Genes & Diseases (2023) 10, 1101–1113

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.keaipublishing.com/en/journals/genes-diseases

FULL LENGTH ARTICLE

Prolonged control of insulin-dependent diabetes via intramuscular expression of plasmid-encoded single-strand insulin analogue

Lu Deng [a,1], Ping Yang [a,1], Caixia Li [b,**], Lifang Xie [a], Wanling Lu [a], Yanhan Zhang [a], Ming Liu [c], Gang Wang [a,*]

[a] *National Engineering Research Center for Biomaterials, Sichuan University, Chengdu, Sichuan 610064, China*
[b] *Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Sichuan 610041, China*
[c] *Department of Abdominal Oncology, West China Hospital, Sichuan University, Chengdu, Sichuan 610044, China*

Received 11 January 2022; received in revised form 27 April 2022; accepted 11 May 2022
Available online 24 May 2022

KEYWORDS
Diabetes;
Gene therapy;
Intramuscular injection;
Plasmid;
Single-strand insulin analogue (SIA);
Synthetic promoter

Abstract Daily insulin injection is necessary for the treatment of the insulin-dependent diabetes. However, the process is painful and inconvenient. Accordingly, we have made exploratory efforts to establish an alternative method for continuous insulin supply via intramuscular injection of a designed plasmid encoding the single-strand insulin analogue (SIA), which provides safe, effective and prolonged control of insulin-dependent diabetes. To generate a SIA, a short flexible peptide was alternatively introduced into the natural proinsulin to replace its original long and rigid C-peptide. Then, the synthetic promoter SP301 was used to drive potent and specific expression of SIA in skeletal muscle cells. By combining the Pluronic L64 and low-voltage electropulse (L/E), the specialized gene delivery technique was applied to efficiently transfer the constructed plasmid into skeletal muscle cells via intramuscular injection. Through these efforts, a plasmid-based intramuscular gene expression system was established and improved, making it applicable for gene therapy. The plasmid-expressed SIA showed biological functions that were similar to that of natural insulin. A single L/E-pSP301-SIA administration provided sustained SIA expression *in vivo* for about 1.5 months. In addition, the diabetic mice treated with L/E-pSP301-SIA were much healthier than those with other treatments. This plasmid-based system was safe for the treatment of diabetes

* Corresponding author.
** Corresponding author.
E-mail addresses: licx@cib.ac.cn (C. Li), wgang@scu.edu.cn (G. Wang).
Peer review under responsibility of Chongqing Medical University.
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.gendis.2022.05.009
2352-3042/© 2022 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

and did not cause immune responses or pathological damage. The results confirmed that, in a mouse model, long-term positive effects were achieved by a single intramuscular L/E-pSP301-SIA injection, which consequently provided reliable experimental basis for its clinical application for the treatment of diabetes mellitus with promising prospects.
© 2022 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

Diabetes mellitus is a type of endocrine dysfunction involving various metabolic disorders.[1] In type 1 diabetes mellitus (T1DM), the dysfunction is largely caused by excessive autoimmune reaction to pancreatic β cells.[2] Recent studies have shown that several therapeutic methods for T1DM, including pancreatic islet transplantation, cell reprogramming, and gene therapy, have brought a glimmer of hope to the long-term control of diabetes without the trouble of daily insulin injections.[3–5] However, pancreatic islet transplantation and cell reprogramming may trigger immune system attack after treatment, and have limitations, such as high expenses, limited sources and complicated operational processes.[6,7] In contrast, gene therapy has the advantages of simple operation, low cost, abundant sources, and a wide applicability among patients, thereby having potential for clinical applications, especially for the treatment of diabetes and cancer.[8–10]

For gene therapy, efficient delivery of the functional gene into target cells is critical. At present, there are a number of viral and non-viral vectors for gene delivery, including adenovirus, adeno-associated virus,[11] retrovirus, lentivirus, and plasmids.[12] Virus-derived vectors have outstanding advantages in gene delivery/expression efficiency, but may cause risks such as triggering immune responses or even oncogenesis.[13] For example, AAV vectors have been considered safer than other viral vectors and have been used in clinical trials. Unfortunately, a decade-long study revealed integration events in genomic DNA, with the possibility of developing cancer.[13] Plasmids can overcome the above-mentioned safety issues, but they still have a major shortcoming of low gene transfer efficiency *in vivo*.

To increase the gene expression level in skeletal muscle cells and avoid the risk of ectopic expression, we constructed a synthetic promoter SP301,[14] which has a significantly higher activity than that of the traditional strong cytomegalovirus (CMV) promoter. Meanwhile, SP301 can drive target gene expression in a muscle-specific manner. To improve the *in situ* gene transfer efficiency into skeletal muscle cells, we established a safe and efficient gene delivery technique by combining Pluronic L64 and low-voltage electropulse (abbreviated as L/E technique), which increased the gene delivery/expression level 11-fold.[15] In this technique, Pluronic L64, an electroneutralized amphiphilic triblock copolymer with a cell membrane-disturbing action,[16,17] was applied to increase the membrane permeability after which the plasmid DNA was subsequently pushed into the cells under the low-voltage electropulse. In addition, to maintain the activity and enhance the synthesis efficiency of insulin in non-β cells, a flexible peptide was introduced to replace the original long and rigid C-peptide in the proinsulin, generating the single-strand insulin analogue (SIA).

Herein, we aim to investigate whether a combination of the L/E delivery technique and the SP301 promoter can continuously produce functional SIA that is sufficient to control diabetes via intramuscular injection of the SIA-encoding plasmid.

Materials and methods

Plasmid construction and structure analysis

The structure of SIA is the B chain-$(GGGGS)_{\times 3}$-A chain. The sequence of the insulin gene was obtained from NCBI (GenBank ID: NM_008386.4). The coding sequence of SIA was synthesized by Tsingke Company (Chengdu, China) and was cloned into plasmids pSP301[14] and pCMV within *Bam*H I/*Eco*R I sites respectively to generate plasmids pSP301-SIA and pCMV-SIA. Enzymes and other reagents for plasmid construction were from TaKaRa (Shiga, Japan). Softwares including the SWISS-MODEL for protein structure prediction and the VMD for protein structure visualization were used in the study.

Cell culture and transfection

C2C12 (mouse muscle myoblasts, ATCC No. CRL-1772), NIH3T3 (mouse embryo fibroblasts, ATCC No. CRL-1658), and HEK293 (human embryonic kidney cells, ATCC No. CRL-1573) cell strains were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China) and cultured according to the ATCC's recommendation. Cells were seeded into 6-well plates and cultured overnight to achieve 90% confluence. Experiments were performed in triplicate. Before transfection, the medium was changed with Opti-MEM (Invitrogen, without FBS and penicillin-streptomycin). Next, cells were transfected with 2 μg plasmid/well using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 4 h, the culture medium was replaced with Dulbecco's Modified Eagle's Medium (DMEM) (high glucose) containing 10% FBS and 1% penicillin-streptomycin.

ELISA test of insulin

C2C12, NIH3T3 and HEK293 cells were transiently transfected with pCMV-SIA or pSP301-SIA plasmids, respectively. The supernatant was collected and cells were lysed 48 h later. The SIA concentration in the supernatant and in the cells was detected using an ELISA kit purchased from MLBIO Co., Ltd. (#ML001983, Shanghai, China). The OD value at 450 nm was measured with a microplate analyzer (BIOTEK, Santa Clara, CA, USA).

Cell uptake test of the glucose analogue

HEK293 cells were transfected with a pCMV-Control plasmid (pCMV-Ctrl group) or the pCMV-SIA plasmid (pCMV-SIA group), respectively. After 24 h, the supernatant was collected and mixed with an auto-fluorescent glucose analogue 2-NBDG, then added to C2C12 cells. Measurement of the cellular uptake of 2-NBDG was carried out using the Screen Quest™ Fluorometric Glucose Uptake Assay Kit (AAT Bioquest, Sunnyvale, CA, USA) following the kit instructions. Fluorescence intensity was measured by a fluorescent spectrometer (BIOTEK, Santa Clara, CA, USA) at an excitation of 488 nm and an emission of 520 nm.

