SUPPORTING INFORMATION

Plasmid DNA Nanoparticles for Nonviral Oral Gene Therapy

S. M. Shatil Shahriar,[†,‡,▽] Jeong Man An,[†,‡,§,▽] Mohammad Nazmul Hasan,[∥] Sachin S. Surwase,[⊥] Yeu-Chun Kim,[⊥] Dong Yun Lee,[#,g] Sungpil Cho[h] and Yong-kyu Lee*[,†,‡,∥,h]*

[†]Department of Chemical and Biological Engineering, Korea National University of Transportation, Chungju-27469, Republic of Korea

[‡]KB Biomed Inc., Chungju 27469, Republic of Korea

[§]Department of Bioengineering, College of Engineering, Hanyang University, Seoul 04763, Republic of Korea

[∥]Department of Green BioEngineering, Korea National University of Transportation, Chungju-27469, Republic of Korea

[⊥]Department of Chemical & Biomolecular Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon-34141, Republic of Korea

[#]Department of Bioengineering, College of Engineering, and BK21 PLUS Future Biopharmaceutical Human Resources Training and Research Team, Hanyang University, Seoul 04763, Republic of Korea

[g]Institute of Nano Science and Technology (INST), Hanyang University, Seoul 04763, Republic of Korea

[h]4D Biomaterials Center, Korea National University of Transportation, Jeungpyeong 27909, Republic of Korea

[▽]These authors contributed equally
*S.P.C. email: sungpilcho74@ut.ac.kr.
*Y.K.L. email: leeyk@ut.ac.kr.

SUPPORTING INFORMATION

A. Experimental Section

1. Materials

Protamine sulfate (salt from salmon, grade X, amorphous powder), branched polyethylenimine 25000 (bPEI), Fluorescein isothiocyanate, 4′,6-Diamidine-2′-phenylindole dihydrochloride, 4-Nitrophenyl chloroformate, Triton X-100, thiazolyl blue tetrazolium bromide, calcium phosphate, HEPES (sodium salt, 99%), D-(+)-glucose solution (10 w/v%), pepsin, and trypsin were purchased from Sigma-Aldrich (USA). Taurocholic acid (TCA, sodium salt hydrate 98%, MW: 515.7058 g/mol) was purchased from ACROS (Belgium). Caco-2, Hep G2, MDCK, and MDCK-ASBT cells were obtained from the Korean Cell Line Bank (Seoul, South Korea). Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), RPMI medium 1640, foetal bovine serum (FBS), penicillin, and 0.05% Trypsin-EDTA was purchased from Gibco by Life Technologies (USA). Dialysis Tubing MWCO 2 kD was purchased from Spectrum Laboratories (USA). Phosphate-buffered saline (PBS) was purchased from BioNieer (Daejeon, Korea). The plasmid purification kit *NucleoBond® Xtra Maxi Plus* was purchased from MACHEREY-NAGEL (Germany).

2. Extraction of GLP1 Plasmid

The cryostock (800 µl bacterial growth and 200 µl of 50% glycerol) of GLP1 plasmid transfected *E. Coli* was kindly provided by Prof. Minhyung Lee, Hanyang University, Seoul 04763, South Korea. The *E. Coli* host strains ($DH5_{alpha}$ cells) were cultured and purified in our laboratory for mass production of GLP1 plasmid according to experimental requirements. The following steps demonstrate the culture and purification of GPL1 plasmid from *E. coli* strains.

2.1. Proliferation of *E.coli* Host Strains

2.1.1. Culture of the *E. coli* Host Strains in LB Agar Plate

Bacterial growth medium containing 1x Agar, 1.7x Luria Broth (LB), and 100 mL dH_2O was sterilized using an autoclave (121°C, 15 min) and subsequently allowed the solution to cool down at room temperature. Next, 50 µg/mL of appropriate selective antibiotic (Ampicillin Sodium salt) was added to the bacterial growth medium to ensure the propagation of the GLP1 plasmid, followed by adding 20 mL of growth media to each 150 mm × 15 mm Petri dish. The plate was solidified at room temperature for 3 h or left in a refrigerator at 4°C with a parafilm wrapping. The GLP1 plasmid transfected *E.Coli* bacteria were scraped on the LB Agar plate, using a sterile plastic loop. After streaking, the plate was inverted (upside down) and placed at a 37°C incubator for 1 day.

2.1.2. Preparation of Starter and Larger Overnight Culture

To prepare the starter culture, 3 mL of sterilized LB medium containing 50 μg/mL working solution of Ampicillin was poured into each 15 mL conical tube, and then a single colony of *E. coli* strain harboring GLP1 plasmid was obtained from a freshly streaked LB Agar plate by sterilized pipette tips and inoculated into the solution. The open-top of the 15 mL conical tubes was sealed by aluminum foil with small holes to ensure the flow of oxygen during culturing, then placed in a shaking incubator (37°C, ~300 rpm, 8 h). We measured the OD_{600} for each sample by an ultraviolet-visible spectrophotometer and then selected the maximum OD_{600} standardized bacterial growth for the large overnight culture. 100 μL of the starter culture was added in a 300 mL LB medium and placed in an incubator (37°C, ~300 rpm, 16 h) to an $OD_{600} = 2$. To collect the GLP1 plasmid transfected *E. Coli,* solutions were centrifuged at 4500 × g for 12 min.

2.2. Purification of GLP1 Plasmid

The GLP1 plasmid was extracted from the pelleted cells using a pDNA purification kit assay according to the manufacturer's protocol (*NucleoBond® Xtra Maxi Plus* EF kit, MACHEREY-NAGEL, USA). The purity concentration of GLP1 plasmid was measured using a full-spectrum ultraviolet-visible spectrophotometer (NanoDrop™ 2000c, Thermo Fisher Scientific, USA). Also, the hydrodynamic radius and surface potential of the GLP1 plasmid were measured by a Zetasizer (Nano-s90, Malvern, UK). GLP1 gene sequence was analyzed from Macrogen, Seoul, Republic of Korea and confirmed by blast matching in the NCBI database.[1]

3. Synthesis and Characterization of PST

For PST synthesis, first, 4-nitrophenyl chloroformate (4-NPC) was chemically linked to the hydroxyl moiety of TCA. In detail, 1 g TCA was completely dissolved in ice-cold temperature at 50 ml DMF, then triethylamine (TEA, 10 molar equivalent) and 4-NPC (5 molar equivalent) were added to the TCA solution. The reaction was continued for 6 hours and maintained at 4 ℃ to avoid heat-induced inactivation of 4-NPC. At the end of the reaction, TCA-NPC was harvested by centrifugation at 12,000 rpm and 4 ℃ for ≥ 6mins. We used a liquid-liquid phase separation method using dH_2O and ethyl acetate (1:1 volume ratio) to extract and separate TCA-NPC from non-bound 4-NPCs and TEAs. Later, the activated TCA (equivalent to 50 moles of PS) was added to the PS solution and stirred for 24 hours at room temperature. The PST was dialyzed using a molecular weight cut-off (MWCO, 3.5 KD) membrane against dH_2O for 48 hours by replacing the dH_2O with fresh media every 3 hours. The lyophilized sample was stored at 4 ℃ for further examinations.

Studies on ^1H NMR (Avance 400FT-NMR, Bruker, USA), FT-IR and Zetasizer (Nano-s90, Malvern, UK) confirmed the successful synthesis of PST. The percentage of conjugation ratios between PS and TCA was measured by performing the trinitrobenzene sulfonic acid (TNBSA) assay. The amount of TCA conjugation to the PS was also quantified.

4. Preparation of pDNA/PST Complex

The pDNA was dissolved in 2M $CaCl_2$ and subsequently incubated at room temperature for 5 mins. Next, PS, bPEI, and PST were dissolved in 2x HEPES buffered saline (HBS) separately. To synthesis pDNA/PS, pDNA/bPEI, or pDNA/PST, pDNA solution was added to PS, bPEI, or PST solutions, followed by 10 minutes incubation at room temperature. Dynamic light scattering, surface potential, and agarose gel electrophoresis (ENDURO™ Gel XL Electrophoresis System, Labnet, USA) analysis confirmed the successful synthesis of pDNA/PS, pDNA/bPEI, and pDNA/PST. The morphology of pDNA/PS and pDNA/PST was observed by a field emission scanning electron microscope (FE-SEM, JSM 7610F, JEOL, Japan) and transmission electron microscope, (TEM, JEM-2100F, JEOL, Japan). The gene encapsulation efficiency of pDNA/PST was quantified employing gene retardation assay.[2]

5. Stability of pDNA/PST under Gastric Conditions

The enzymatic and pH stabilities of pDNA/PST were measured under gastric conditions and compared with pDNA/PS. The pDNA/PST was dissolved into pepsin (0.5 mg/mL, pH 2.0) or trypsin (140 g/mL, pH 5.5) solutions.[3] The stability of the complex was performed by the measurement of hydrodynamic diameter (nm), polydispersity index (PDI), and surface charge (mV) through dynamic light scattering and Zeta potential analysis during predetermined gastric emptying and duodenum digestion times. Subsequently, the structural integrity of pDNA/PST was also measured in the gastric fluid of leptin-protein mutant Lep^{OB}/Lep^{OB} mice. In short, mice (n = 5) were fasted overnight to remove food particles from their stomach. After 12 hours of fasting, the pylorus of the anesthetized mice was ligated, the mice were administered with 500 ul of ice-cold physiological saline, and then the junction in their esophagogastric was closed. Stomach fluid was collected within half an hour after the stomachs were carefully removed. Finally, the pDNA/PST was dissolved in the gastric fluid and the time-dependent degradation of the PDNA was measured using agarose gel electrophoresis.

6. MTT Assay

The cytotoxicity of PS, TCA, and PST in Caco-2, Hep G2, and MDCK-ASBT cells was analyzed performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to our established protocol.[4] The cells were grown in 96-well plate (5×10^4 cells/well) and incubated for 24 hours at 37 °C with 5% CO_2, followed by treatment with 0.1, 0.25, 0.5, and 1.0 mg/ml of PS, TCA, or PTSA for 48 hours. After incubation with MTT solution, the absorbance of chromogenic colors was measured at 590 nm wavelength, and the following equation was used to calculate the cellular cytotoxicity.[4]

Cell viability (%) = (absorbance of nanoparticle treatment cells /absorbance of control cells) × 100

7. CCK-8 Assay

Here, we have used HEK293 cells to compare the cellular cytotoxicity of pDNA/PST with pDNA/bPEI nanocomplex employing the CCK-8 assay. HEK293 cells were seeded in a 96-well microtiter plate (5×10^5 per well) and subsequently treated with pDNA/PST or pDNA/bPEI NP. Each cell containing well was treated with 10 μL of CCK-8 solution and incubated for 4 hours at 37°C incubator supplemented by 5% CO_2. The cell viability was measured according to the manufacturer's protocol (Sigma-Aldrich, USA).

