene transfer studies demonstrated therapeutic benefit of VIP gene delivery in several autoimmune diseases, but the development of an effective gene transfer vector carrying VIP faces several limitations. First, the clinical efficacy of plasmid DNA transfer is low. The therapeutic efficacy of adenoviral vectors is restricted because these vectors are perceived as an antigen by the immune system, and can only provide transient gene expression [42-45]. AAV has low transduction efficacy and limited cargo capacity [46-49]. Conversely, lentiviral vectors can provide long-term gene expression without toxicity [50], and they do not possess an oncogenic risk due to self-inactivation following insertion. Thus, an HIV-based lentivirus vector was chosen

Fig. 7 a Internal tissue distribution profile of LentiVIPinjected C57BL/6J obese mice (n = 5/group). **b** VIP immunohistochemistry analyses of abdominal organs highly transduced with lentivirus vectors (pancreas, liver and spleen n = 5/group). Control indicates tissue sections of HFD rats without VIP primary antibody treatment



to deliver VIP transgene (LentiVIP) for the studies described herein.

HFD-fed, low-dose STZ-injected mice are a well-defined model of T2DM [51], prompting its use to test the therapeutic efficacy of LentiVIP [27, 52]. We saw a statistical difference in body weight between SD-fed vs. HFD-fed mice after 8 weeks on the HFD and blood glucose levels of HFD-fed mice were higher than SD-fed mice, but it was not high enough to be considered diabetes. The DIO mice did display insulin resistance and glucose intolerance—traits considered to be prediabetic indications associated with abdominal fat deposition. STZ injection (150 mg/kg) was needed to induce diabetes in both obese and non-obese mice. Higher blood glucose levels and beta cell loss as revealed by the immunohistochemistry analysis indicated that pancreatic islets of HFD-fed mice were more sensitive

to STZ injection, correlating with the severity of diabetes, than those of SD-fed mice.

Before we started the gene transfer studies, we wanted to define the endogenous VIP and VIP receptor expression profiles on pancreatic islets of HFD-fed/low-dose STZinjected diabetic mice. Pancreatic islets of SD- or HFD-fed mice displayed low VIP expression, no detectable VPAC1 expression, but significant VPAC2 expression. Immunohistochemistry analysis revealed VIP expression in pancreatic islets was drastically reduced both in obese and non-obese diabetic mice following STZ injections. Since VPAC2 is a natural VIP receptor expressed extensively in pancreatic islets [53], VIP gene delivery was expected to function similar to VPAC2 selective agonists. Potential therapeutic effects of VIP regarding T2DM may include insulinotropic action, which is essential to improve



Fig. 8 a IP injection of LentiVIP in HFD-fed C57BL/6J mice (n = 4/ group) increased serum VIP levels in blood (p < 0.0001, Two-way ANOVA). PBS-injected C57BL/6J obese mice were used as controls. **b** LentiVIP injection (n = 10) but not LentiLacZ (n = 14) altered insulin sensitivity in C57B/6J mice (p < 0.0001, Two-way ANOVA; Bonferroni's post tests: SD vs. HFD LentiLacZ, p < 0.001; SD vs. HFD LentiLacZ (n = 14) injected animals (p < 0.0001, Two-way ANOVA; Bonferroni's post tests: SD vs. HFD LentiLacZ, p < 0.001; SD vs. HFD LentiLacZ (n = 14) injected animals (p < 0.0001, Two-way ANOVA; Bonferroni's post tests: SD vs. HFD LentiLacZ, p < 0.001; SD vs. HFD LentiVIP, p > 0.05). SD-fed mice (n = 11) were used as controls in insulin resistance and glucose tolerance tests

glucose tolerance, ability to induce islet cell proliferation and differentiation and to protect islet cells from apoptosis [14].

