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Enhancing the Therapeutic Efficacy of GLP-1 for Hyperglycemia Treatment: Overcoming Barriers of Oral Gene Therapy with Taurocholic Acid-Conjugated Protamine Sulfate and Calcium Phosphate

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ABSTRACT: Activating the glucagon-like peptide-1 (GLP-1) receptor by oral nucleic acid delivery would be a promising treatment strategy against hyperglycemia due to its various therapeutic actions. However, GLP-1 receptor agonists are effective only in subcutaneous injections because they face multiple barriers due to harsh gastrointestinal tract (GIT) conditions before reaching the site of action. The apical sodium bile acid transporter (ASBT) pathway at the intestinal site could be an attractive target to overcome the problem. Herein, we used our previously established multimodal carrier system utilizing bile salt, protamine sulfate, and calcium phosphate as excipients (PTCA) and the GLP-1 gene as an active ingredient (GENE) to test the effects of different formulation doses against diabetes and obesity. The carrier system demonstrated the ability to protect the GLP-1 model gene encoded within the plasmid at the GIT and transport it *via* ASBT at the target site. A single oral dose, regardless of quantity, showed the generation of GLP-1 and insulin from the body and maintained the normoglycemic condition by improving insulin sensitivity and blood sugar tolerance for a prolonged period. This oral gene therapy approach shows significantly higher therapeutic efficacy in preclinical studies than currently available US Food and Drug Administration-approved GLP-1 receptor agonists such as semaglutide and liraglutide. Also, a single oral dose of GENE/PTCA is more effective than 20 insulin injections. Our study suggests that oral GENE/PTCA formulation could be a promising alternative to injection-based therapeutics for diabetics, which is effective in long-term treatment and has been found to be highly safe in all aspects of toxicology.

KEYWORDS: *oral gene delivery, nonviral gene delivery, glucagon-like peptide-1 (GLP-1), apical sodium bile acid transporter (ASBT), engineered nanoparticles, gene therapy, diabetes*

1. INTRODUCTION

Around 200 million people globally rely on daily insulin injections to manage hyperglycemia, a chronic disease affecting 470 million individuals worldwide.[1] The need to fast before each dose and pain from multiple injections leading to inflammation and diabetes wounds are common complications. Missing even a few doses daily can result in an imbalance in glycemic goals.[2] Previous studies have shown that long-term treatment with insulin can cause cardiovascular disease, cancer, and all-cause mortality compared to other drugs for hyperglycemia.[3]

Glucagon-like peptide-1 (GLP-1) receptor activation is a promising treatment for hyperglycemia due to its various therapeutic actions.[4] However, GLP-1 receptor agonists (GLP-1RA) are only effective in subcutaneous injections, and the only approved oral dosage form, Rybelsus, is expensive and requires a high daily dose.[5,6] To address these problems, ongoing efforts are dedicated to developing a weekly oral dosage form of a therapeutic gene. However, challenges remain due to the stomach's acidic pH, proteolytic enzymes in the small intestine, and the rapid degradation of therapeutic genes.[7] Previous studies have focused on developing many innovative needle-based gene therapies that could be alternative treatment modalities but may not overcome the problems associated with injection-based dosage forms.[8] Existing research on novel oral gene delivery systems has not yet produced a feasible solution due to the high dose of a therapeutic gene required and the complexity of the synthesis protocol, making large-scale production difficult.[9]

The apical sodium bile acid transporter (ASBT) is an active transporter found in the ileum of the small intestine that transports hepatic bile salts (BSs).[10,11] Our original research on ASBT-mediated drug delivery systems showed that they could

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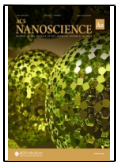