8. Apoptosis Assay

The apoptotic profile of pDNA/PST was analyzed employing the Annexin V apoptosis assay according to the previously established protocol.[4] In brief, cells (Caco-2, HepG2, and MDCK-ASBT) were treated with pDNA/PST (5 mg/mL) for 12 h and then washed 2 times with PBS. The resuspended cellular samples were incubated with the same volume of Annexin V & Dead Cell reagent. After 20 min of incubation at room temperature, we quantitatively analyzed early and late apoptosis, live, and dead cells using a cell analyzer.

9. Cellular Uptake Assay

We used FITC-conjugated pDNA/PST to determine the contribution of bile salt (TCA) in the cellular uptake and translocation studies in Caco-2 ($ASBT^+$ intestinal epithelial cells), HepG2 ($NTCP^+$ hepatocytes), and ASBT-expressing or ASBT-knockout MDCK (MDCK-ASBT or MDCK) cells. This study was performed according to our previously reported protocol.[4] Using a light microscope (Axio Observer D1 Inverted Phase Contrast Fluorescence Microscope, ZEISS, Germany), we confirmed no contamination in the cells before treating our samples. The sample treated cell lines were incubated in a 37°C incubator in the presence of 5% CO_2 for 4 hours. Next, the nuclei of cells were counterstained by DAPI-blue dye (Vectashield H-1200, Vector Laboratories Inc., Burlingame, USA).[2] The number of $FITC^+$ cells was measured by the Fluorescence-activated cell sorting (FACS) analysis (Amnis® CellStream® Flow Cytometer, Luminex Corporation, USA). The cellular uptake of FITC-conjugated pDNA/PS or pDNA/PST was also quantified by measuring the fluorescence of FITC in the cells.[4]

10. Competitive Cellular Uptake Assay

To determine the ASBT mediated cellular uptake of pDNA/PST-FITC, we performed a competitive cellular uptake analysis in Caco-2, Hep G2, MDCK, and MDCK-ASBT cells. After pre-treatment of the cells with various concentrations of free TCA (0.1 ~ 1.0 mg/ml) for 30 min, the cells were treated with pDNA/PST-FITC for 4 hours. The competitive cellular uptake profile for pDNA/PST was quantified by measuring the fluorescence intensity of FITC (Synergy HTX, BioTek, USA) in samples prepared by lysing the cells with 0.3% Triton X100 solution.

11. Transepithelial Transport Study

To measure the transepithelial permeability of pDNA/PST, we performed a transwell migration assay with a standard protocol of a Millicell-ERS (Millipore, USA) system.[2] After verification of the integrity of the monolayered intestinal Caco-2 cells, we treated the cells with either pDNA/PST-FITC or pDNA/PS-FITC. After 0.5, 1, 2, 4, 6, 12, or 24 hours of treatment, the fluorescence intensity of the samples collected from the bottom of the transwell chamber was measured by a fluorescence spectrometer (FS-2, Scinco, Daejeon, South Korea). We have also checked the TEER value to ensure whether the Caco-2 monolayer was intact during the experiments as shown in Figure S11.

12. Nuclear Transport Study

The gene transfection efficiency of a nonviral nanocarrier depends on its ability to import the treated gene from the cytoplasm to the nucleus. To study the nuclear transportability of PST, MDCK-ASBT cells were treated with FITC-labeled pDNA/PST at different time points, such as 30 mins and 2 hours. Subsequently, the medium of cells was discharged, followed by washing the cell line with PBS to remove the free pDNA/PST. The nuclei of the cells were fixed with 4 % paraformaldehyde and counterstained with DAPI (Vectashield H-1200, Vector Laboratories Inc., Burlingame, USA). Finally, nuclear transportability of FITC-conjugated pDNA/PS or pDNA/PST was visualized using a high-resolution confocal microscope (DE/LSM-510, Carl Zeiss, Germany).

13. *In vitro* EGFP Gene Expression Study

The Hep G2 ($NTCP^+$ hepatocytes) cells were cultured in EMEM growth medium supplemented with 2mM glutamine, 1% NEAA (non-essential amino acid), 10% (volume/volume) FBS, and 1% streptomycin. Cells (5×10^4 / well) were seeded into microtiter (96-well) plates. The mediums were replaced with serum-free mediums after 12 h incubation at 37°C and 5% CO_2. Next, the cells in each well were treated with the enhanced green fluorescence protein (EGFP) pEGFP-N1 vectors loaded with PS, bPEI, lipofectamine 2000 (Lipo, a model transfection agent), or PST. After 4 hours of incubation, the mediums were placed with fresh and complete growth mediums, followed by incubation at 37°C and 5% CO_2 for the next 24 hours. The EGFP gene transfection efficiencies were visualized with a fluorescence microscope (LSM, Carl Zeiss, Germany).

14. *In vitro* Luciferase Gene Transfection Assay

To analyze the cellular gene transfection of the PST complex into the Caco-2 and MDCK-ASBT cells, we measured the luciferase gene expression by luciferase assay (Luciferase Reporter Assay Substrate Kit – Firefly #ab228530, Abcam, Cambridge, UK). In brief, MDCK-ASBT and Caco-2 cells were prepared in 96 well cell culture plates at the cell density of 5×10^4 cells/well. After the treatment of the cells with PST or bPEI complexed with luciferase plasmids for 18 hours in a 37 °C incubator under 5% CO_2, luciferase assay was performed by the measurement of cellular

luminescence intensity using a luminometer (Orion II Microplate Luminometer, Titertek-Berthold, Pforzheim, Germany).

15. *In vitro* GLP1 Gene Transfection Study

To study the *in vitro* therapeutic efficacy of pDNA/PST, we next examined the GLP1 Transfection efficiency in the MDCK-ASBT cells ($NTCP^+$ hepatocytes). The MDCK-ASBT cells were grown in a complete growth medium (RPMI + 10% FBS + 100 units / mL streptomycin) prior to the study. The cells were seeded into 96-well plates (5×10^4 / well) and treated with pDNA/bPEI or pDNA/PST gene complexes at 37°C supplemented with 95% air and 5% CO_2. After 24 hours of treatment, the supernatants from each treatment group including control were collected and the ELISA kit assay was employed according to the manufacturer's protocol (Human GLP-1 ELISA Kit, MyBiosource, USA).

16. Animal Study

All animal studies were performed under the regulation of IACUC (Institutional Animal Care and Use Committee) of Hanyang University (Seoul 04763, Republic of Korea) for the care and use of laboratory animals. In this research, 6-week old female ICR mice were used (Daehan Bio Link, Inc., Republic of Korea) to perform oral absorption, biodistribution, and *in vivo* EGFP and luciferase genes transfection studies, and 7-week old female SD rats (Daehan Bio Link, Inc., Republic of Korea) were used to study the hemolysis profile and complete blood count test.

Next, male C57BL/6J mice (7-week old) were purchased from Daehan Bio Link, Inc. (Chungbuk, Republic of Korea) and maintained a high-fat diet to produce a high-fat diet-induced diabetic (DIO) mouse model. To examined the therapeutic efficacy in more progressed and spontaneous diabetic strains, we purchased leptin protein-mutant Lep^{OB}/Lep^{OB}, and leptin receptor-deficient $Lepr^{DB}/Lepr^{DB}$ mice (male, 6-week old) from Gempharmatech, Nanjing, China. Normal C57BL/6J mice were used in this study as a standard non-diabetic model. All mice were divided into several experimental groups according to experimental design and housed in a controlled photocycle (light: 12h on and 12h off) room with free access to food and water.

Moreover, the safety profile and hypoglycemic properties of pDNA/PST were investigated in both normal and diabetic non-human primates (*Macaca mulatta, female, further* details can be found in Table S4-5). The animals were housed in a controlled room temperature (20-24 °C) and photoperiod (light: 12h on and 12h off) during experiments with free access to regular food supplemented with fresh vegetables and fruits. Animals were kept in close observation and examined their physical condition at least twice daily over the experimental periods. After a single oral administration of pDNA/PST, food uptake, body weight, blood glucose response, and changes in serum biochemical parameters were examined at predetermined time points. All animal experiments were conducted according to the guidlines of the National Institutes of Health (NIH) guidelines.[5]

17. Oral Absorption and Biodistribution Studies

The FITC-labeled pDNA/PS and pDNA/PST complexes were administered to the fasted 6-week female ICR mice ($N = 5$) by oral gavage. After predetermined times, the mice were euthanized to harvest organs. In order to perform cryosectioning, the intestinal ileum tissues were excised from GIT after 4 hours of administration, washed with cold PBS, and fixed with 4% paraformaldehyde. Next, the ileum tissues were embedded in OCT medium, and frozen tissue sections were obtained of 10-micron thickness with a cryo-microtome (Leica Microsystems Inc., USA). DAPI (Vectashield H-1200, Vector Laboratories Inc., Burlingame, USA) was used to stain cell nuclei. Thin slices were mounted in a mounting solution and examined with a laser scanning confocal microscope (CLSM 710 NLO, Carl Zeiss, Germany). For the analysis of the biodistribution profiles of pDNA/PST-FITC and pDNA/PS-FITC, samples were prepared by homogenization of the harvested organs in cold lysis buffer, and the total fluorescence intensity from the samples was measured by a fluorescence spectrometer (FS-2, Scinco, Daejeon, South Korea).

18. Hemolysis Assay

The hemolytic activity of pDNA/PS and pDNA/PST was measured by using a modified method from the previous report.[6] 500 μL PBS suspension of red blood cells (RBC) was obtained from a healthy female SD rat (7 weeks old, Daehan Bio Link, Inc. Republic of Korea). Thereafter, the samples were incubated with the same volume of 0.125, 0.25, 0.5, or 1.0 mg/ml of either pDNA/PS or pDNA/PST in PBS solution for 2 hours. After 2 hours of incubation at 37 ℃, the absorbance of supernatant from the centrifugation of the samples for 3 minutes at 10,000 rpm was measured by UV-spectrometer (OPTIZEN IV, Mecasys Co. Ltd., South Korea) at the 570 nm wavelength.

19. Complete Blood Count Test

To examine the toxicity of pDNA/PST in blood, we performed a complete blood count (CBC) test by an automated hematology analyzer (Sysmex XN-9000, Japan).[2] Briefly, the maximum available dose of pDNA/PST (100x) was orally administered to the female SD rats ($N = 5$) once weekly for 4 consecutive weeks. PBS-treated rats ($N = 5$) were used as the experimental control group. After the treatment, we collected 5ml of whole blood from each rat and performed CBC to check blood parameters such as white blood cells (WBC), hemoglobin (Hb), hematocrit (HCT), and platelet (PLT).

20. ECP-Mediated *In vivo* EGFP Gene Transfection Efficiency

Female ICR mice ($N = 5$) were used to study the *in vivo* ECP-riding gene transfection efficiency of PST. This study was conducted according to previously established protocols with slight modifications.[7] Overnight fasting mice were administered orally by EGFP plasmid pEGFP-N2, pEGFP-N2/PS, or pEGFP-N2/PST. After 24 hours of treatment, the mice were sacrificed and their organs, such as ileum and liver, were amputated to determine the gene expression kinetics of the

test NPs in $ASBT^+$ ileum and $NTCP^+$ liver tissues. The *in vivo* EGFP gene transfection efficiency of PST in ileum and liver tissues is shown in Figure 2E using an *in vivo* imaging system (IVIS, Xenogen, Alameda, CA, USA).