We generated lentivirus vectors pseudo-typed with Vesicular Stomatitis Virus G glycoprotein carrying VIP. VIP expression and secretion were confirmed in HepG2 liver carcinoma cells using immunocytochemistry and ELISA assays. In vitro functional analysis of LentiVIP was carried out using Min6 pancreatic beta cells. Functional properties of LentiVIP vector were confirmed using cAMP production and GSIS tests. Before continuing with in vivo therapeutic applications, it was pertinent to show serum VIP levels could be raised by IP injection of LentiVIP in HFDfed mice. LentiVIP injection into obese mice reversed insulin resistance and improved glucose tolerance. In addition, LentiVIP protected obese mice from STZ-induced diabetes. Normoglycemia was maintained during the entire follow-up period, and correlated with the protection of beta cell mass as revealed by immunohistochemistry studies. While LentiLacZ-injected obese mice displayed a drastic reduction in serum insulin levels leading to hyperglycemia following STZ injection, normal serum insulin levels were maintained in LentiVIP-injected obese mice compared to PBS-injected obese mice. Liver is one of the major organs playing key roles in lipid metabolism concerning fatty acid synthesis and lipid circulation [54]. Since both VPAC1 and VPAC2 expressions are present in liver, high VIP expression in liver following LentiVIP gene delivery might affect lipid metabolism of obese diabetic animals. However, the mechanism of how LentiVIP gene delivery influences blood lipid profile remains to be studied.

In conclusion, our results suggest VIP gene delivery mediated by an HIV-based third generation lentivirus has the capacity to reverse obesity-related prediabetic complications and protect pancreatic islets from STZ-induced destruction in obese C57BL/6J mice.

Materials and methods

Animal handling and care

C57BL/6 mice are regarded as an obesity prone animal species, leading them to be considered as the gold standard for studies where DIO is involved [55]. Because male C57BL/6J mice are more susceptible to DIO than females [56, 57], we used C57BL/6 male mice in our study. Male C57BL/6J mice were obtained at 4 weeks of age and housed in a humidity and temperature controlled room under a 12-h light/dark cycle. Diets and tap water were provided ad libitum and sterilised bedding was provided by Animal Care Laboratory of Akdeniz University Hospitals. At 8-weeks of age C57BL/6 mice were maintained on a regular diet (SD) or switched to a high-fat diet (HFD) where 60% of the calories come from fat. After 8 weeks, SD- and HFD-fed mice received intraperitoneal injection of STZ (150 mg/kg

Days	HFD $(n = 8)$	HFD STZ LentiLac $(n = 9)$	HFD STZ LentiVIP $(n = 10)$
0	131.9 ± 7.1	128.7 ± 6.2	164.5 ± 14.3
3	137.0 ± 10.3	210.8 ± 49.4	188.9 ± 19.3
7	154.0 ± 10.6	323.5 ± 44.3	188.8 ± 19.2
10	143.5 ± 10.4	378.0 ± 56.3	224.3 ± 31.4
14	159.3 ± 14.9	337.6 ± 27.6	240.6 ± 41.26
21	165.3 ± 11.6	321.3 ± 14.4	194.5 ± 22.9
28	157.3 ± 14.6	326.1 ± 19.8	224.5 ± 25.5

 Table 1 Blood glucose measurements^a

^aData are reported as means ± SEM

body weight; Sigma, St. Louis, MO). Cages were changed twice a week after STZ injection to provide dry bedding for polyuric animals. Age-matched, non-diabetic C57BL/6J mice were used as control. Mice were handled in accordance with the regulations of the Institutional Animal Care and Use Committee of the Akdeniz University School of Medicine (Approval Protocol Number B.30.2. AKD.0.05.07.00/05). Blood samples were collected via intracardiac puncture, and blood serum samples were obtained using Serum Separator Vacutainer (Vacuaette, Greiner bio-one, Cat. No. 454343) by centrifugation for 15 min at 3000 rpm. Blood serum samples were stored at -20° C until use. Insulin sensitivity test was performed using the fast-acting insulin analogue Lispro (HUMALOG®).