Competitive binding of insulin antibody by SIA and insulin

A 6-well plate was coated with 0.05 mg/mL of insulin overnight at 4 °C. HEK293 cells were transiently transfected with the plasmid pCMV-SIA and the supernatant was collected 24 h later. A horseradish-peroxidase (HRP)-labeled insulin antibody was diluted with either 2% (w/v) BSA or the supernatant, added to the insulin-coated plate, respectively, and then incubated for 1 h at 37 °C. Then, medium was discarded and cells were rinsed 3 times with PBST, then 100 μL TMB one-component color solution was added and incubated for 10 min at 37 °C. The reaction was terminated by adding sulfuric acid and the OD value at 450 nm was measured with a microplate analyzer (BIOTEK, Santa Clara, CA, USA).

Type 1 diabetic mouse model construction and local gene delivery

Animal care and experimental operations were conducted according to national and local animal experimental guidelines. In brief, six-week-old male C57BL/6 J mice with an average body weight of 20—22 g were purchased from the Experimental Animal Center of Sichuan Province (approval No. KS2019070). Streptozotocin (STZ, Sigma, Saint Louis, MO, USA, #S0130) was dissolved in citrate buffer (pH 4.2—4.5). For establishing the type 1 diabetic mouse model, mice were administered STZ by a single intraperitoneal injection (200—220 mg/kg body weight).[18,19] Mice in the control group received an equal volume of citrate buffer only. After 3 days of STZ induction, the blood glucose level was detected from the vein of mouse tail using the Roche blood glucose instrument (ACCU-CHEK, Mannheim, Germany). When the blood glucose level was over 16.8 mmol/L and maintained for 12 days, the mouse was considered diabetic.[18,19] Mice with blood glucose levels between 20 and 28 mmol/L were selected for further experiments.

The mice were divided into an NC group (normal mice without treatment) and diabetic groups: L/E-SP301 group (pSP301-control plasmid transferred by L/E technique), L/E-CMV-SIA group (pCMV-SIA transferred by L/E technique), L/E-SP301-SIA group (pSP301-SIA transferred by L/E technique), SP301-SIA group (pSP301-SIA injection) and CMV-SIA group (pCMV-SIA injection). For local gene delivery, 100 μg plasmid in 30 μL TE buffer (pH 7.0) was mixed with 30 μL of 0.2% (w/v, in saline) Pluronic L64 (L64; Sigma—Aldrich, St. Louis, MO, USA). The compound was incubated for 5 min at room temperature and injected equally into both sides of tibialis anterior (TA) muscles. An hour later, the electropluse was applied as described using a clinical SDZ-V Nerve and Muscle Stimulator.[20]

Intraperitoneal glucose tolerance tests (IPGTT)

Intraperitoneal glucose tolerance tests (IPGTT) tests were performed on the day 12, 36, and 60 after the intramuscular injection, respectively. After a night of fasting, mice were injected intraperitoneally with a 25% glucose solution at 2 g/kg body weight. In each mouse, the blood glucose level was measured every 30 min for 3 h.

Muscle SIA and insulin measurement

For the collection of tissue samples, experiments were repeated at least independently in triplicate to acquire solid confirmation of the data. In brief, mice were anesthetized by inhalation of isoflurane and sacrificed by cervical dislocation, and washed with PBS. The TA muscles acquired plasmid injection were harvested and muscle tissues were homogenized and lysed with lysate buffer. After diluting the sample solution into a certain proportion, levels of SIA and insulin were determined using an ELISA kit. The total protein concentration was measured with a Pierce™ BCA Protein Assay Kit (Thermo Fisher) using the Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher).

Gene expression analysis

The SIA concentration in blood was determined using an ELISA kit from MLBIO Co., Ltd (#SU-B1163, Shanghai, China). For SIA mRNA analysis, total RNA was extracted from the injected TA muscles using Trizol Reagent (Thermo, Carlsbad, CA, USA) and reverse transcription was performed using RevertAid First Strand cDNA Synthesis kit (Thermo, Carlsbad, CA, USA). Quantitative PCR (qPCR) was designed based on the MIQE guidelines[21] and conducted in triplicate using EvaGreen Master Mix (Bio-rad, Hercules, CA, USA) on a CFX96 machine (BioRad, Hercules, CA, USA). The expression of SIA mRNA was normalized to the expression of β-actin mRNA and calculated using the Pfaffl method.[21,22] Data are expressed as the fold change compared with the CMV-SIA group. Primers used for qPCR were as follows: mouse β-actin-F: 5′-GGCTGTATTCCCCTCCATCG-3′ and R: 5′-CCAGTTGGTAACAATGCCATGT-3’; human β-actin-F: 5′-CATGTACGTTGCTATCCAGGC-3′ and R: 5′-CTCCTTAATGTCACGCACGAT-3’; SIA-

F: 5′-CACTTCCTACCCCTGCTGG-3′ and SIA-R: 5′-ACCACAAAGATGCTGTTTGACA-3’.

Body weight, food intake, water intake, and survival rate

Body weight, food intake, and water intake were measured at designated time points over 72 days. The food intake or water intake was the amount difference between the start and end point during each measurement duration (24 h). At the start of the experiment, there were 12 mice per group, and the survival rates were calculated at the end of the experiment.

GHb and glycogen detection

The concentration of mouse glycated hemoglobin (GHb) was determined on the day 0, 45, and 60, respectively, using an ELISA kit purchased from Shanghai Zhuo Cai Technology Co. Ltd (ZC-38711, Shanghai, China). For glycogen determination, 200 mg of muscle or liver was separated from each mouse (3 mice per group) and washed with PBS buffer, then the glycogen concentration was measured following the instructions of the glycogen detection kit (#BC0345, Solarbio) on day 12, 36, and 60, respectively.

Histological analysis

At the end of treatment, mice were euthanized, and their heart, liver, spleen, lungs, kidneys, pancreas, and TA muscles were harvested. The tissues were fixed by 4% fresh neutral paraformaldehyde solution, and embedded in paraffin for section preparation. Sections were stained with hematoxylin and eosin. Images were taken by a microscope at 100×magnification (Leica DMI 4000 B, Weztlar, Germany).

Immunohistochemical and immunofluorescent detection

Both the pancreas and TA muscles were harvested from euthanized mice, washed three times with PBS and fixed in 4% fresh neutral paraformaldehyde solution. For immunohistochemical analysis, the samples were marked with anti-mouse insulin primary antibody (#PAA448Mu01, Cloud-Clone Corp, China) followed by goat anti-rabbit secondary antibody (#SP-9001, Zsbio Commerce Store, China). Images were taken by a microscope (BA400Digital, MOTIC CHINA GROUP CO., LTD). For immunofluorescence analysis, sections were stained with DAPI, primary CD68 antibody (Abcam) followed by Cy3-labeled goat anti-mouse secondary antibody (#GB21301, ServiceBio) and FITC-CD3e (BD Biosciences). Images were taken by a confocal laser scanning microscope (Leica TCS SP5, Weztlar, Germany).

Measurement of serum IgG and IgM

Serum was collected from both diabetic mice (L/E-SP301-SIA group) and healthy mice (NC group) before or after one or two weeks of treatment. The serum collected from mice before treatment was used as a control and set as week 0. Serum was stored at −80 °C before measurement. Serum IgG and IgM levels were measured using ELISA kits purchased from Solarbio (Cat#SEKM-0098 Lot No. 20210918 for IgG and Cat#SEKM-0102 Lot No. 20210918 for IgM).

Statistical analysis

Data were presented as the mean ± SEM and were analyzed by unpaired *t*-test, one-way ANOVA and two-way ANOVA using GraphPad Prism (Version 7.0). $P < 0.05$ was considered significant.

Results

Characteristics of the SP301 and SIA *in vitro*

Before *in vivo* application, *in vitro* assays were performed to investigate whether the functions of SIA were similar to those of natural insulin. It has been considered that structure is the basis of function. Thus, the structures of proinsulin, SIA, and insulin were predicted and compared (Fig. 1A). Proinsulin has a long and rigid C peptide chain, while SIA contained a $(GGGGS)_{\times 3}$ flexible peptide linker to connect the B chain and A chain. In comparison with the structure of proinsulin, the structure of SIA was more similar to that of the natural insulin because the $(GGGGS)_{\times 3}$ flexible linker could give SIA adequate space to be stretchable, thereby contributing to the formation of disulfide bonds. More importantly, the area around PheB24 is critical for the function of insulin, especially for the binding of insulin to its receptor.[23] In SIA, there was enough space between the linker and three α-helixes to fully expose the PheB24 area. Therefore, the predicted structures provided certain theoretical support for insulin-like function of SIA.