21. *In vivo* Luciferase Gene Expression Study

Overnight fasting mice were randomly divided into three separate groups, with 5 mice in each group. Subsequently, the mice were administered orally by luciferase plasmid PLuc, PLuc/PS, or PLuc/PST using oral feeding needles. After 24 hours of treatment, the mice were sacrificed, and then major organs such as the stomach, small intestine (SI), liver, heart, lungs, kidneys, spleen, and pancreas were harvested. The major organs were ground and homogenized into cell culture lysis reagent. Thereafter, the cell suspensions were centrifuged at 10000 rpm for 10 min. Finally, the luciferase activity was detected according to the previous section as described in section 2.14.

22. *Ex vivo* Tissue Expression Kinetics of pDNA/PST

We further tested the expression of the GLP1 gene in the leptin receptor-deficient $Lepr^{DB}/Lepr^{DB}$ mice ($N = 5$) treated with a single oral dose of pDNA, pDNA/PS, and pDNA/PST. Each oral formulation contained 0.1 mg/kg GLP1 gene. The major organs such as the stomach, small intestine, heart, liver, lungs, kidneys, spleen, and pancreas were amputated from $Lepr^{DB}/Lepr^{DB}$ mice after 24 hours of treatment. Subsequently, the 5 μm thick tissues were deparaffinized with xylene and rehydrated through gradient ethanol immersion. After antigen retrieval, the tissue sections were blocked by 20% goat serum and subsequently treated by the rabbit polyclonal anti-GLP1 immunoglobulin (Abcam #ab23468, USA) at ice control temperature for overnight. The tissues were then incubated with secondary anti-rabbit antibodies (AlexaFluror® 488, 1:1000, Invitrogen, USA) at dark. The tissues were then washed with PBS and counterstained with DAPI medium. Finally, the tissue expression kinetics of the test NPs were visualized using a high-resolution fluorescence microscope (Eclipse TE2000-s, Nikon, Japan).

23. Dose-response Study

For the dose-response study, $Lepr^{DB}/Lepr^{DB}$ mice were divided into 5 treatment groups ($N = 5$), excluding the experimental control group, and administered with a single oral dose of 1, 0.7, 0.5, 0.1, or 0.05 mg/kg pDNAwith PST complex. The diabetic and experimental normal control groups were administered with a similar volume of PBS. On day -7 to 0, the blood glucose level was measured for all experimental groups to confirm whether their blood glucose exhibited a consistent base level (Figure S15). The non-fast blood glucose responses of $Lepr^{DB}/Lepr^{DB}$ mice-treated with pDNA/PST were measured in a regular manner (24 hourly) according to previously reported protocol.[8] The second, third, and fourth drops of tail vein blood were used to check blood glucose levels with a handheld glucometer.

SUPPORTING INFORMATION

24. Monitoring of Anti-diabetic Effect

Three different T2DM mouse models such as DIO mice, Lep^{OB}/Lep^{OB} mice, and $Lepr^{DB}/Lepr^{DB}$ mice were used in this study. 7 weeks old C57BL/6J mice maintained a high-fat diet (rodent diet with 65% of kilocalories contributed by fat) for 12 weeks to induce blood glucose at a hyperglycemic range before starting the experiment. In contrast, Lep^{OB}/Lep^{OB} mice and $Lepr^{DB}/Lepr^{DB}$ mouse models were accommodated in laboratory conditions for 12 weeks until the blood glucose level reaches an extreme hyperglycemic range.

25. Comparative Anti-diabetic Effects

To study the comparative analysis of pDNA/PST's anti-diabetic effects in the different formulation conditions and conventional drugs, each diabetic mouse model was randomly assigned to several groups (N = 5) according to the studies. For the first experiments, pDNA with PST formulated with or without CaP was administered by oral gavage to the 6 hours fasted diabetic mice (DIO, Lep^{OB}/Lep^{OB}, $Lepr^{DB}/Lepr^{DB}$). For second experiments, pDNA, PST, pDNA/PS, pDNA/PST, or pDNA/PST with the equivalent amount of PST were orally administered to fasted diabetic mice. The non-fast blood glucose responses of these three different diabetes mouse models were monitored in a daily manner over 8 days (day 0 to 7). The second, third, and fourth drops of tail vein blood were used to check blood glucose levels with a handheld glucometer. Comparisons of the anti-diabetic effect between groups measured by the non-fast blood glucose response and normalized blood glucose AUC after a single administration.

26. Pleiotropic Effects of pDNA/PST

To study the actual pleiotropic effects of pDNA/PST against T2DM after a single oral administration, mice (DIO, Lep^{OB}/Lep^{OB}, and $Lepr^{DB}/Lepr^{DB}$) were fasted for 6 hours prior to treatment and subsequently administered with pDNA/PST complex or an equivalent amount of PBS through oral administration. Male C57BL/6J mice aged 7 weeks were used as the normal control in these studies. The therapeutic efficacy in terms of hypoglycemic activities was measured regularly. The blood glucose level in non-fast condition was checked up to day 7 post-administration as described in the previous section. The total blood glucose exposure (AUC_{TOTAL}) was measured according to the daily blood glucose level and normalized with diabetic control. We have also investigated how treatment outcome alters daily food consumption following body-weight.

27. Glucose Challenge with IPGTT

In order to further confirm whether pDNA/PST could contribute to a sustained glycemic improvement for 7days, we studied an IPGTT in DIO mice. A single oral dose of pDNA/PST complex or an equivalent volume of PBS was administered to DIO mice. After 48 hours of treatment, a D-(+)-glucose solution (10 w/v%) was intraperitoneally injected to 4 hours fasted DIO

mice at a dose of 1000 mg/kg body weight. After intraperitoneal glucose challenge, the blood glucose response was measured according to predetermined time points such as 0, 30, 45, 60, 90, 120, and 180 minutes. On day-4 post-administration, we further studied IPGTT as previously described.

28. Long-term Therapeutic Efficacy

Finally, to evaluate the potency of pDNA/PST in the long-term treatment of T2DM, we used the most progressive T2DM model ($Lepr^{DB}/Lepr^{DB}$ mice) in this study. Male $Lepr^{DB}/Lepr^{DB}$ mice (N = 5) aged 12 weeks were administered orally by pDNA/PST once a week for 20 weeks of the treatment period. PBS-treated $Lepr^{DB}/Lepr^{DB}$ mice (N = 5) and untreated C57BL/6 mice (N = 10) were served as diabetes and normal control, respectively. The mice were kept under close observation throughout the study period. Post-administrative non-fast blood glucose levels and body weight changes (%) were measured in a daily manner as described before. Normalized blood glucose AUC of experimental mice receiving pDNA/PST complex was measured and compared with that of the PBS (diabetic control) group. Moreover, body weight changes of mice were measured periodically throughout the experiment. At the end of the study, the biochemical measurement of HbA1C (hemoglobin combined with blood glucose, %HbA1C baseline >8.0) was performed using ELISA kits according to the manufacturer's protocols (Mouse HbA1c Kit, Crystal Chem#80310).

29. Measurement of Serum GLP1 and Insulin

During the weekly oral administration of a multiple-dose of pDNA/PST to $Lepr^{DB}/Lepr^{DB}$ mice for 20 weeks, blood samples were collected from mouse tail vein and stored in the VACUETTE® blood collection tubes (Greiner Bio-One, Germany). The level of GLP1 and insulin in serum was measured using ELISA kits according to the manufacturer's protocols.

30. Immunohistochemistry (IHC)

The IHC of selected tissues was performed according to our previously established protocols.[2] In brief, the pancreases were harvested from $Lepr^{DB}/Lepr^{DB}$ mice-treated with a single oral dose of pDNA/PST, fixed in 10 % formalin, embedded in paraffin, and excised into 5-µm-close parts. The selected tissue sections were lapped on glass plates, followed by deparaffinized with xylene and rehydrated through ethanol immersion. Next, the tissue parts from the pancreases were treated with mouse monoclonal anti-insulin (1:1200; Abcam #ab6995, USA) and rabbit polyclonal anti-GLP1 immunoglobulins (1:1200; Abcam #ab23468, USA) at ice control temperature for overnight. The samples were then incubated with secondary goat anti-mouse (AlexaFluror® 574, 1:1000, Invitrogen, USA) and anti-rabbit (AlexaFluror® 488, 1:1000, Invitrogen, USA) antibodies at dark. After 1.5 h of incubation at 25 °C, the tissue slides were counterstained by a nuclear staining dye, DAPI (Vectashield H-1200, Vector Laboratories Inc., Burlingame, USA). Finally, the expressions of insulin and GLP1 in the pancreases were assessed using a high-resolution fluorescence

microscope (Eclipse TE2000-s, Nikon, Japan) with the excitation and emission filters of 575 and 488 nm, respectively.

31. Tissue Histology

After 20 weeks of treatment with 0.1 mg/kg (1x), 10x, or 80x of pDNA/PST in $Lepr^{DB}/Lepr^{DB}$ mice, we performed tissue histopathological analysis of major organs such as heart, lung, liver, kidney, spleen, stomach, small intestine, and pancreas according to our previously reported methodology[2,4]. The tissues were fixed with 4% paraformaldehyde, dehydrated in C_2H_5OH, embedded in paraffin, cut into 20-μm-thick sections, and mounted on glass slides. The rehydrated tissue sections were then stained with hematoxylin & eosin. Finally, the pathological assessment of each tissue section was examined with a microscope (AxioScan Z1, Zeiss, Germany).

32. Measurement of Pro-Inflammatory Cytokines

The $Lepr^{DB}/Lepr^{DB}$ mice ($N = 5$) were administered orally by 0.1 mg/kg (1x), 10x, 50x, or 100x of pDNA/PST. Mice ($N = 5$) were treated with an equal volume of PBS used as a control in this experiment. After 72 hours of oral pDNA/PST administration, the blood serum of $Lepr^{DB}/Lepr^{DB}$ mice was collected and the pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-12, and interferon (IFN)-γ were measured by mouse magnetic Luminex assays according to the previously published protocol.[9]

33. Evaluation of Safety Profile in a Healthy Rhesus Macaque

A normal rhesus monkey (*Macaca mulatta*, sex: female, age: 10 years) was treated with an oral dose of pDNA/PST containing 0.3 mg/kg pDNA. 7 days before and after the treatment, *Macaca mulatta* was anesthetized with ketamine, and blood sample was collected by venipuncture to analyze the following hematological and biochemical markers: total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin (TB), direct bilirubin (DBIL), total protein (TP), albumin (ALB), blood urea nitrogen (BUN), serum creatinine (CR-S), creatine kinase (CK), and blood glucose level (BGL). The serum biochemical analysis methodology was performed according to the previously established protocol.[10] Furthermore, we measured the amount of daily food consumption in the pDNA/PST treated monkey over a period of 15 days to examine whether oral pDNA/PST was associated with GIT adverse effect.