Generation of lentivirus vectors encoding human vasoactive intestinal peptide

The open reading frame (ORF; 513 bp) of human vasoactive intestinal peptide was obtained from Invitrogen ORF clone library (Invitrogen, IOH80326) and cloned into an entry vector pENTR(tm)221 carrying a Kanamycin resistance gene. Gateway Technology was employed to generate the lentiviral expression clone (pLentiVIP). For this purpose, the VIP transgene was cloned into pLenti6.3/V5-DEST Gateway Vector using Virapower Hiperform Lentiviral Gateway Expression Kit (Invitrogen, K5330-00). To generate the expression clone (the transfer plasmid), LR recombination reaction was carried out in the presence of Gateway LR Clonase II Enzyme Mix as suggested by the manufacturer. The reaction mixture was transformed into OneShot Stb13 Chemically Competent E. coli. Following plasmid isolation, EcoRI and XhoI double restriction enzyme digests were performed to identify colonies carrying the right size of inserts. The orientation and the sequence information of the vector were confirmed by DNA sequencing analysis using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Cat No. 4336917) with ABI Prism 310 Genetic Analyser.

Lentivirus production

pLentiVIP transfer vector was constructed as detailed above. Three additional plasmids (pMDLg/pRRE, HIV-1 pGag-Pol 12251), Rev-Plasmid (pRSV-Rev 12253) and pMD2.G (pVSV-G 12259; Addgene) were used to provide trans-acting factors required for packaging. While LV-RFP plasmid (Plasmid 26001) was used to optimise lentivirus production process, LV-Lac plasmid (Plasmid 12108) was used to generate LentiLacZ vector encoding beta galactosidase. To produce lentivirus vectors, 293T cells (ATCC) were first expanded in medium containing Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/l D-glucose, 4 mM L-glutamine, sodium pyruvate) with 10% FBS, 1% Pen + Strep using CellStar[®] T-175 cm² flasks (Cat No: 660175, Greiner Bio-One GMbH, Frickenhausen, Germany). Then, 80×10^6 293T cells were passaged into Cell MasterTM roller bottles with 1700 cm² ribbed surface (Cat No: 681 062, Greiner Bio-One GMbH, Frickenhausen, Germany). In all, 293T cells were allowed to adhere to the roller bottle surface by incubating at 0.3 rpm for 24 h and then the rolling speed was raised to 1 rpm for an additional 12-16 h in a HERAcell 240i CO₂ incubator (Thermo Scientific, Waltham, MA, USA). The cell expansion media (4.5 g/l Dglucose, 4 mM L-glutamine, sodium pyruvate) was replaced 1 h before the transfection with 180 ml of transfection media (Iscove's Modified Dulbecco's Medium (IMDM), Sigma, Cat No. I7633) containing 10% FBS, 1% Pen + Strep and 25 µM Chloroquine (Sigma, Cat No. C6628). The roller bottle was placed back into 37 °C, 5% CO₂ incubator. For transient transfection, 176 µg pGag-Pol, 68 µg pRSV-Rev, 95 µg pVSV-G and 270 µg pLentiVIP were mixed in a 50 ml conical tube, then the volume was raised to 10.5 ml with $0.1 \times \text{TE:dH}_2\text{O}$ (2:1 v:v). In addition, 1.5 ml 2 M CaCl₂ in ddH₂O was added to the mixture to obtain a final concentration of 0.25 M CaCl₂. A 5 ml serological pipet was used to expel bubbles into 12 ml 2 × hepes buffered saline (HBS, pH 7.0) transferred into a new 50 ml conical tube. The DNA/CaCl₂ mixture was added dropwise into the bubbling 2×HBS solution. Following incubation at room