Furthermore, experiments were conducted to test whether SIA can promote cellular uptake of glucose (the most important function of insulin), and bind to insulin antibodies. When SIA expressed by HEK293 was added to cultured C2C12 cells, the uptake of glucose analogue 2-NBDG in C2C12 cells was significantly increased (1218.6 *vs* 641.8 & 639.6, $^{**}P < 0.01$) (Fig. 1B). If SIA could bind to the insulin antibody, it consequently inhibited the binding of insulin to the antibody. As predicted, when SIA was first incubated with an insulin antibody, then mixed with insulin, the amount of insulin-bound HRP-labeled insulin antibody was significantly reduced (1.7 *vs*. 3.9 & 3.9, $^{***}P < 0.001$) (Fig. 1C), thereby indicating competitive binding of SIA to the insulin antibody. Therefore, the results demonstrated that SIA has structural and functional similarities to natural insulin.

Although the promoter SP301 could drive reporter gene expression with high efficiency and muscle specificity,[15] it is still necessary to identify if it has the same effects on the expression of SIA. The expression levels of SIA and SIA mRNA regulated by the promoters of SP301 and CMV were compared among muscle-derived and non-muscle derived cells. In muscle-derived C2C12 cells, SP301-driven SIA in both supernatant and cell lysates was significantly increased when compared with CMV-driven SIA (Fig. 1D, $^{***}P < 0.001$).

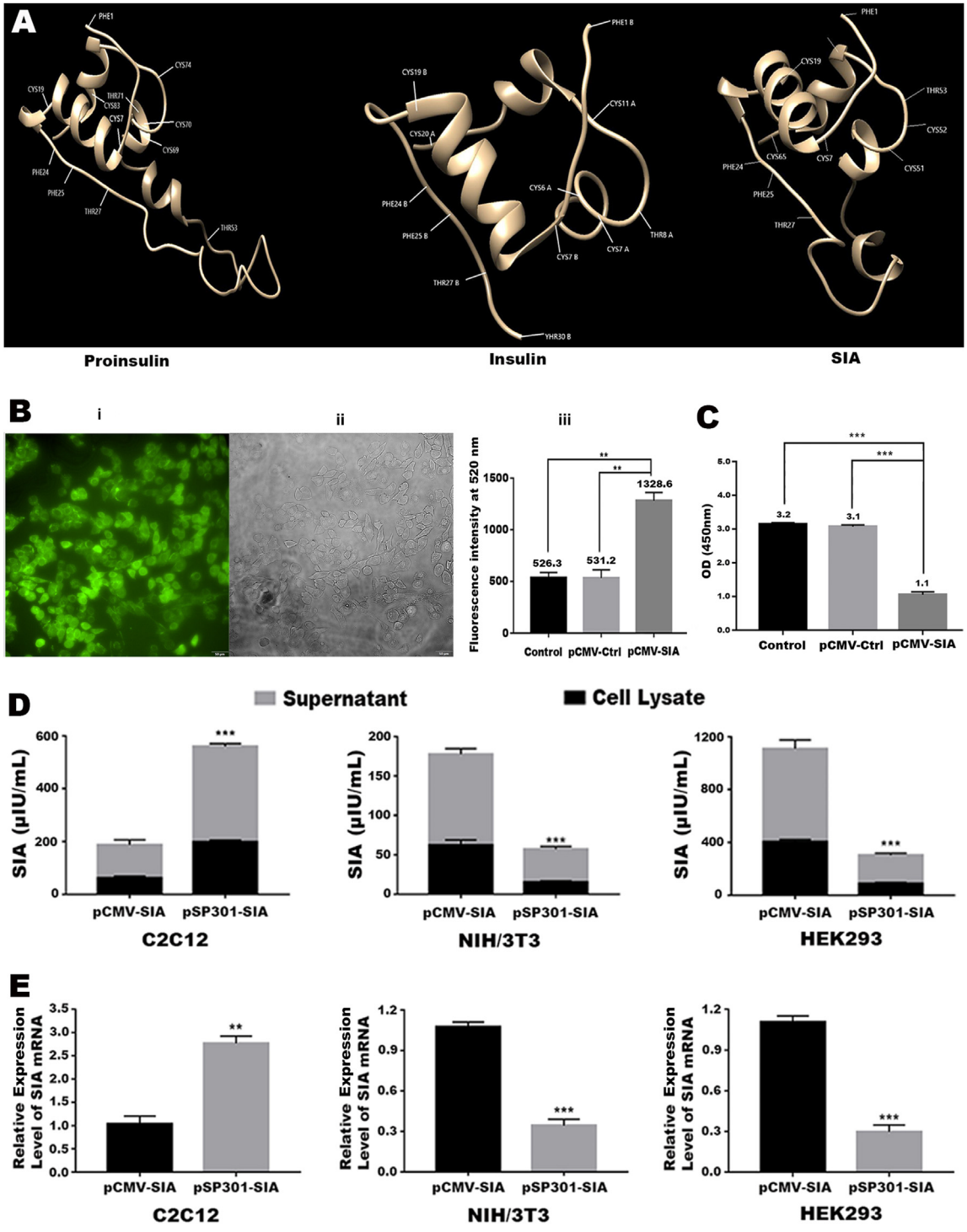

Figure 1 Evaluation of the function of SP301 and single-strand insulin analogue *in vitro*. **(A)** The predicted structures of proinsulin, insulin, and single-strand insulin analogue (SIA). **(B)** Promotion of the glucose analogue uptake via expressed SIA: (**i**) uptake of the glucose analogue displayed in the fluorescence field; (**ii**) cells in bright field; (**iii**) quantitative analysis of the glucose uptake by measuring the OD_{520} value. Control: normally cultured cells without transfection. pCMV-Ctrl: cells transfected with an empty pCMV-Control plasmid. pCMV-SIA: cells transfected with a pCMV-SIA plasmid. **(C)** Competitive binding of insulin antibody by SIA and insulin. Insulin-coated wells were incubated with control medium containing insulin antibody +2% BSA (Control), conditioned medium of pCMV-Ctrl (insulin antibody + supernatant from HEK293 cells transfected with pCMV-Control), or conditioned medium of pCMV-SIA (insulin antibody + supernatant from HEK293 cells transfected with pCMV-SIA), respectively. OD_{450} was measured to evaluate the amount of insulin antibody bound to insulin. $n = 5$. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* pCMV-SIA or indicated counterpart. **(D)** Expression of SIA in supernatants and cell lysates. $n = 3$. **(E)** Expression of SIA mRNA in different cell strains. $n = 3$.

In contrast, in non-muscle derived cells HEK293 and NIH/3T3 the opposite was found, i.e., SP301-driven SIA was significantly lower than those of CMV-driven SIA in the supernatant and cell lysate (Fig. 1D, $^{***}P < 0.001$). Correspondingly, SIA mRNA levels were consistent with the expression patterns of SIA in these cell strains. That is, the expression of SP301-driven SIA mRNA was higher in C2C12 cells (Fig. 1E, $^{**}P < 0.01$) but lower in HEK293 and NIH/3T3 cells (Fig. 1E, $^{***}P < 0.001$) when compared with the mRNA expression of CMV-driven SIA. Thus, the results showed that while driving SIA expression, the promoter SP301 maintained its advantages of high efficiency and muscle specificity, which was consistent with its ability to drive the expression of reporter genes.

Expression of SIA in diabetic mice via different administration of plasmids

The above-mentioned experimental results demonstrated that plasmid-encoded SIA has important functions that are similar to insulin (Fig. 1B, C). Furthermore, it is important to identify if the L/E-SP301-SIA system can express enough SIA to regulate the blood glucose level *in vivo*. To address this, the levels of SIA in the serum, as well as SIA protein and SIA mRNA in the TA muscle were continuously measured over 60 days. As expected, the levels of serum SIA, muscular SIA, and SIA mRNA in the L/E-SP301-SIA group were the highest in all diabetes/treatment groups (Fig. 2A–C). Moreover, the level of serum SIA in the L/E-SP301-SIA group was even higher than the insulin level in normal mice (NC group) from day 12 to day 24, and remained at the same level thereafter (Fig. 2A) ($P > 0.05$). The expression level of SIA in the L/E-CMV-SIA group was significantly higher than that in L/E-SP301, SP301-SIA, and CMV-SIA groups, but still lower than that in the L/E-SP301-SIA group (Fig. 2A–C), thereby indicating that: (1) the SP301 promoter was stronger than the CMV promoter for SIA expression *in vivo*; (2) the L/E gene delivery technique was necessary in this plasmid-based gene therapy system.