34. Hypoglycemic Properties Evaluation in Diabetic Monkeys

Diabetes rhesus monkeys (*Macaca mulatta*, sex: female, age: 10-24 years) were administered with a 0.3 mg/kg oral dose of pDNA/PST or an equivalent amount of ligand solution. The plasma glucose was measured at predetermined time intervals such as day 0, 1, 2, 3, 4, 8, 12, and 14.

35. Statistical Analysis

In this study, data were shown as mean ± standard deviation from three to five independent experiments as indicated. Statistical analyses were performed using analysis of variance (ANOVA) in GraphPad Prism 8.4.1 between two or more groups. *P*-values of <0.05 were considered statistically significant.

B. Supplementary Figures

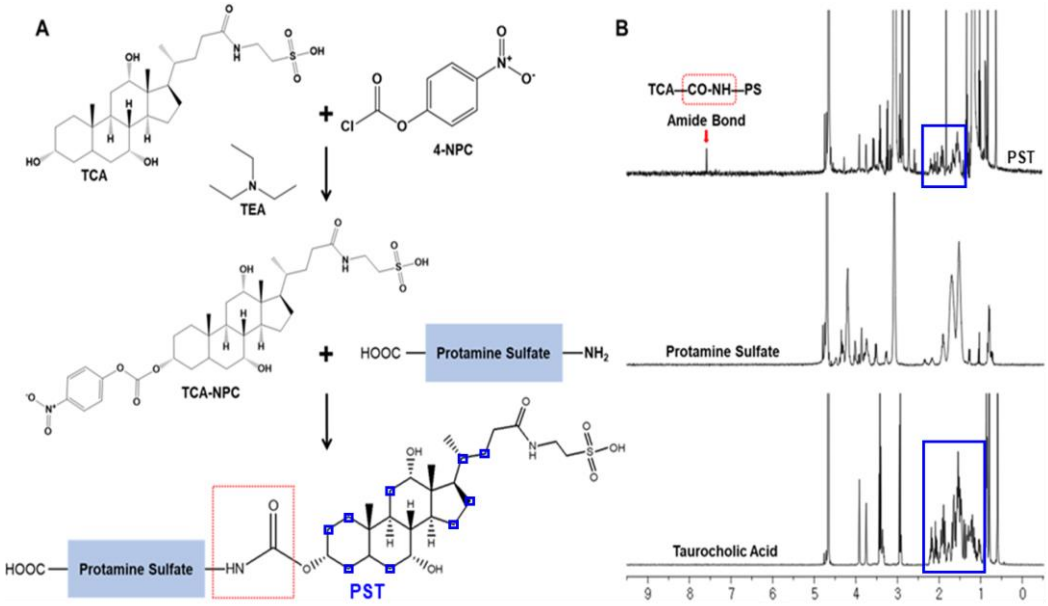

Figure S1. Synthesis and Characterization of pDNA/PST. (A) A chemical synthesis scheme of PST for oral gene delivery. (TCA: taurocholic acid, TEA: triethylamine, PS: protamine sulfate). First, TCA-NPC conjugates were prepared by coupling the hydroxyl group of TCAs to 4-NPC. Subsequently, PST was synthesized by reacting the active ester group of TCA-NPC with the primary amine of PS. (B) Confirmation of the PST synthesis by 1H NMR analysis. The attachment of TCA to the PS was evident by observation of the 1H NMR peaks of an amide bond (red rectangular box) and TCA moieties (blue rectangular boxes).

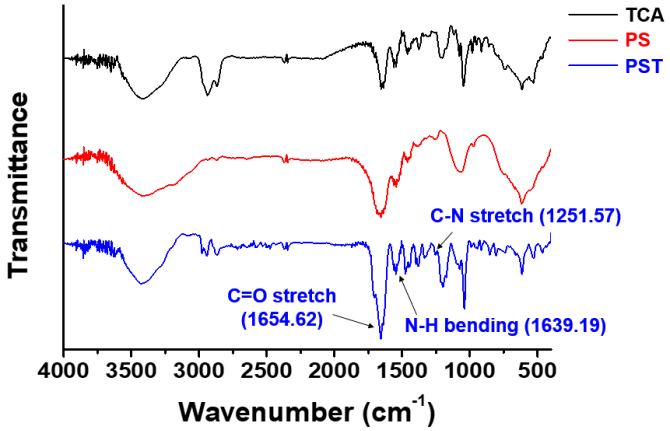

Figure S2. FT-IR analysis of a PST conjugate. The functional group signals at 1654.62, 1639.19, and 1251.57 cm−1 confirm the successful formation of a PST conjugate with C=O stretch, N-H bending, and C-N stretch, respectively.

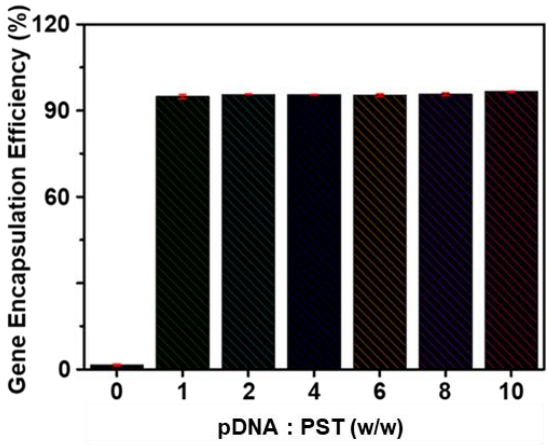

Figure S3. Gene encapsulation efficiency assay supports the shielding of pDNA (GLP1 plsmid) inside the carrier. The 1:1 weight ratio of pDNA/PST found as optimum with 100% loading efficiency. Mean ± SD, $N = 5$.

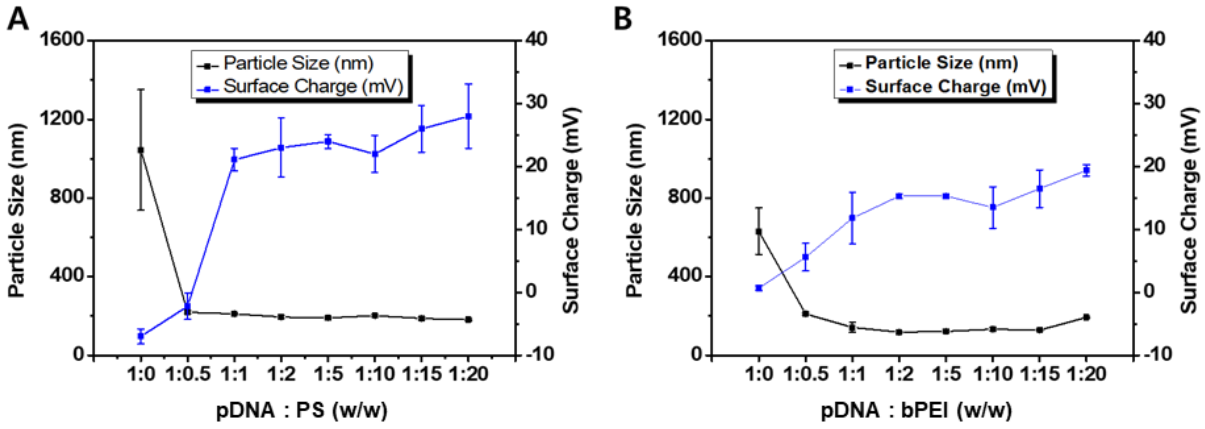

Figure S4. (A-B) Effect of different weight ratios of pDNA/PS and pDNA/bPEI gene complexes on their hydrodynamic diameters and surface charges, respectively. Data are expressed as mean ± SE, $N = 3$.

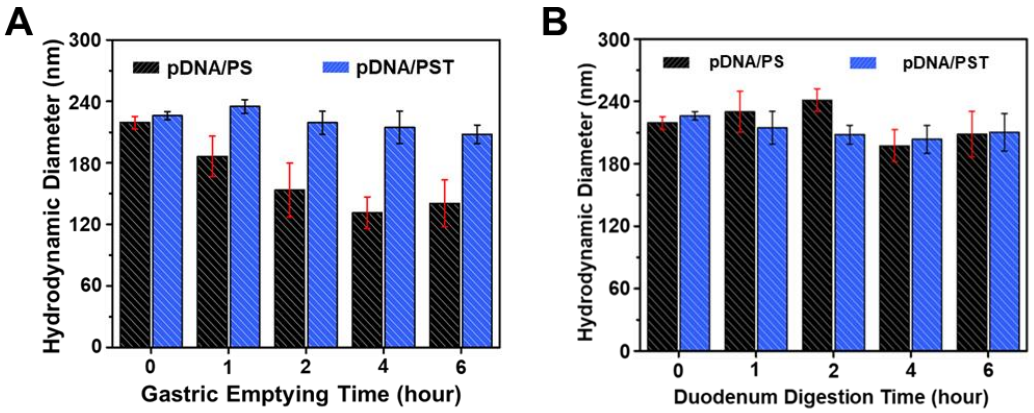

Figure S5. (A-B) Time-dependent size stability of pDNA/PS and pDNA/PST under the simulated gastric emptying times and duodenum digestion times, respectively. To test the integrity of the pDNA/PST under gastric conditions, we measured the changes in the hydrodynamic diameter of the test NPs, which were incubated under the simulated gastric and duodenum juices in the presence of pepsin and trypsin, for 6-hour of total duodenum digestion time. Data are expressed as mean ± SE, $N = 5$.

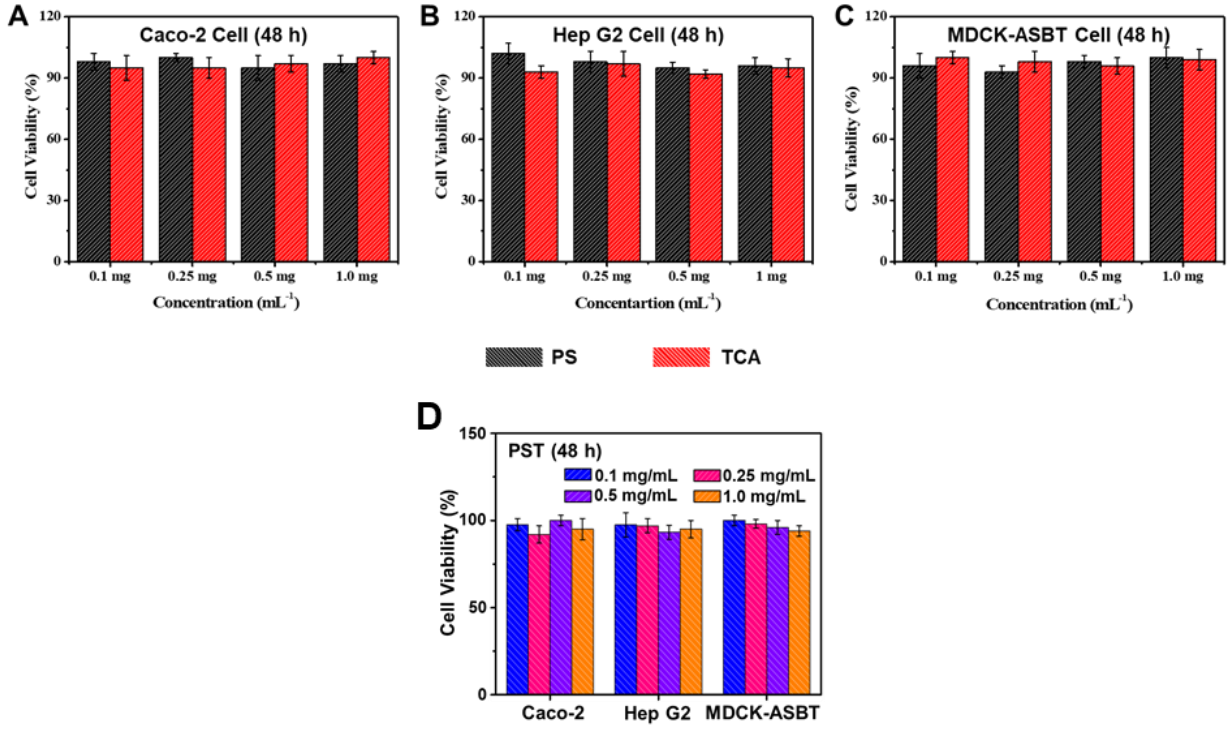

Figure S6. (A-C) Cell viability assay to evaluate the cytotoxicity of PS and TCA in Caco-2, Hep G2 and MDCK-ASBT cells, respectively, after 48 hours incubation. (D) Cellular toxicity of PST measured by MTT assay in Caco-2, Hep G2, and MDCK-ASBT cell lines after 48 hours of PST treatment. Mean ± SD, $N = 3$.