Fig. 9 a LentiVIP injection protected obese mice from developing STZ-induced diabetes. HFD-fed obese mice received IP injection of either LentiVIP (n = 10) or LentiLacZ (n = 9). One month after gene delivery, both groups received IP injection of 150 mg/kg STZ to induce diabetes (p < 0.0001, Two-way ANOVA; Bonferroni's post tests: HFD STZ LentiLacZ vs. HFD Control, p < 0.001; HFD STZ LentiLacZ vs. HFD Control vs. HFD STZ LentiVIP, p > 0.05; HFD Control vs. HFD STZ LentiVIP, p > 0.05). **b** Insulin immune-staining of pancreatic

temperature for 5 min, the roller bottle was removed from the incubator and the mixture was added into the transfection media. The roller bottle was placed back into the incubator and kept at 0.3 rpm for 6–8 h. After the removal of the transfection media by aspiration, 200 ml collection media (OPTI-MEM, Invitrogen,) with 10% FBS, 1% Pen + Strep were added to the cells and incubated for additional 2.5 days at 1 rpm, 37 °C, 5% CO₂ for the lentiviral vector production process.

Viral supernatants were harvested, centrifuged at $2000 \times g$ for 15 min to remove the cell debris and filtered through 0.45 µm prior to concentration. Unconcentrated virus solution was aliquoted into ultracentrifuge tubes (pollyallomer ultracentrifuge tubes, Beckman Coulter, Cat No.326823). Virus purification was done using a 10% sucrose solution cushion ultracentrifuged at 25,000 rpm, 4 °C for 2.5 h using Beckman SW28 rotors in a Beckman Coulter Optima L-

sections to reveal pancreatic beta cell content (n = 6/group). Insulin (+) area is given in panel **c** (n = 6/group, LentiLacZ vs. (SD or HFD) p < 0.0001; LentiLacZ vs. LentiVIP p = 0.001; LentiVIP vs. (SD and HFD) nonsignificant p > 0.05. **d** Serum insulin levels of SD-fed (n =5) and HFD-fed (n = 5) C57BL/6J mice compared to STZ-injected LentiVIP (n = 5) or LentiLacZ (n = 9) delivered obese mice as determined by Mouse Insulin ELISA (p = 0.0003, One-way ANOVA)

90K ultracentrifuge. After aspiration of the supernatant, tubes were allowed to drain upside-down on a clean paper towel for the removal of the residual supernatant. HBSS (100 μ l) was added to each pellet, and parafilm-sealed tubes were left for incubation overnight at 4 °C. The pellets were resuspended by pipetting and collected into microcentrifuge tubes. Viral stock was stored at -80 °C in aliquots of 50–100 μ l prior to titering. In all, 3×10^8 TU LentiVIP or LentiLacZ was used for in vivo therapeutic applications concerning insulin sensitivity, glucose tolerance, induction of STZ-induced diabetes and blood lipid profile.

p24 ELISA for physical titering of lentiviral vectors

The QuickTiterTM HIV Lentiviral Quantitation Kit (HIV p24 ELISA-CellBiolabs, Inc., Cat No. VPK-108-H) was used to titre lentiviral stocks.



Fig. 10 Effect of gene delivery on blood lipid profile of C57BL/6J mice. Plasma triglyceride (**a**, p < 0.0001, One-way ANOVA) and cholesterol levels (**b**, p < 0.0001, One-way ANOVA) of SD-fed (n = 5) and HFD-fed (n = 5) C57BL/6J mice compared to STZ-injected LentiVIP (n = 5) or LentiLacZ (n = 9) delivered obese mice