To identify the source of SIA, levels of insulin (NC group) and SIA (L/E-CMV-SIA & L/E-SP301-SIA groups) in the pancreas and TA muscle were measured by immunohistochemistry and data were compared on day 48. Signals were strong in the pancreases of normal mice (shown in brown, Fig. 2D) but undetectable in the pancreases of L/E-CMV-SIA and L/E-SP301-SIA mice, thereby indicating that pancreatic β-cells were almost completely destroyed in diabetic model mice (Fig. 2D). Moreover, SIA signals were clearly visible in TA muscles of L/E-CMV-SIA and L/E-SP301-SIA mice, especially in the latter, and signals were obviously weaker in NC mice, suggesting that the SIA was expressed in TA muscles via the plasmids administration (Fig. 2D).

The expression level of serum SIA is the determinant factor of physiological functions, and is a critical indicator to evaluate the applicable potential of this plasmid-based gene therapy system. Taken together, the data have demonstrated the potential of the L/E-SP301-SIA system for the treatment of diabetes in the mouse model since it can continuously provide the highest level of SIA.

Effects of intramuscularly expressed SIA on the syndromes of diabetes

Blood glucose is the most direct and important indicator for diagnosis and treatment of diabetes. To determine whether the blood glucose level is considered normal, a reference range is needed. However, the normal reference range of blood glucose in male C57BL/6 J mouse has been reported to be different.[24] In a previous study, the upper limit of blood glucose in 6-week male C57BL/6 J mouse was 10 mmol/L.[24] According to this value, the blood glucose was considered normal after a single dose of L/E-SP301-SIA treatment from day 9 to day 52. Especially, from day 15–39, blood glucose levels were identical to those of normal mice (Fig. 3A).[24] Furthermore, in L/E-CMV-SIA-treated mice, the blood glucose level was also significantly decreased, but in most cases, it was still higher than the normal range. In comparison, the blood glucose level was not reduced in L/E-SP301, SP301-SIA, and CMV-SIA groups (Fig. 3A).

Glycated hemoglobin (GHb) is formed by slow, continuous and irreversible glycation reactions via the combination of hemoglobins (in red blood cells, RBCs) and carbohydrates (in serum). Its content depends on the concentration of blood glucose and the contact time between blood glucose and hemoglobin. In general, GHb is stable during the 120-day lifetime of RBCs.[25] Therefore, unlike blood glucose, which may fluctuate over time, the concentration or percentage of GHb is constant in healthy individuals, making it a commonly-used convincing clinical indicator to monitor the average blood glucose level within 2–3 months.[26] In this study, the results showed that mice that received either L/E-SP301-SIA or L/E-CMV-SIA treatment acquired effective or even complete recovery of the GHb concentration on day 45 and 60 (not significant, *vs* the NC group) (Fig. 3B). In contrast, the GHb concentration in mice that received either L/E-SP301 or SP301-SIA treatment was still higher than that of mice in the NC group on day 45 ($^{*}P < 0.05$, $^{**}P < 0.01$). Mice in both L/E-SP301 and SP301-SIA groups were gradually dead on the day 60 because of uncontrolled persistent hyperglycemia (Fig. 3B). Thus, these results showed that L/E-SP301-SIA treatment can effectively control the GHb concentration, which is routinely used as objective and reliable evidence for the diagnosis and treatment of diabetes.

Next, IPGTT assays were carried out on the day 12, 36, and 60, respectively. In the NC group, the blood glucose level increased to about 15 mmol/L (Fig. 3C, D), and subsequently decreased to the normal level within several hours (NC group, Fig. 3C, D). In the 5 groups of diabetic mice, blood glucose levels rapidly increased to about 30 mmol/L half an hour after receiving a glucose injection (Fig. 3C, D). However, blood glucose levels in L/E-SP301-SIA treated diabetic mice quickly and significantly decreased within 150–180 min on the day 12 and 36, and levels were close to that of mice in the NC group (Fig. 3C, D), thereby indicating the capability and sensitivity of SIA to regulate the blood glucose level in a timely manner. That was identical to the H&E staining showing the high expression level of SIA and its effective physiological role in the

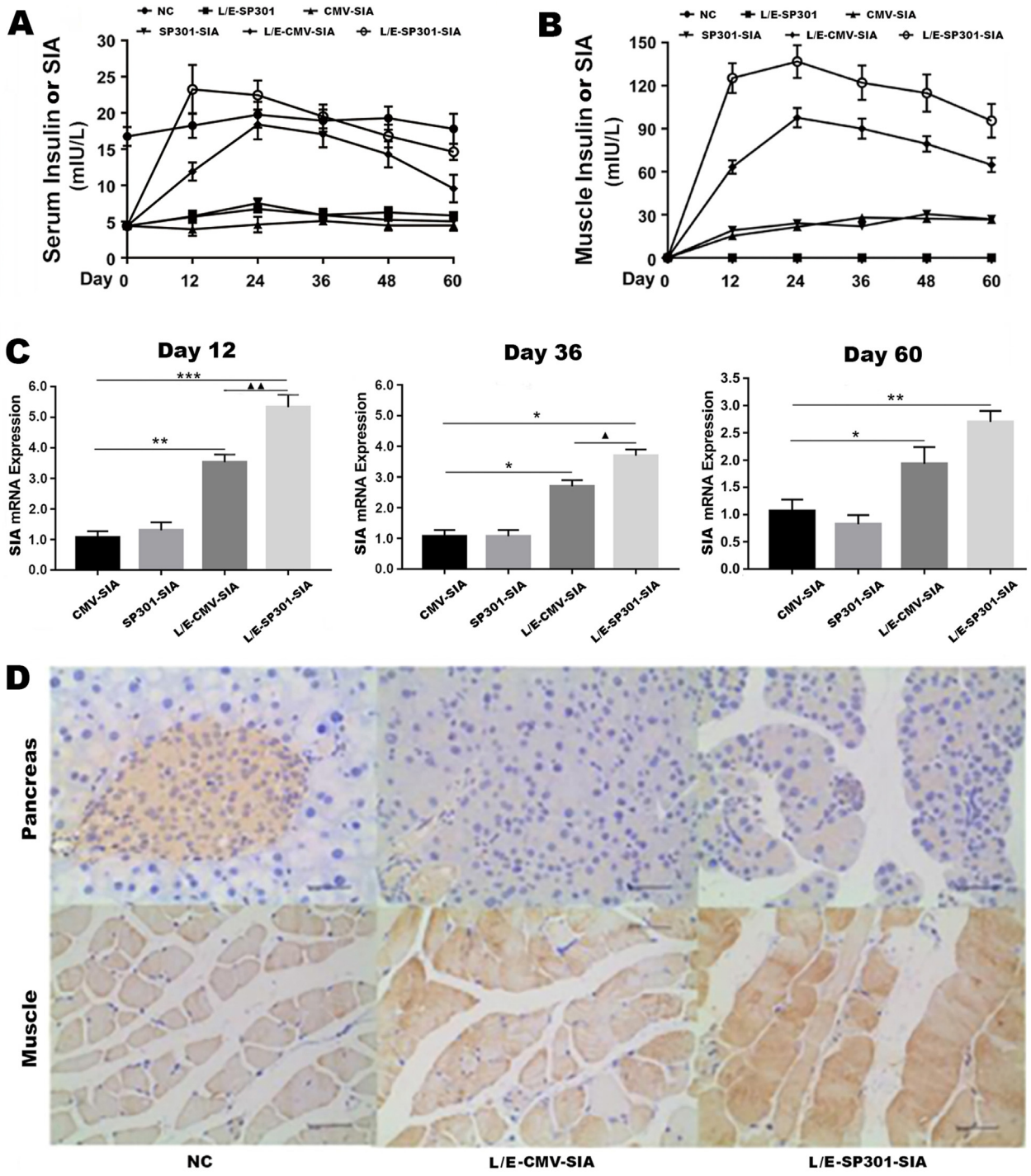

Figure 2 *In vivo* single-strand insulin analogue expression within 60 days after a single administration of different plasmids in diabetic mice. Expression of insulin in the normal control (NC) group and single-strand insulin analogue (SIA) in other treatment groups were measured by ELISA in serum **(A)** and TA muscle **(B)** within 60 days. $n = 6$ mice per group per point time. **(C)** Relative expression level of SIA mRNA in TA muscle was analyzed by qPCR and expression in Group CMV-SIA on day 12, 36 and 60, respectively, was compared. $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* CMV-SIA & SP301-SIA; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *vs* L/E-CMV-SIA. **(D)** Insulin (NC group) and SIA (L/E-CMV-SIA & L/E-SP301-SIA groups) in the pancreas and TA muscle were determined by immunohistochemistry (shown in brown) on the day 48. $n = 3$. Scale bar: 40 μm.