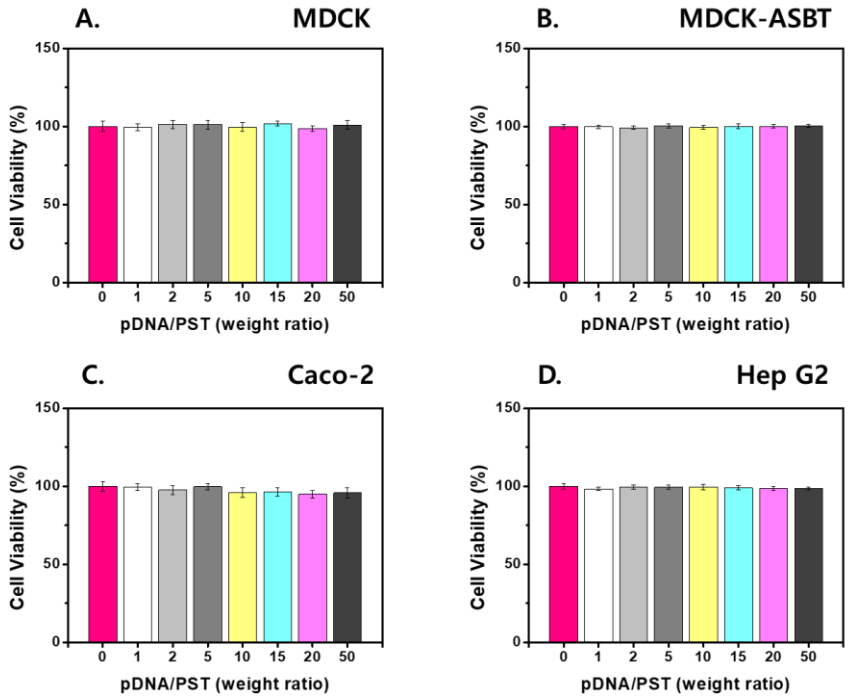

Figure S7. (A-D) Dose dependent cytotoxicity of pDNA/PST in MDCK, MDCK-ASBT, Caco-2, and Hep G2 cell lines, respectively. Data were presented as mean± SD, $N = 5$.

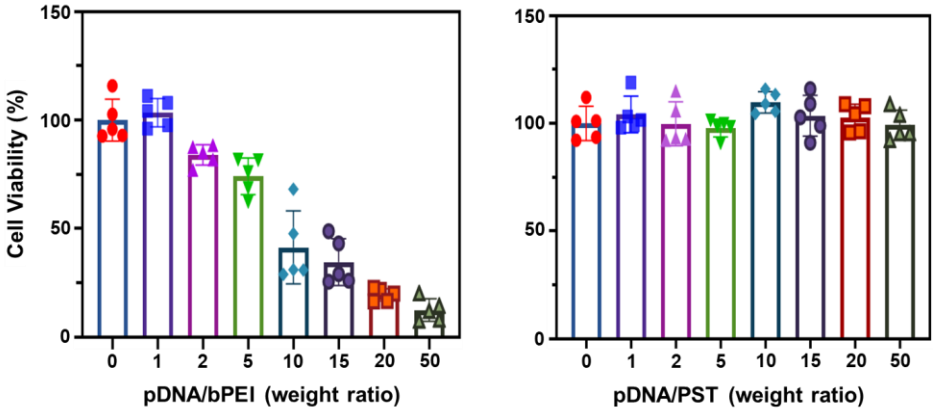

Figure S8. The nanoparticle-based therapeutic delivery system can cause kidney damage as the kidneys are often exposed to therapeutic or toxic molecules. Hence, to test the acceptability of our gene delivery system in a clinical setting, we examined the safety profile of pDNA/PST in the HEK293 cell line by employing the cell count kit-8 (CCK-8) assay. The cell viability assay indicated no cellular toxicity in the various pDNA/PST concentrations with the complexation ratio of up to 1:50 (GLP1: PST), while GLP1/bPEI, a conventional cationic polymer complex for gene delivery, exhibited observable cellular toxicity when the amount of bPEI exceeded the equal amount of GLP1.

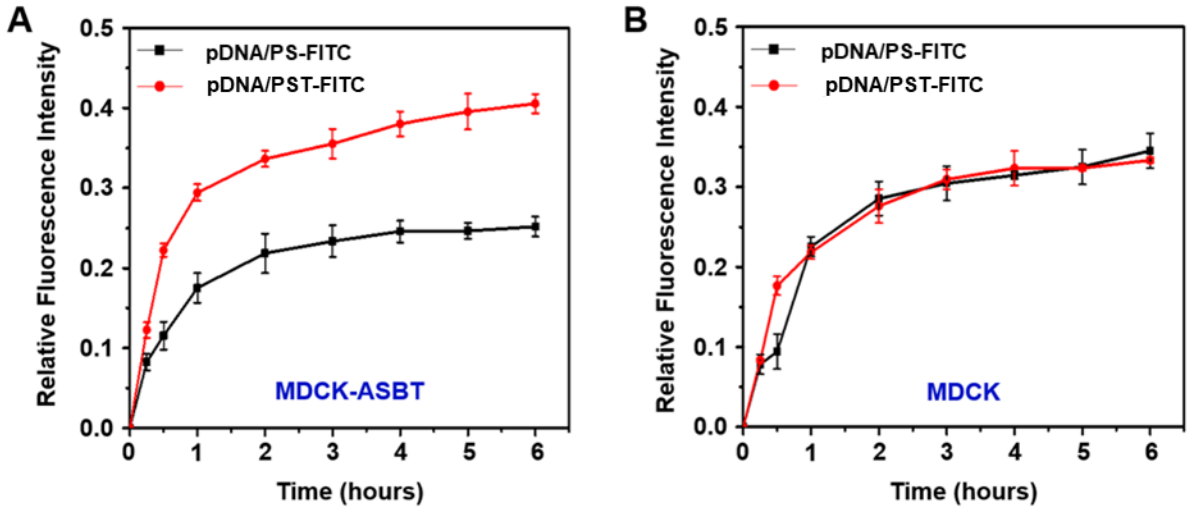

Figure S9. (A-B) Quantification of fluorescence intensity in the MDCK-ASBT (A) and MDCK after treatment with pDNA/PS-FITC or pDNA/PST-FITC (mean ± SD, $N = 4$). These studies clearly demonstrated the role of TCA in ASBT-mediated endocytosis.

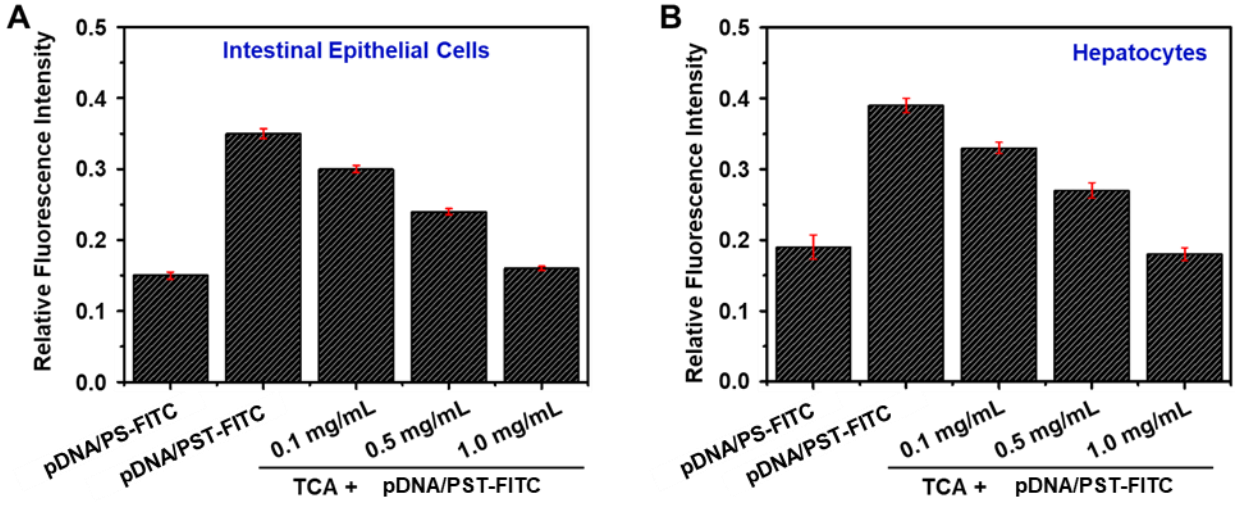

Figure S10. TCA-assisted cellular internalization assay of pDNA/PST**.** Cells were preincubated with different concentrations of TCA, and then competitive cellular intake analysis of pDNA/PST-FITC was performed in both Caco-2 (A) and HepG2 (B) cell lines (mean ± SD, $N = 4$). The results suggest that pDNA/PST can be absorbed in the intestinal ileal region through the interaction of the TCA moieties in the pDNA/PST to the ASBT transporter on the ileum, transported to the liver through enterohepatic recycling of the bile acids, and accumulated into the liver tissue by the NTCP transporter.

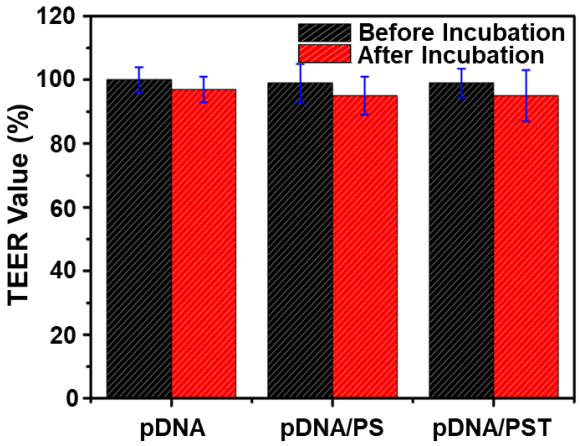

Figure S11. TEER values of Caco-2 monolayer before and after incubation with naked pDNA, pDNA/PS, and pDNA/PST. Data were presented as mean ± SD, $N = 5$.