Functional titration of lentiviral vectors using realtime PCR

HT1080 cells (ATCC[®] CCL-121TM) were seeded into 24well plates (50,000 cell/well) the day before transduction. Cells were then transduced with increasing amounts of the lentivirus stock $(10^{-4}, 10^{-2}, 1, 2, 4, 8 \mu l)$ in the presence of polybrene (6 µg/ml). Lentivirus-transduced HT1080 cells were further incubated for 3 days at 37 °C in a 5% CO₂ incubator. The culture medium was removed and 100 µl/ well lysis buffer was added for 2 min at 96 °C, after which the samples were centrifuged at 14,000 rpm for 2 min. Samples were then incubated on ice or stored at -20 °C until the reaction setup. For real-time (RT-)PCR reactions, WPRE primers (Fwd: 5'-CCGTTGTCAGGCAACGTG-3'; Rev: 5'-AGCTGACAGGTGGTGGCAAT-3') were used for the quantification of viral genome, while Albumin pri-5'-GCTGTCATCTCTTGTGGGCTGT-3'; (Fwd: mers

Rev: 5'-ACTCATGGGAGCTGCTGGTTC-3') were used for the internal control. A standard curve was established using different concentrations of albumin plasmid (Addgene). The RT-PCR reaction was carried out using Quantitect SYBR Green PCR Kit (Qiagen, Cat No: 204143) in a ABI 7500 Fast Real-Time PCR System.

Histological studies

To determine the extent of pancreatic beta cell destruction within islets, mice were sacrificed 30 days after STZ injection. The pancreas was fixed in formalin and then embedded in paraffin. Sections were cut and hematoxylin and eosin (H&E) staining was performed, as well as insulin staining using polyclonal rabbit anti-insulin antiserum (Abcam, Cat No. ab63820). Images of the beta cell area on each section were captured on Olympus IX81 motorised inverted fluorescent microscope. The insulin positive and total pancreas area were quantified with Image J software (National Institutes of Health, Bethesda, MD; http://rsb. info.nih.gov/ij/) to reveal relative ratio of insulin positive areas to the entire pancreas. Both serum insulin and plasma lipid levels were measured in blood samples collected post mortem (30 days after STZ injection).

VIP and receptor profiles in pancreatic islets of T2DM animal model

Pancreata were harvested from animals sacrificed 2 months after feeding HFD and 1 month after STZ injections. Antigen retrieval was performed in TRIS-EDTA pH 9.0. EXPOSE Rabbit specific HRP/DAB detection IHC kit (Abcam, ab80437) was used for peroxide and protein blocking on tissue sections. Immunohistochemical analysis was performed by using five different primary antibodies to evaluate insulin (Abcam, Cat No. ab63820; 1/2000), glucagon (Abcam, Cat No. ab18461; 1/400), VIP (Abcam, Cat No. ab101959; 1/100), VPAC1 (Millipore, Cat No. AB2265; 1/250) and VPAC2 (Bioss, Cat No. bs-0197R; 1/ 250) expression profiles in pancreas. Stained sections were evaluated using Olympus IX81 motorised inverted fluorescent microscope. Assessment scores were given as described previously [58, 59]. VIP and receptor profiles of tissues highly transduced with lentiviral vectors (pancreas, liver and spleen) in animals sacrificed 1 month after STZ injection were revealed using above described method.

VIP enzyme immunoanalysis

HepG2 cells (ATCC) were seeded at 50,000 cells/well in 24-well plates and incubated 24 h at 37 °C. Cells were infected with lentivirus at increasing multiplicity of infection (MOI) in the presence of polybrene ($6 \mu g/ml$) for 24 h



Fig. 11 Immunohistochemistry analysis (n = 5/group) of VPAC2 (a) and VPAC1 (b) expressions in liver and spleen following LentiVIP or LentiLacZ gene delivery. Control represents sections from HFD-fed animals stained in the absence of VIP primary antibody

at 37 °C prior to medium exchange. VIP levels in culture supernatants were determined by ELISA according to the manufacturer's instructions (Peninsula Laboratories, LLC, Bachem; Cat. No. S-1183, San Carlos, CA 94070, USA).