utilization, catabolism, and conversion of blood glucose. On the day 60, the IPGTT assays were not as effective as those on the day 12 and 36, because of the decrease of SIA expression (Fig. 2A–C). In IPGTT assays of L/E-SP301, SP301-SIA, and CMV-SIA groups, mice almost had no capability to deal with the highly-increased blood glucose levels

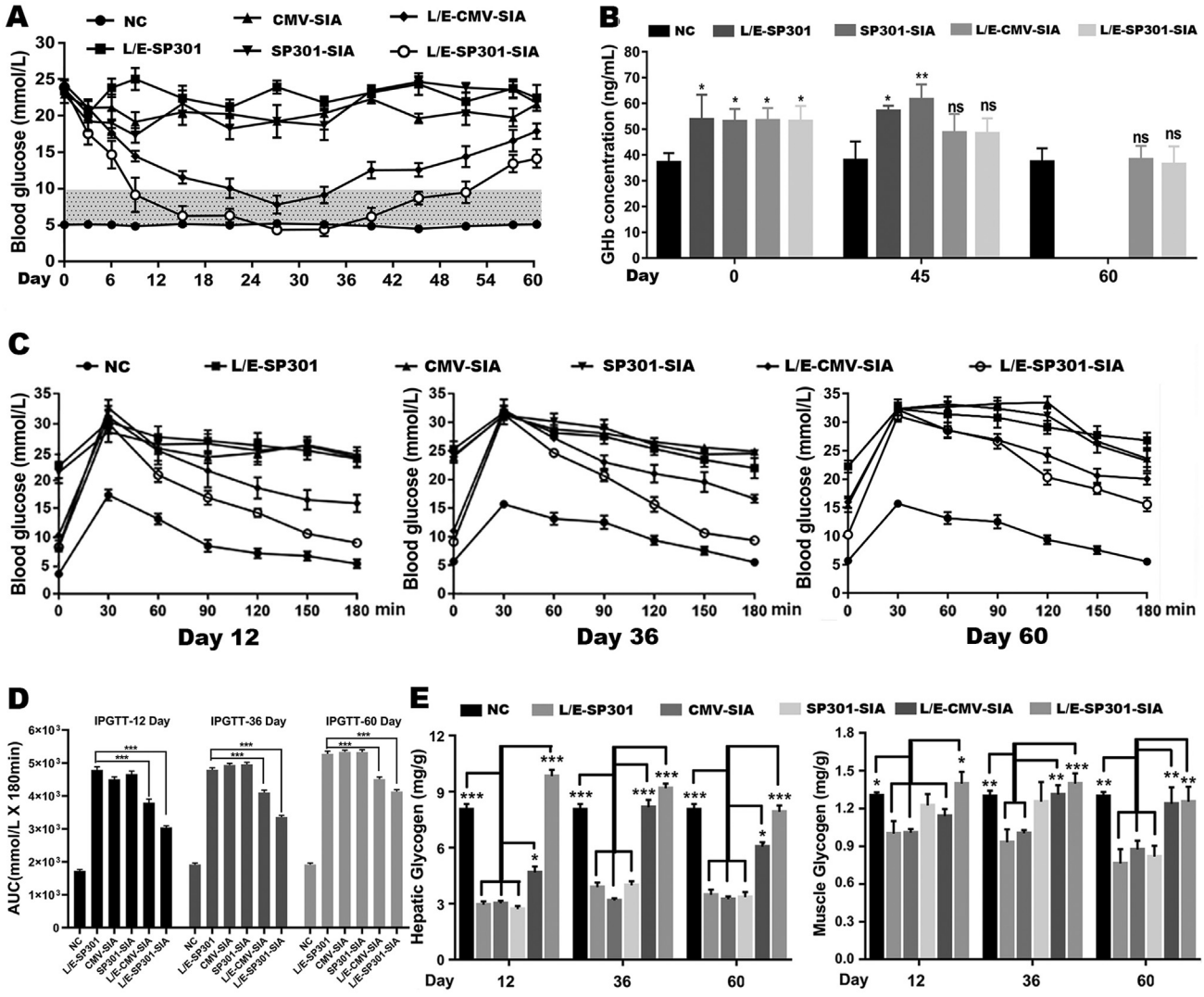

Figure 3 Effects of expressed single-strand insulin analogue on blood glucose, GHb, IPGTT, and glycogen synthesis within 60 days after plasmid administration. $n = 6$. **(A)** Blood glucose. **(B)** Glycated hemoglobin (GHb). $^{*}P < 0.05$, $^{**}P < 0.01$, ns, no significant *vs* NC. Normal physiological blood glucose is indicated by a gray background. **(C)** IPGTT tests. **(D)** Area under curve for IPGTT tests. **(E)** Hepatic glycogen and muscle glycogen. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

on the day 12, 36, and 60 (Fig. 3C, D), because of the lack of SIA (L/E-SP301) or low expression of SIA (SP301-SIA and CMV-SIA). In the L/E-CMV-SIA group, IPGTT results were not as good as those of the L/E-SP301-SIA group, which was consistent with the SIA expression levels of the two treatments (Fig. 2A—C).

An important function of insulin is to promote glycogen synthesis with redundant glucose.[27] Hence, the contents of hepatic and muscular glycogens were measured on the day 12, 36, and 60, respectively. The results showed that the contents of both types of glycogens in L/E-SP301-SIA-treated mice were identical to those in NC mice (Fig. 3E). Moreover, the contents of both glycogens in L/E-SP301-SIA and NC groups were significantly higher than those of L/E-SP301, CMV-SIA, and SP301-SIA groups ($^{***}P < 0.001$ for hepatic glycogen; **** for muscle glycogen. Fig. 3E). In addition, L/E-CMV-SIA treatment increased the contents of hepatic and muscle glycogens, but it was not as good as the L/E-SP301-SIA treatment (Fig. 3E). Therefore, it is convincible that L/E-SP301-SIA treatment significantly promotes the synthesis of hepatic and muscle glycogens by SIA expression in diabetic mice.

Certain symptoms are commonly seen in diabetes, including increased food intake, water intake, and body weight loss.[1] In each group of diabetic mice after intraperitoneal injection of STZ, the average body weight decreased while food and water intake increased. Only in the L/E-SP301-SIA group, the average body weight gradually recovered to the same weight as that of mice in the NC group from the day 24 to day 42, but slowly decreased thereafter (Fig. 4A). The results suggested that if a secondary injection was given at an appropriate time, the therapeutic effects could be sustained or be even better. However, food intake and water intake were not recovered to the normal level in any group ($P < 0.05$ or 0.01, *vs* NC group), and L/E-SP301-SIA treatment significantly ameliorated these symptoms ($P < 0.05$ or 0.01, *vs* L/E-