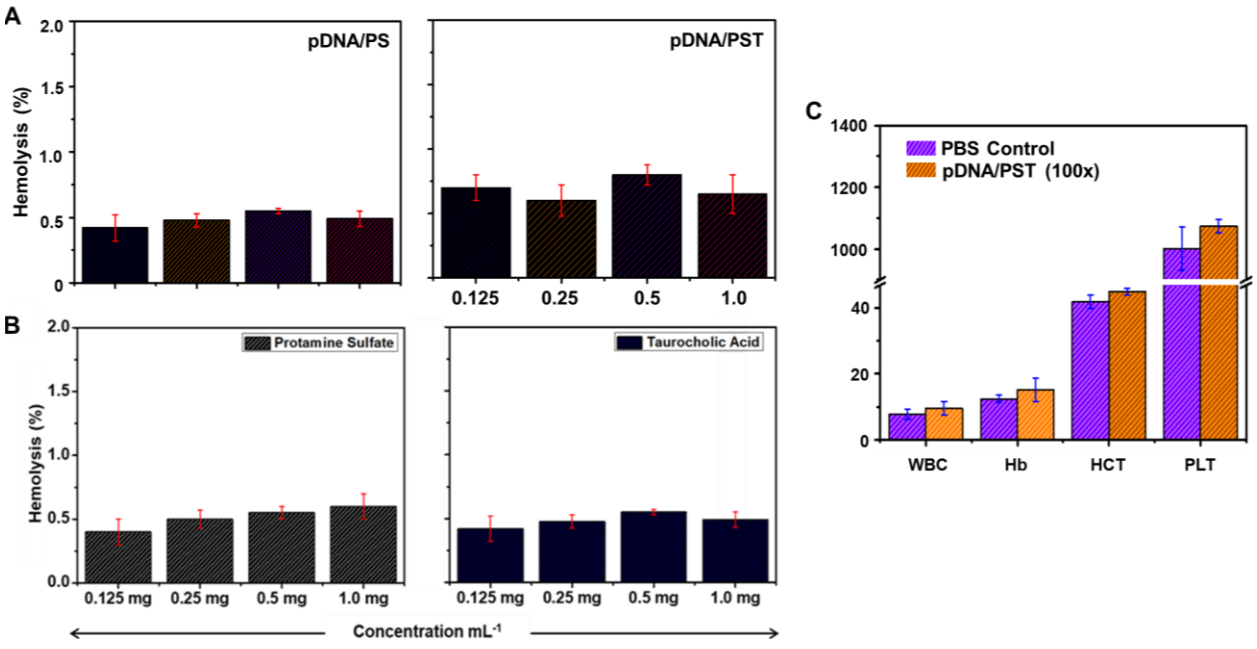

Figure S12. (A-B) Hemocompatibility profile of pDNA/PS, pDNA/PST, protamine sulfate (PS) and taurocholic acid (TCA). RBCs were isolated from the whole blood of a healthy SD rat, followed by incubation with different concentrations of PS and TCA. PS and TCA showed a negligible hemolysis profile. (C) Assessment of the blood toxicity by measuring complete blood count (CBC) the rat blood after oral administration of pDNA/PST. pDNA/PS was used as experimental control. Units: WBC, 10^9/L; Hb, g/dL; HCT, %; PLT, 10^9/L. Mean ± SD, $N = 3$.

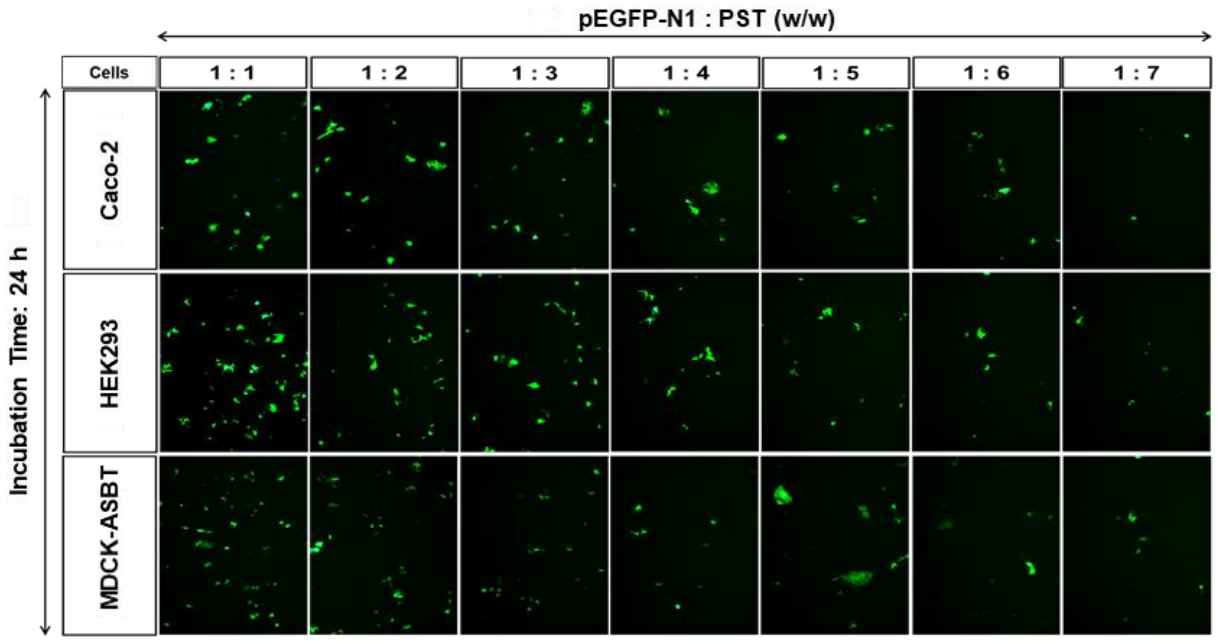

Figure S13. The transfection efficiency of pEGFP-N1/PST in Caco-2, HEK293, and MDCK-ASBT cell lines. Cells were treated with different ratios of PST containing the same amount of pEGFP-N1. After 24 hours of incubation, the transfection efficiency of pEGFP-N1/PST was measured in all cell lines. As the ratio of PST with plasmids increased, the gene expression efficiency of pEGFP-N1/PST was gradually decreased in all cell lines, indicating that the optimal ratio of the gene/vector complex should be less than or equal to 1:2. Because the presence of lysine residues in PS structure contributes to reducing the binding affinity with pDNA resulted in the induction of transfection-minimizing activity.[11]

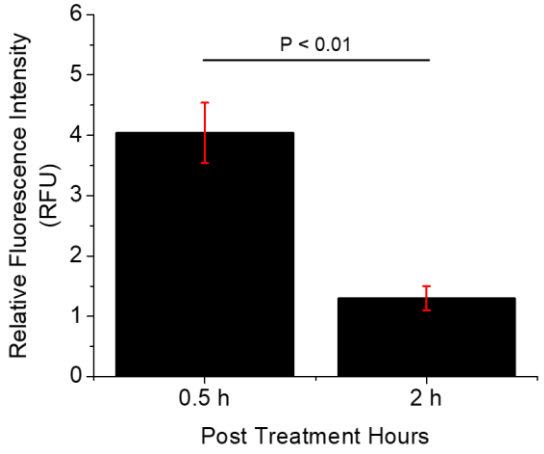

Figure S14. Measurement of fluorescence intensity of FITC-labeled pDNA/PST in the cytoplasm of MDCK-ASBT cell line by ImageJ (mean ± SD).

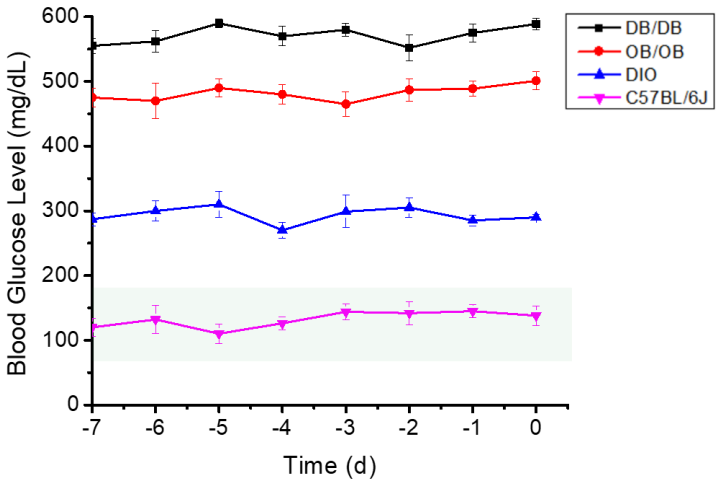

Figure S15. Blood glucose levels in DB/DB ($Lepr^{DB}/Lepr^{DB}$), OB/OB ($Lepr^{OB}/Lepr^{OB}$), DIO, and C57BL/6J mice before starting the dose-response study. The shaded region indicates the range of normoglycemia. Data are presented as mean ± SD