VIP immunocytochemistry

LentiVIP-transduced HepG2 cells were washed with PBS, and then fixed with %4 paraformaldehyde prior to treatment with 1% Triton-X 100 prepared in phosphate buffered saline with Tween-20 (PBST). After blocking with 1% bovine serum albumin (BSA), cells were incubated with rabbit polyclonal antibody developed against VIP (Abcam, ab8556). After overnight incubation at 4 °C, cells were washed with PBS again and horseradish peroxidaseconjugated goat anti-rabbit IgG was applied as a secondary antibody (Abcam, ab97051). Cells were visualised using EXPOSE Rabbit Specific HRP/DAB detection IHC kit (Abcam, ab80437) and images were captured by Olympus IX81 inverted fluorescent microscope.

In vitro functional analysis of LentiVIP vector

MIN6 pancreatic beta cell line (kindly provided by Prof. Jun-Ichi Miyazaki, Japan) were transduced with VIPencoding lentiviral vectors in increasing doses at MOIs of 5, 25 and 100. One-hundred MOI LentiLacZ-transduced cells were used as control. Following transduction, cAMP levels were measured with cAMP Direct Immunoassay Kit (ab65355) according to the manufacturer's protocol. Glucose-stimulated insulin secretion (GSIS) of LentiVIPtransduced MIN6 cells were conducted in combination with insulin ELISA following glucose stimulation. To do this, pancreatic beta cell line MIN6 cells were plated in 96-well culture plates at 1×10^5 MIN6 cells/well overnight. MIN6 cells were then transduced with LentiVIP at increasing MOIs (5, 25 and 100) of the vector in the presence of polybrene (6 µg/ml). The day after the transduction the medium was removed, and replaced with 250 µl DMEM without polybrene. Lentivirus-transduced MIN6 cells were further incubated for 7 days at 37 °C in a 5% CO₂ incubator. Then, the supernatant was harvested for ELISA analysis and stored at -20 °C. To perform GSIS assay, lentivirustransduced MIN6 cells were washed with PBS, then treated with Krebs Ringer Buffer (KRB) without glucose. All wells were preincubated in KRB containing 2.8 mM glucose for 30 min at 37 °C. The medium was then removed and cells were washed with glucose-free KRB again. Cells were incubated with 250 µl KRB supplemented with 0, 2.8 or 25 mM glucose for 1 h at 37 °C. Supernatants were collected and stored at -20 °C prior to detection of secreted insulin. Insulin levels of culture supernatants collected from lentivirus-transduced MIN6 cells and blood samples drawn after in vivo gene delivery were detected using an Ultra-Sensitive Mouse Insulin ELISA kit (CrystalChem, Cat No: 90080, Downers Grove, IL USA) according to the manufacturer's instructions.

Detection of blood lipid profile of LentiVIP or LentiLacZ-injected animals

Lipid measurements were made using blood drawn 1 month after STZ injection. Plasma triglyceride levels were measured using EnzyChrom Triglyceride Assay Kit (BioAssay Systems, Cat No. ETGA-200). Cholesterol/Cholesteryl Ester Quantitation Kit (Abcam, #ab65359) was used to determine blood cholesterol levels.

Tissue tropism of lentivirus vectors as revealed by genome integration studies

Following lentivirus production, LentiVIP vectors were administrated to obese C57BL/6J mice 2 months after feeding HFD by intraperitoneal injection. Animals were sacrificed one month after STZ injection. Then, pancreas, liver, spleen, kidney and heart tissue were dissected and stored at -80 °C. DNA isolation was performed using DNeasy Blood & Tissue Kit (Qiagen, 69506), and quantitative PCR reactions were performed using WPRE primers (Fwd: 5'-CCGTTGTCAGGCAACGTG-3'; Rev: 5'-AGCTGACAGGTGGTGGCAAT-3'). RT-PCR reactions were carried out with Quantitect SYBR Green PCR Kit (Qiagen, Cat No.: 204143).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data are presented as means +/- SEM. A *p*-value of 0.05 was considered as significant.

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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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