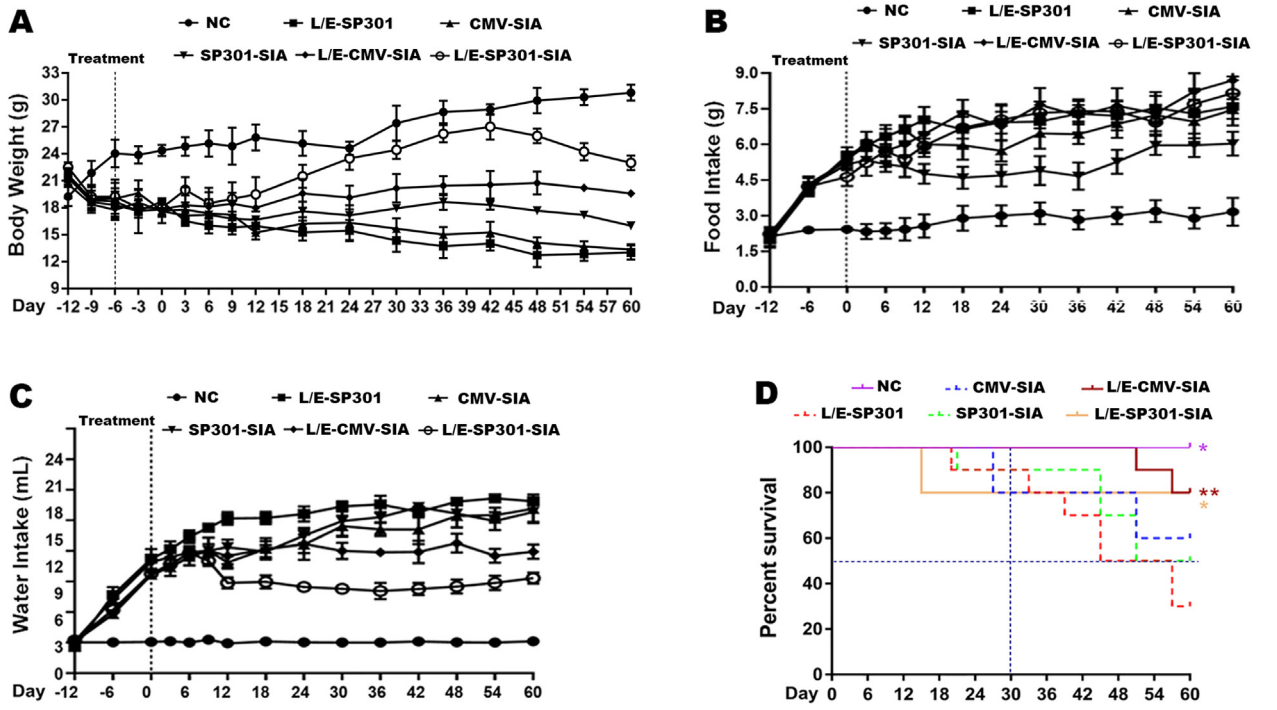

Figure 4 Body weight, food intake, water intake, and survival percentage of mice. Continuous tests of body weight **(A)**, food intake **(B)** and water intake **(C)** of mice for 72 days since STZ injection. $n = 6$. **(D)** Survival percentage of mice within 60 days after treatment. $n = 12$.

SP301, CMV-SIA or SP301-SIA group) (Fig. 4B, C). In addition, diabetic mice in L/E-SP301, CMV-SIA and SP301-SIA groups maintained an unhealthy status and appeared with thin and hairless bodies and a sleepy state, whereas mice in L/E-SP301-SIA group showed less of these symptoms and looked more similar to the healthy mice in the NC group.

The survival rate on day 60 was 100% in NC group, and 83.3% in L/E-SP301-SIA and L/E-CMV-SIA groups. However, for L/E-SP301, SP301-SIA, and CMV-SIA groups, the survival rate on day 60 was only 33.3%, 50%, and 58.3%, respectively (Fig. 4D). Two L/E-CMV-SIA-treated mice died on day 51 and 57, which should be due to an increase in the blood glucose level (Fig. 3A) and a decrease in the expression of SIA (Fig. 2A—C). An unexpected event was the death of two L/E-SP301-SIA-treated mice on day 15. We noticed that just before day 15, the blood glucose levels of these 2 mice were slightly lower than those of NC mice (data not shown), implying that the death might be due to hypoglycemia. Correspondingly, the serum SIA concentration reached a maximum on day 12 (Fig. 2A), which likely induced the hypoglycemia.

Safety evaluation of different treatments

On day 60 after treatment, pathological evaluation was performed by H&E staining of tissue sections on the heart, liver, spleen, lungs, kidneys, pancreas, and TA muscle of mice in the 4 groups. The results showed that the heart, liver, spleen, lungs, and kidneys in mice in the L/E-SP301, L/E-CMV-SIA, and L/E-SP301-SIA diabetic groups were almost identical to those of mice in the NC group, without obvious pathological changes (Fig. 5A). However, the pancreas of mice in the 3 diabetic groups was seriously damaged by STZ induction (Fig. 5A), which was consistent with functional detection by immunohistochemistry (Fig. 2D). TA muscles of L/E-SP301-treated diabetic mice showed severe lesions, including atrophy, necrosis, and infiltration of inflammatory cells. In contrast, from the anatomy appearance (not shown) and H&E staining results, diabetic mice treated with L/E-CMV-SIA and L/E-SP301-SIA had TA muscles that were as healthy as those of NC mice (Fig. 5A).

The immune safety of L/E-SP301-SIA treatment should be considered more carefully and thoroughly. Firstly, the SIA is not completely the same as natural insulin. To a certain extent, SIA can be regarded a new protein, and it is still possible to induce the production of antibodies and other immune responses. Secondly, in T1DM, as pancreatic β-cells are attacked by their immune system, it is necessary to detect these novel insulin-producing cells in TA muscles.[28] In recent years, many studies on T1DM have focused on long-term treatment to replace the daily insulin injection, such as pancreatic islet transplantation and cell reprogramming. Those studies also showed the possibility of triggering attacks by the immune system after treatments.[29—31] In this study, on day 3 (the 15th day after STZ induction) and day 60 (the 72nd day after STZ induction), there were no detectable immunological immunofluorescent signals in TA muscle section from NC mice and L/E-SP301-SIA-treated mice (Fig. 5B). The immunofluorescent signal included T lymphocytes via CD3e-immunostaining and macrophages via CD68-immunostaining, respectively. Moreover, the total levels of IgM and IgG in both healthy mice (NC group) and