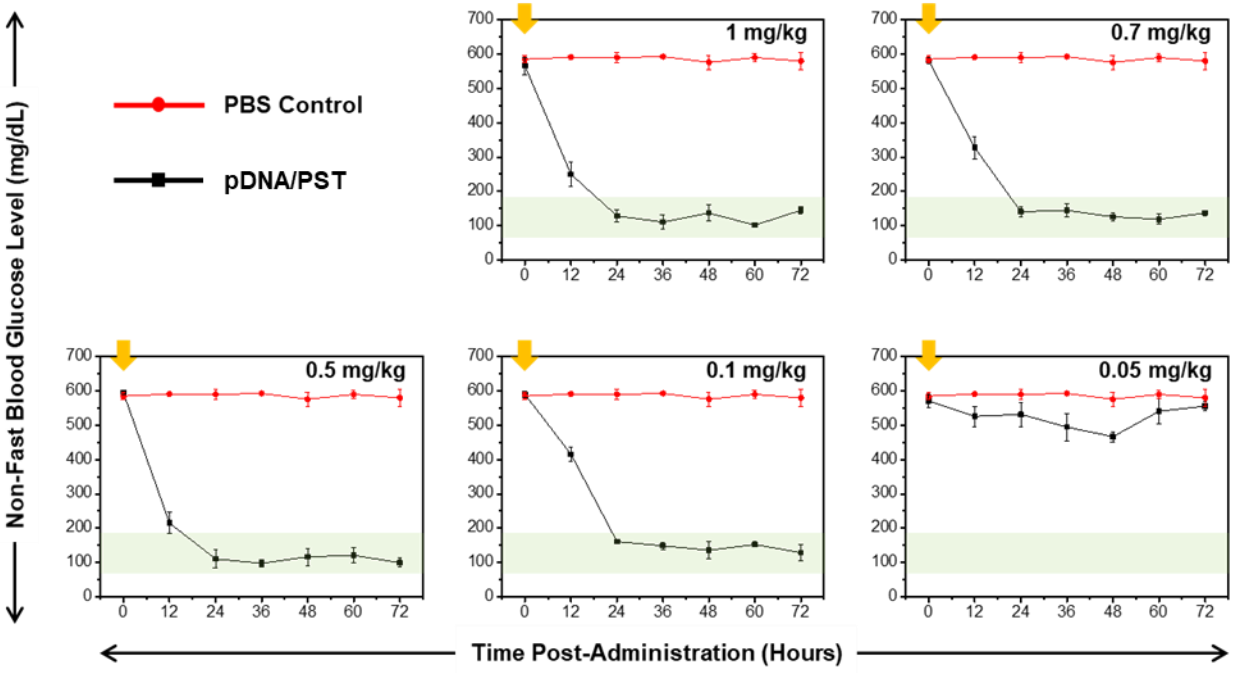

Figure S16. Dose-response study of pDNA/PST in $Lepr^{DB}/Lepr^{DB}$ mice. The post-administrative non-fast blood glucose levels in $Lepr^{DB}/Lepr^{DB}$ mice-treated with a single oral dose of 1, 0.7, 0.5, 0.1, or 0.05 mg/kg pDNA with PST complex. The diabetic control mice were treated with an equal volume of PBS. pDNA/PST treated $Lepr^{DB}/Lepr^{DB}$ mice showed a significant reduction in non-fast blood glucose levels for 1 to 0.1 mg/kg doses and maintained normoglycemia for the entire treatment period after a single oral administration, indicating transgenesis-based therapeutic effects of pDNA/PST, unlike release kinetics-based action. The yellowish arrow indicates the

dosing frequency of pDNA/PST or PBS. The shaded areas indicate the level of normoglycemia (mean ± SD, $N = 5$).

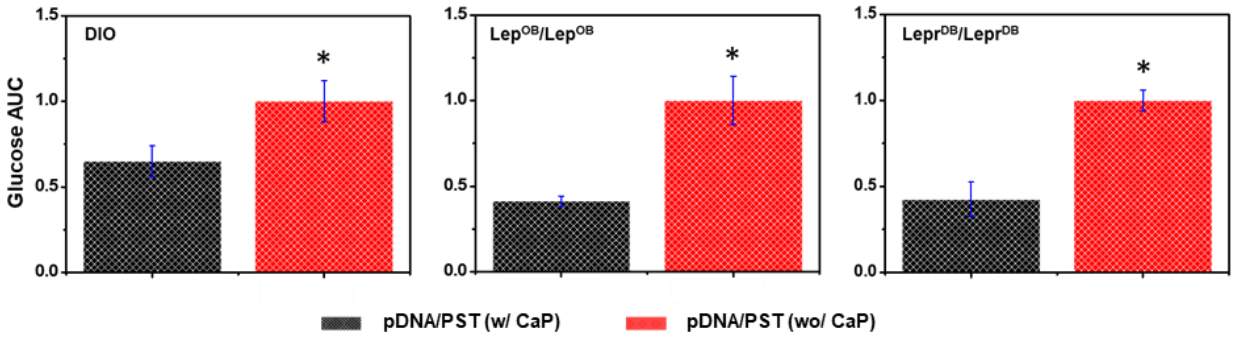

Figure S17. Glucose AUC was calculated over 8 days in three different diabetic mouse models after a single oral delivery of pDNA/PST with or without CaP. The total blood glucose exposures in pDNA/PST (w/ CaP) treated mice were normalized to the pDNA/PST (wo/ CaP) treatment groups. Data were presented as mean ± SE, $N = 5$, *$p<0.01$.

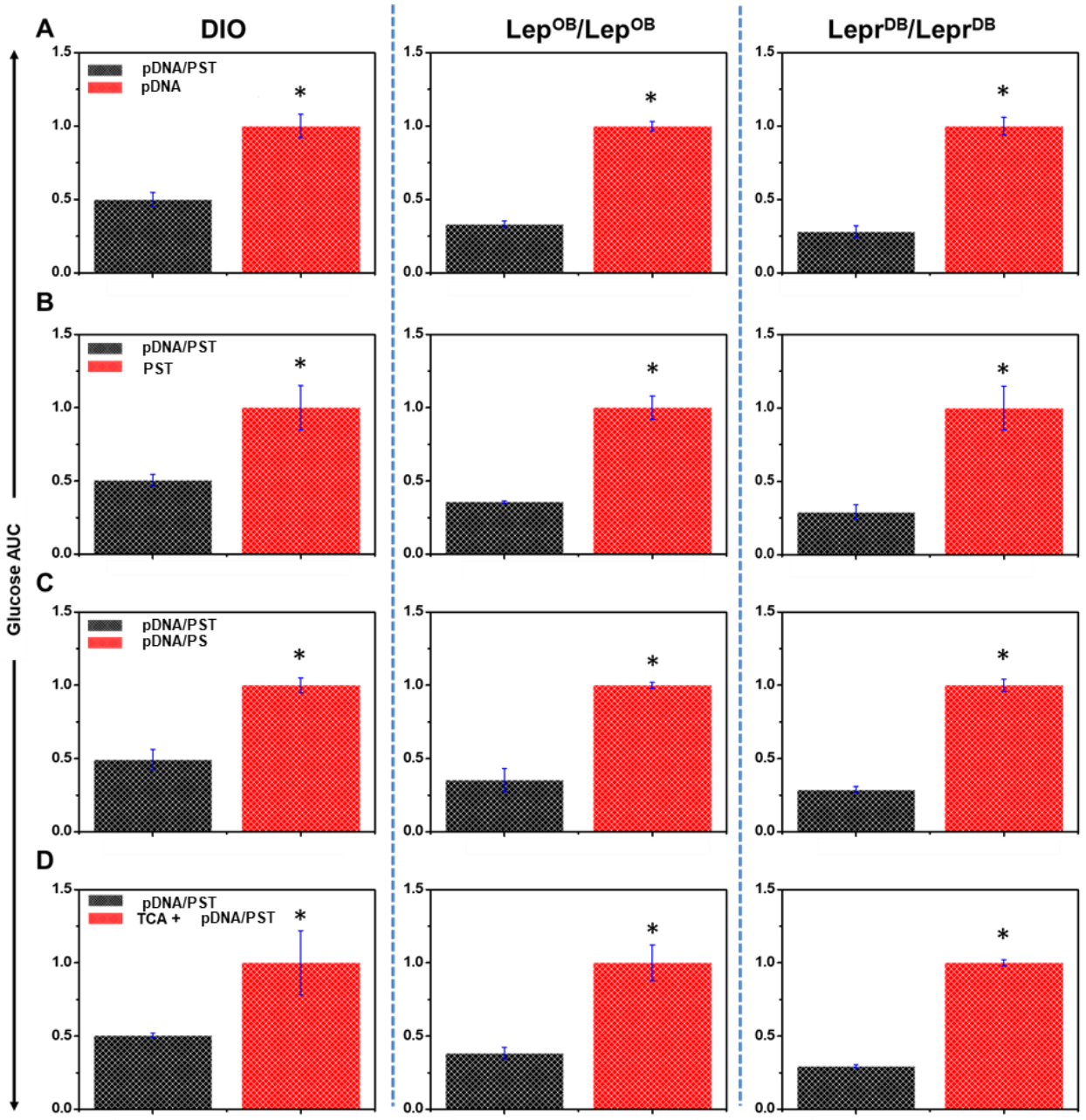

Figure S18. Glucose AUC was measured in all mouse models treated with pDNA/PST and normalized to the pDNA (A), PST (B), pDNA/PS (C), or TCA + pDNA/PST (D) treatment groups. Mean ± SE, $N = 5$, *$p<0.01$.

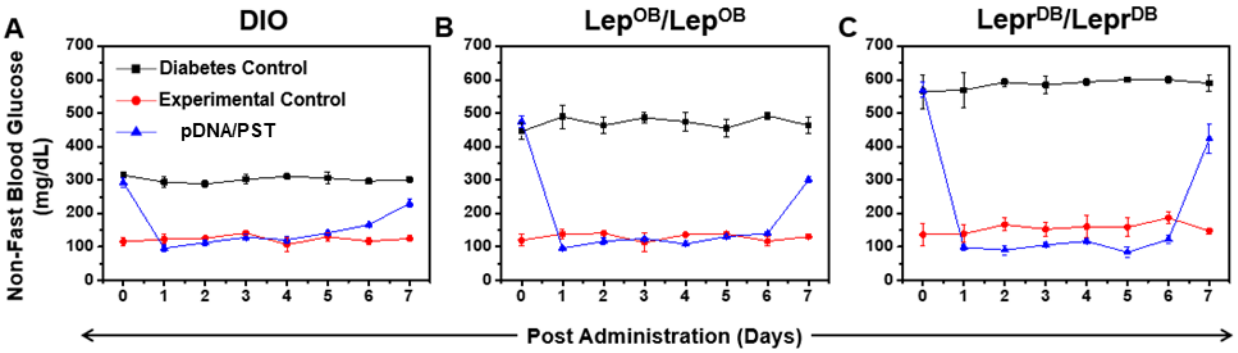

Figure S19. (A-C) Physiological responses of DIO (A), $Lepr^{OB}/Lepr^{OB}$ (B), $Lepr^{DB}/Lepr^{DB}$ (C) mice after a single oral administration of pDNA/PST (mean ± SD, $N = 5$).

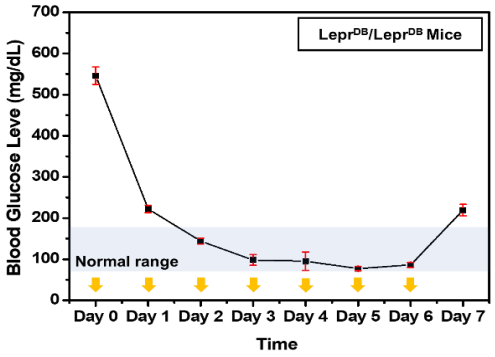

Figure S20. Evaluation of the antihyperglycemic activity of liraglutide in $Lepr^{DB}/Lepr^{DB}$ mice. Mice were daily injected with 18 μg of liraglutide for 7 days. The shaded region indicates the normal blood glucose level. Data are presented as mean ± S.D. ($N = 7$).

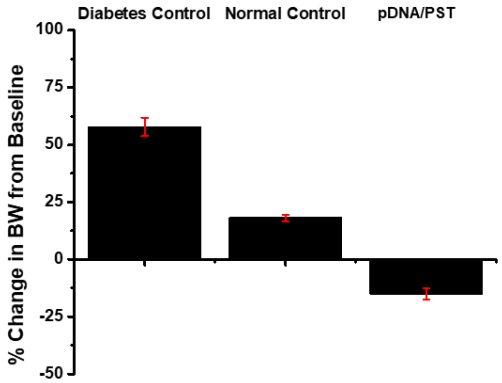

Figure S21. The $Lepr^{DB}/Lepr^{DB}$ mice were treated with oral pDNA/PST weekly for 150 days. The change in body weight (%) from baseline was measured at the end of the study. Data were presented as mean ± S.D. ($N = 5$).