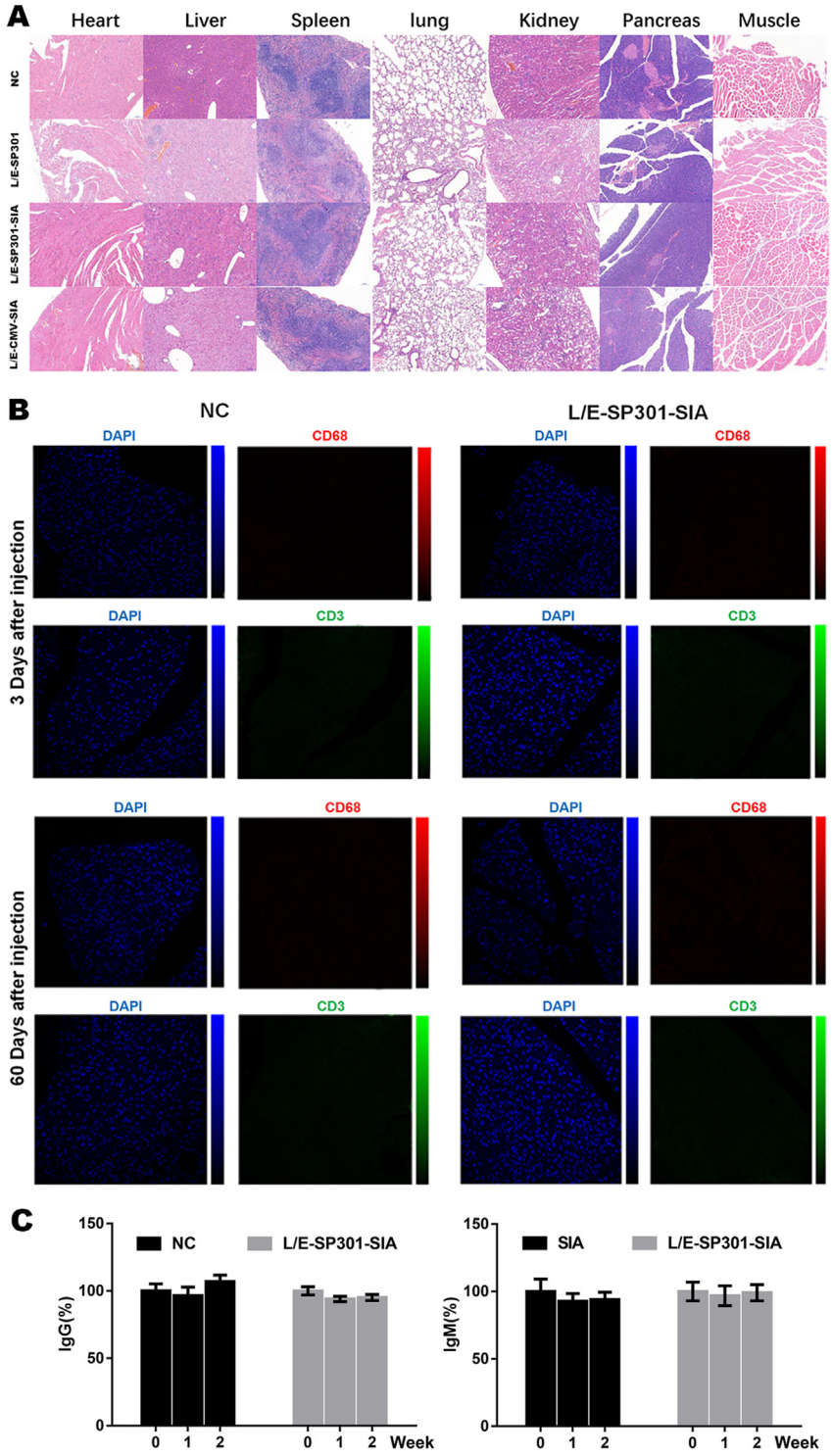

Figure 5 Pathological analysis and immunological assays of mice. **(A)** The pathological conditions of heart, liver, spleen, lungs, kidneys, pancreas, and muscle in four groups detected using HE stain on day 60. Scale bar: 100 μm. $n = 3$. **(B)** Immunological tests of TA muscles on the day 3 and 60, respectively. Scale bar: 50 μm. CD3e for lymphocytes (green), CD68 for macrophages (red), DAPI for nuclei (blue). $n = 3$. **(C)** Measurement of serum IgG and IgM by ELISA. Data are presented as the mean ± SD ($n = 5$).

diabetic mice (L/E-SP301-SIA group) were stable during the 2-week treatment period (Fig. 5C). The results confirmed the immune safety of L/E-SP301-SIA treatment. From another perspective, the sustained therapeutic effects in diabetic mice also provided indirect but convincible evidence regarding immune safety.

Discussion

Skeletal muscles have characteristics of absorbing exogenous plasmids and keeping the intramuscular expression of target genes for months.[20,32] Meanwhile, they are widely distributed throughout the body and highly vascularized,[33,34] making them conducive to protein synthesis and secretion.[35,36] Therefore, skeletal muscles are a promising "factory" for ectopic expression and secretion of functional proteins.[37,38] In previous studies, the potential of intramuscular expression of insulin analogs for diabetes treatment has been demonstrated.[39,14] However, they had different obstacles or shortcomings. Firstly, viral vectors were obvious obstacles for further clinical applications.[39] Secondly, applying proinsulin or preproinsulin would greatly reduce the therapeutic effects because the activity of proinsulin is only 3%—5% that of insulin.[27,40] Skeletal muscle cells are unable to remove the C-peptide from the proinsulin, which mainly depends on specific enzymes that are only located in pancreatic β cells.[40] Thirdly, in plasmid-based cases, direct injection of plasmids usually generated extremely low gene transfer efficiency[41,42]; thus, gene expression may not be sufficient to provide satisfactory biological effects. Finally, the plasmid promoters lack tissue specificity, and there may be a risk of ectopic gene expression after intramuscular injection.[43,44] Herein, to overcome the above-mentioned shortcomings and obstacles, we constructed a safe, effective, economical, and comfortable plasmid-based gene therapy method, which provided prolonged self-supply of SIA for insulin-deficient diabetes.

The IPGTT results of mice in the L/E-SP301-SIA group were still not as good as those in healthy mice. Physiologically, insulin is synthesized by pancreatic β cells and reserved in specialized vesicles within β cells. When the blood glucose level increased, the insulin reserved in the vesicles would be rapidly released to reduce the blood glucose in several minutes; thus, the blood glucose level would not increase to a very high level (about 15 mmol/L; Fig. 3C, D). Subsequently, the blood glucose level decreased to the normal level within several hours (NC group; Fig. 3C, D). However, in diabetic model mice, the synthesis of SIA started from gene transcription in skeletal muscle cells, and then SIA was released and secreted into the circulation. This procedure is more time-consuming than the direct secretion of insulin from pancreatic β cells in healthy mice. Therefore, blood glucose levels in the 5 groups of diabetic mice rapidly increased to about 30 mmol/L half an hour after receiving a glucose injection (Fig. 3C, D). Blood glucose levels in L/E-SP301-SIA treated diabetic mice significantly decreased within 180 min on day 12, 36, and 60, but the levels were still not as satisfied as that of mice in the NC group (Fig. 3C, D). However, the decrease extent of blood glucose levels in L/E-SP301-SIA treated diabetic mice were even greater than that of mice in the NC group, illustrating that the SIA had the ability to efficiently decrease blood glucose levels. The unsatisfied IPGTT results in L/E-SP301-SIA treated diabetic mice were mainly due to the high level of blood glucose (about 30 mmol/L) caused by the delayed action of the SIA. These results suggest that the application of rapid-acting SIAs with less tendency of self-aggregation may result in better IPGTT results.[45,46]

We noticed the accidental death of two L/E-SP301-SIA-treated mice on day 15. The unexpected death exposed the potential defect of the L/E-SP301-SIA therapeutic program, i.e., uncontrolled and sustained production of SIA resulted in higher level of SIA in L/E-SP301-SIA-treated mice than that of insulin in NC mice (Fig. 2A, B), thereby bringing lower blood glucose level on day 27 and 33 (Fig. 3A). It also had the risk of hypoglycemia or even more severe outcomes.[47] To acquire safer therapeutic results, a simple and direct way is to reduce the injection amount of the plasmid. Since we only injected the plasmid once during the whole procedure, improvements could be made by multiple injections of a reduced dosage of the plasmid in a suitable time interval to maintain the normal SIA concentration. It has been generally considered that recovery of the blood glucose level is usually due to instability and degradation of free extrachromosomal plasmids. In order to obtain safe and stable effects on blood glucose control, the combination of the reduced dosage of the plasmid and the injection time interval should be optimized to sustain a safe and functional SIA supply. More importantly, a practical and reliable strategy is to construct a blood glucose-responsive or glucose-regulated SIA-expressing plasmid.[24,48]

Conclusions

In this study, we introduced a novel plasmid-based gene therapy system for the sustained production of a designed SIA. The system combines the strong muscle-specific promoter SP301 with an L/E *in situ* gene delivery technique. The L/E-SP301-SIA system is efficient, economical, and safe for prolonged and comprehensive control of insulin-deficient diabetic mice via a single intramuscular administration. Furthermore, it is safe enough to avoid pathological damage to skeletal muscles and immune responses to the SIA and SIA-producing cells. Taken together, this study provides a convincing experimental foundation for the treatment of diabetes by this system, which has promising prospects in more clinical applications due to its advantages of biosafety, effectiveness, and low cost.

Ethics declaration

This study was performed with the approval of the Medical Ethics Committee of Sichuan University. All procedures were strictly conducted in accordance with the code of ethics. Significant efforts were made to minimize the number of animals used and their suffering.

Author contributions

G. W., C. X. L., P. Y. and L. D. designed the project; P. Y. and L. D. performed the experiments with assistance from L. F. X., W. L. L., Y. H. Z., and M. L.; G. W., L. D. and C. X. L. analyzed the data and wrote the paper.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This study was supported by the National Natural Science Foundation of China (No. 31971390) and the International Cooperative Project of Sichuan Province on Science and Technology Innovation (China) (No. 2021YFH0142).