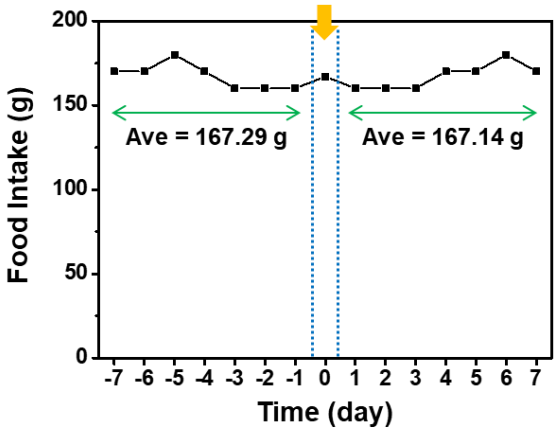

Figure S22. A normal and healthy rhesus monkey was administered orally by pDNA/PST, and subsequent treatment changes in diet behavior were observed to evaluate the gastrointestinal (GI) adverse events of our third generation oral pDNA/PST formation.

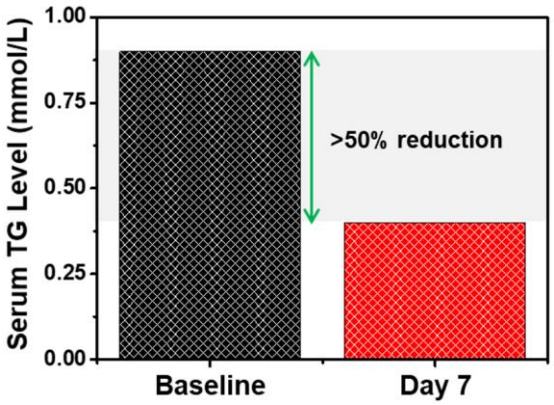

Figure S23. A single oral administration of pDNA/PST significantly reduced serum triglyceride level in *Macaca mulatta* by more than 50%. Since a high level of triglycerides is associated with an increased risk of atherosclerosis and therefore causes coronary artery disease and stroke, GLP1 expression in the heart increases cardiac protection by lowering triglyceride levels.

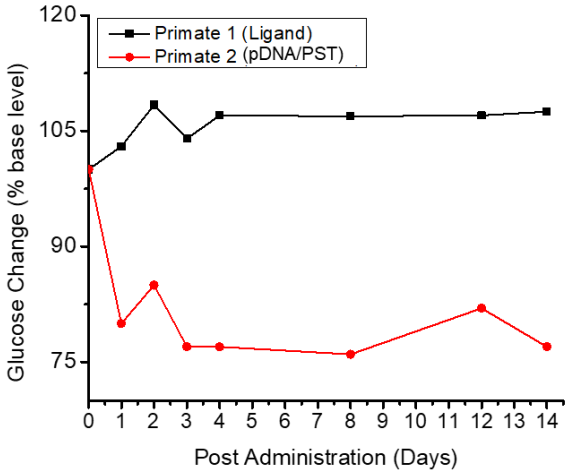

Figure S24. Blood glucose responses of diabetic rhesus macaque monkeys to pDNA/PST or an equivalent amount of the ligand, each treated by a single oral dose.

C. Supplementary Tables

Table S1: Conjugation of protamine sulfate (PS) to 4-nitrophenyl chloroformate-conjugated taurocholic acid (TCA-NPC).

Molar Ratio	Protamine Sulfate	TCA-NPC
1:10	100 mg	151.1 mg
1:30	100 mg	453.3 mg
1:50	100 mg	755.6 mg
1:70	100 mg	1057.8 mg
1:90	100 mg	1360 mg

Table S2: The average hydrodynamic diameter, polydispersity index and surface charge of pDNA, PST, pDNA/PST (1:1), pDNA/PS (1:0.5) and pDNA/bPEI (1:5). Data are presented as mean ± S.D., $N = 5$ with triplicate independent experiments.

Nanoparticle	Diameter (nm)	PDI	Surface Charge (mV)
pDNA	1044.1 ± 307.7	0.351	-7.0 ± 1.2
PST	53 ± 6.8	0.127	6 ± 4.1
pDNA/PST	226.1 ± 4.1	0.225	-3.1 ± 1.0
pDNA/PS	219.3 ± 6.2	0.269	-2.2 ± 2.1
pDNA/bPEI	117.8 ± 4.0	0.311	15.3 ± 0.4

Table S3: Evaluation of the apoptotic profile of pDNA/PST in Caco-2, Hep G2, and MDCK-ASBT cell lines.

Caco-2 Cell	Cell Concentration (cells/ml)	% Gated
Live	2.61E+05	96.55 %
Early Apoptotic	2.16E+03	0.80 %
Late Apop./ Dead	2.57E+03	0.95 %
Debris	4.60E+03	1.70 %
Total Apoptotic	4.73E+03	1.75 %

Hep G2 Cell	Cell Concentration (cells/ml)	% Gated
Live	3.27E+05	98.35 %
Early Apoptotic	1.83E+03	0.55 %
Late Apop./ Dead	1.66E+03	0.50 %
Debris	2.00E+03	1.60 %
Total Apoptotic	3.50E+03	1.05 %

MDCK-ASBT Cell	Cell Concentration (cells/ml)	% Gated
Liv	2.48E+05	91.40 %
Early Apoptotic	4.81E+03	1.80 %
Late Apop./ Dead	7.74E+03	2.85 %
Debris	1.07E+04	3.95 %
Total Apoptotic	1.26E+04	4.65 %

SUPPORTING INFORMATION

Table S4: Information for the non-human primate used in acute toxicity test.

Species	*Macaca mulatta*	
Animal Model	Normal, healthy	
Sex	Female	
Age	10 years	
Weight	~ 4.5 kg	
Schedule	Schedule No.	1935SDT
	Observatio Time	15 days
Dose	ROA	Oral
	Frequency	Single
	Amount	0.3 mg/kg
	Concentration	0.313 (mg/ml)
	Volume	4.3 ml

Table S5: Information for non-human primates used in the evaluation of hyperglycemic activity of oral pDNA/PST.

Species	*Macaca mulatta*	
Animal Model	Type 2 diabetes mellitus	
Sex	Female	
Age	10-24 years	
Weight	~ 11.1 kg	
Schedule	Observation Time	21 days
Dose	ROA	Oral
	Frequency	Single
	Amount	0.3 mg/kg
	Concentration	0.313 (mg/ml)

References

(1) Hasan, M. N.; Hwang, Y. H.; An, J. M.; Shahriar, S. M. S.; Cho, S.; Lee, Y. Oral GLP1 Gene Delivery by an Antibody-Guided Nanomaterial to Treat Type 2 Diabetes Mellitus. *ACS Appl. Mater. Interfaces* **2020**. https://doi.org/10.1021/acsami.0c09814.

(2) Nurunnabi, M.; Lee, S.-A.; Revuri, V.; Hwang, Y. H.; Kang, S. H.; Lee, M.; Cho, S.; Cho, K. J.; Byun, Y.; Bae, Y. H.; Lee, D. Y.; Lee, Y. Oral Delivery of a Therapeutic Gene Encoding Glucagon-like Peptide 1 to Treat High Fat Diet-Induced Diabetes. *J. Control. Release* **2017**, *268*, 305–313. https://doi.org/10.1016/j.jconrel.2017.08.035.

(3) An, J. M.; Shahriar, S. M. S.; Hwang, Y. H.; Hwang, S. R.; Lee, D. Y.; Cho, S.; Lee, Y. Oral Delivery of Parathyroid Hormone Using a Triple-Padlock Nanocarrier for Osteoporosis via an Enterohepatic Circulation Pathway. *ACS Appl. Mater. Interfaces* **2021**, acsami.0c22170. https://doi.org/10.1021/acsami.0c22170.

(4) Kang, S. H.; Revuri, V.; Lee, S.-J.; Cho, S.; Park, I.-K.; Cho, K. J.; Bae, W. K.; Lee, Y. Oral SiRNA Delivery to Treat Colorectal Liver Metastases. *ACS Nano* **2017**, *11* (10), 10417–10429. https://doi.org/10.1021/acsnano.7b05547.

(5) *Guide for the Care and Use of Laboratory Animals*; National Academies Press: Washington, D.C., 2011. https://doi.org/10.17226/12910.

(6) Wu, Q.; Niu, M.; Chen, X.; Tan, L.; Fu, C.; Ren, X.; Ren, J.; Li, L.; Xu, K.; Zhong, H.; Meng, X. Biocompatible and Biodegradable Zeolitic Imidazolate Framework/Polydopamine Nanocarriers for Dual Stimulus Triggered Tumor Thermo-Chemotherapy. *Biomaterials* **2018**, *162*, 132–143. https://doi.org/10.1016/j.biomaterials.2018.02.022.

(7) Lin, P.-Y.; Chiu, Y.-L.; Huang, J.-H.; Chuang, E.-Y.; Mi, F.-L.; Lin, K.-J.; Juang, J.-H.; Sung, H.-W.; Leong, K. W. Oral Nonviral Gene Delivery for Chronic Protein Replacement Therapy. *Adv. Sci.* **2018**, *5* (8), 1701079. https://doi.org/10.1002/advs.201701079.

(8) Luginbuhl, K. M.; Schaal, J. L.; Umstead, B.; Mastria, E. M.; Li, X.; Banskota, S.; Arnold, S.; Feinglos, M.; D'Alessio, D.; Chilkoti, A. One-Week Glucose Control via Zero-Order Release Kinetics from an Injectable Depot of Glucagon-like Peptide-1 Fused to a Thermosensitive Biopolymer. *Nat. Biomed. Eng.* **2017**, *1* (6), 0078. https://doi.org/10.1038/s41551-017-0078.

(9) Han, X.; Lu, Y.; Xie, J.; Zhang, E.; Zhu, H.; Du, H.; Wang, K.; Song, B.; Yang, C.; Shi, Y.; Cao, Z. Zwitterionic Micelles Efficiently Deliver Oral Insulin without Opening Tight Junctions. *Nat. Nanotechnol.* **2020**, *15* (7), 605–614. https://doi.org/10.1038/s41565-020-0693-6.

(10) Yu, W.; Hao, X.; Yang, F.; Ma, J.; Zhao, Y.; Li, Y.; Wang, J.; Xu, H.; Chen, L.; Liu, Q.; Duan, S.; Yang, Y.; Huang, F.; He, Z. Hematological and Biochemical Parameters for Chinese Rhesus Macaque. *PLoS One* **2019**, *14* (9), e0222338. https://doi.org/10.1371/journal.pone.0222338.

(11) Sorgi, F.; Bhattacharya, S.; Huang, L. Protamine Sulfate Enhances Lipid-Mediated Gene Transfer. *Gene Ther.* **1997**, *4* (9), 961–968. https://doi.org/10.1038/sj.gt.3300484.