References

1. Yao D, GangYi Y, QiNan W. Autophagic dysfunction of β cell dysfunction in type 2 diabetes, a double-edged sword. *Genes Dis*. 2020;8(4):438—447.
2. Tanabe M, Motonaga R, Terawaki Y, et al. Prescription of oral hypoglycemic agents for patients with type 2 diabetes mellitus: a retrospective cohort study using a Japanese hospital database. *J Diabetes Investig*. 2017;8(2):227—234.
3. Crunkhorn S. Gene therapy: reprogramming α-cells reverses diabetes. *Nat Rev Drug Discov*. 2018;17(2):93.
4. Pathak V, Pathak NM, O'Neill CL, et al. Therapies for type 1 diabetes: current scenario and future perspectives. *Clin Med Insights Endocrinol Diabetes*. 2019;12:1179551419844521.
5. Lebreton F, Lavallard V, Bellofatto K, et al. Insulin-producing organoids engineered from islet and amniotic epithelial cells to treat diabetes. *Nat Commun*. 2019;10(1):4491.
6. Qiu YH, Deng FY, Li MJ, et al. Identification of novel risk genes associated with type 1 diabetes mellitus using a genome-wide gene-based association analysis. *J Diabetes Investig*. 2014;5(6):649—656.
7. Bhattacharya S, Bank S, Maiti S, et al. The control of hyperglycemia by estriol and progesterone in alloxan induced type I diabetes mellitus mice model through hepatic insulin synthesis. *Int J Biomed Sci*. 2014;10(1):8—15.
8. Every AL, Kramer DR, Mannering SI, et al. Intranasal vaccination with proinsulin DNA induces regulatory CD4+ T cells that prevent experimental autoimmune diabetes. *J Immunol*. 2006;176(8):4608—4615.
9. Yoon JW, Jun HS. Recent advances in insulin gene therapy for type 1 diabetes. *Trends Mol Med*. 2002;8(2):62—68.
10. Handorf AM, Sollinger HW, Alam T. Insulin gene therapy for type 1 diabetes mellitus. *Exp Clin Transplant*. 2015;13(Suppl 1):37—45.
11. Chen YH, Keiser MS, Davidson BL. Viral vectors for gene transfer. *Curr Protoc Mol Biol*. 2018;8(4):e58.
12. Flotte TR, Solow R, Owens RA, et al. Gene expression from adeno-associated virus vectors in airway epithelial cells. *Am J Respir Cell Mol Biol*. 1992;7(3):349—356.
13. Nguyen GN, Everett JK, Kafle S, et al. A long-term study of AAV gene therapy in dogs with hemophilia A identifies clonal expansions of transduced liver cells. *Nat Biotechnol*. 2021;39(1):47—55.
14. Liu Y, He Y, Wang Y, et al. Synthetic promoter for efficient and muscle-specific expression of exogenous genes. *Plasmid*. 2019;106:102441.
15. Liu S, Ma L, Tan R, et al. Safe and efficient local gene delivery into skeletal muscle via a combination of Pluronic L64 and modified electrotransfer. *Gene Ther*. 2014;21(6):558—565.
16. Chen J, Luo J, Zhao Y, et al. Increase in transgene expression by pluronic L64-mediated endosomal/lysosomal escape through its membrane-disturbing action. *ACS Appl Mater Interfaces*. 2015;7(13):7282—7293.
17. Song H, Liu S, Li C, et al. Pluronic L64-mediated stable HIF-1α expression in muscle for therapeutic angiogenesis in mouse hindlimb ischemia. *Int J Nanomed*. 2014;9:3439—3452.
18. Chaudhry ZZ, Morris DL, Moss DR, et al. Streptozotocin is equally diabetogenic whether administered to fed or fasted mice. *Lab Anim*. 2013;47(4):257—265.
19. Deeds MC, Anderson JM, Armstrong AS, et al. Single dose streptozotocin-induced diabetes: considerations for study design in islet transplantation models. *Lab Anim*. 2011;45(3):131—140.
20. Tevz G, Pavlin D, Kamensek U, et al. Gene electrotransfer into murine skeletal muscle: a systematic analysis of parameters for long-term gene expression. *Technol Cancer Res Treat*. 2008;7(2):91—101.
21. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009;55(4):611—622.
22. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res*. 2002;30(9):e36.
23. Mirmira RG, Tager HS. Role of the phenylalanine B24 side chain in directing insulin interaction with its receptor. Importance of main chain conformation. *J Biol Chem*. 1989;264(11):6349—6354.
24. Xie M, Ye H, Wang H, et al. β-cell-mimetic designer cells provide closed-loop glycemic control. *Science*. 2016;354(6317):1296—1301.
25. Miller KM, Beck RW, Foster NC, et al. HbA1c levels in type 1 diabetes from early childhood to older adults: a deeper dive into the influence of technology and socioeconomic status on HbA1c in the T1D exchange clinic registry findings. *Diabetes Technol Ther*. 2020;22(9):645—650.
26. Kilpatrick ES, Bloomgarden ZT, Zimmet PZ. International Expert Committee report on the role of the A1C assay in the diagnosis of diabetes: response to the International Expert Committee. *Diabetes Care*. 2009;32(12):e159.
27. Cahill Jr GF. Insulin and proinsulin. *N Engl J Med*. 1970;283(14):762.
28. Realsen JM, Chase HP. Recent advances in the prevention of hypoglycemia in type 1 diabetes. *Diabetes Technol Ther*. 2011;13(12):1177—1186.
29. Kang HM, Kim J, Park S, et al. Insulin-secreting cells from human eyelid-derived stem cells alleviate type I diabetes in immunocompetent mice. *Stem Cell*. 2009;27(8):1999—2008.
30. Xiao X, Guo P, Shiota C, et al. Endogenous reprogramming of alpha cells into beta cells, induced by viral gene therapy, reverses autoimmune diabetes. *Cell Stem Cell*. 2018;22(1):78—90.
31. Wang MY, Dean ED, Quittner-Strom E, et al. Glucagon blockade restores functional β-cell mass in type 1 diabetic mice and enhances function of human islets. *Proc Natl Acad Sci U S A*. 2021;118(9):e2022142118.
32. Boisgerault F, Mingozzi F. The skeletal muscle environment and its role in immunity and tolerance to AAV vector-mediated gene transfer. *Curr Gene Ther*. 2015;15(4):381—394.
33. Blaveri K, Heslop L, Yu DS, et al. Patterns of repair of dystrophic mouse muscle: studies on isolated fibers. *Dev Dyn*. 1999;216(3):244—256.
34. Frontera WR, Ochala J. Skeletal muscle: a brief review of structure and function. *Calcif Tissue Int*. 2015;96(3):183—195.
35. Lu QL, Bou-Gharios G, Partridge TA. Non-viral gene delivery in skeletal muscle: a protein factory. *Gene Ther*. 2003;10(2):131—142.
36. Kessler PD, Podsakoff GM, Chen X, et al. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc Natl Acad Sci U S A*. 1996;93(24):14082—14087.

37. Olfert IM, Breen EC, Mathieu-Costello O, et al. Skeletal muscle capillarity and angiogenic mRNA levels after exercise training in normoxia and chronic hypoxia. *J Appl Physiol*. 2001;91(3):1176—1184.
38. Tripathy SK, Svensson EC, Black HB, et al. Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector. *Proc Natl Acad Sci U S A*. 1996;93(20):10876—10880.
39. Hou WR, Xie SN, Wang HJ, et al. Intramuscular delivery of a naked DNA plasmid encoding proinsulin and pancreatic regenerating III protein ameliorates type 1 diabetes mellitus. *Pharmacol Res*. 2011;63(4):320—327.
40. Vakilian M, Tahamtani Y, Ghaedi K. A review on insulin trafficking and exocytosis. *Gene*. 2019;706:52—61.
41. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle in vivo. *Science*. 1990;247(4949 Pt 1):1465—1468.
42. Wells DJ. Gene therapy progress and prospects: electroporation and other physical methods. *Gene Ther*. 2004;11(18):1363—1369.
43. Rincon MY, Sarcar S, Danso-Abeam D, et al. Genome-wide computational analysis reveals cardiomyocyte-specific transcriptional cis-regulatory motifs that enable efficient cardiac gene therapy. *Mol Ther*. 2015;23(1):43—52.
44. Escobar H, Schöwel V, Spuler S, et al. Full-length dysferlin transfer by the hyperactive sleeping beauty transposase restores dysferlin-deficient muscle. *Mol Ther Nucleic Acids*. 2016;5(1):e277.
45. Home PD. The pharmacokinetics and pharmacodynamics of rapid-acting insulin analogues and their clinical consequences. *Diabetes Obes Metab*. 2012;14(9):780—788.
46. Heinemann L, Heise T, Wahl LC, et al. Prandial glycaemia after a carbohydrate-rich meal in type I diabetic patients: using the rapid acting insulin analogue [Lys(B28), Pro(B29)] human insulin. *Diabet Med*. 1996;13(7):625—629.
47. Garg SK, Wernicke-Panten K, Wardecki M, et al. Efficacy and safety of insulin aspart biosimilar SAR341402 versus originator insulin aspart in people with diabetes treated for 26 weeks with multiple daily injections in combination with insulin glargine: a randomized open-label trial (GEMELLI 1). *Diabetes Technol Ther*. 2020;22(2):85—95.
48. Zhou Y, Kong D, Wang X, et al. A small and highly sensitive red/far-red optogenetic switch for applications in mammals. *Nat Biotechnol*. 2022;40(2):262—272.