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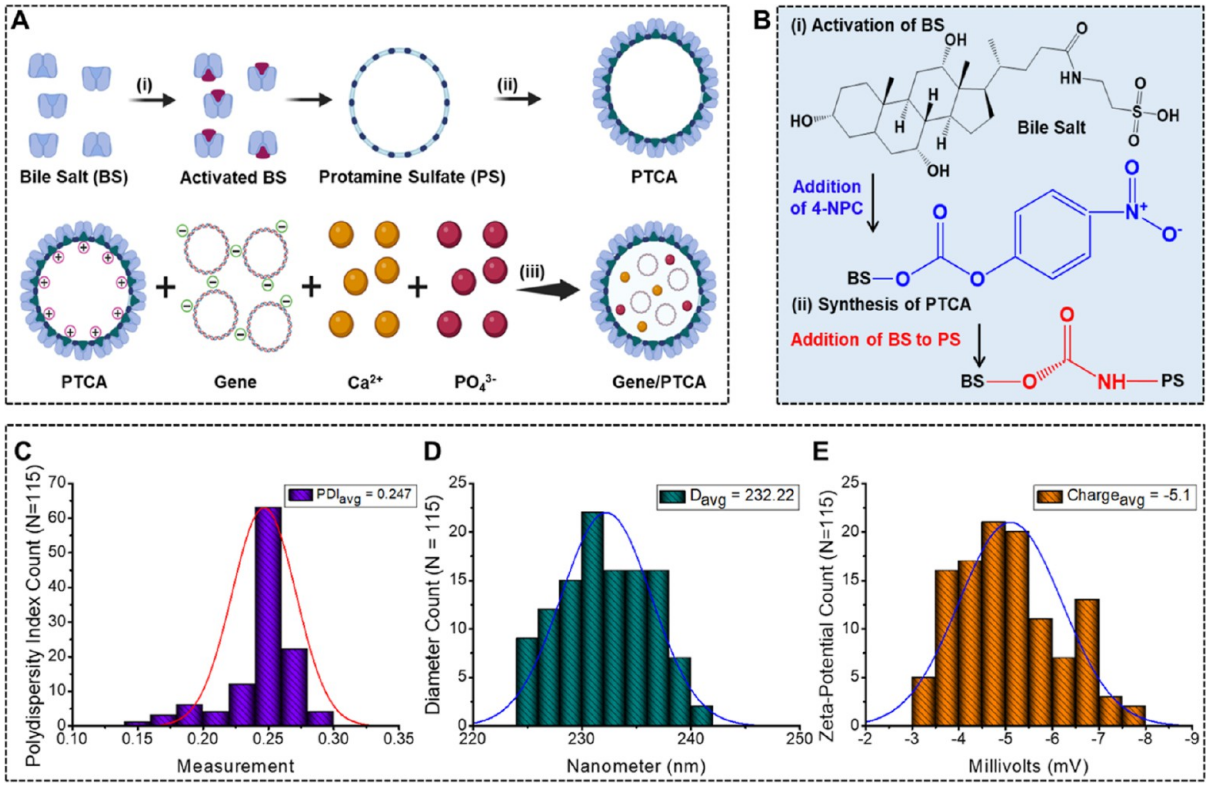

Figure 1. Preparation and characterization of GENE/PTCA. (A) The schematic illustrates the production procedure of the pharmaceutical oral dosage form of the GLP-1 gene. (i) The BSs are activated *via* 4-NPC, an intermediate that has an affinity to the primary amine groups. (ii) Next, the activated BS is added to the PS solution. (iii) Finally, the GENE/PTCA is prepared by electrostatic interaction between the therapeutic gene, calcium phosphate, and PTCA. (B) Chemical scheme represents the preparation of PTCA. (C–E) Polydispersity index, diameter, and zeta-potential analysis of GENE/PTCA, respectively.

transport a high percentage of BSs that reach the ileum.[12] Inspired by this, we hypothesized that modifying the surface of an oral nanoformulation containing a plasmid encoding the GLP-1 gene could mimic bile acid physiology.[13] We previously reported proof-of-principle studies on this oral GLP-1 gene formulation and have now demonstrated its ability to treat diabetes and morbid obesity with a minimal therapeutic dose.[13] In this article, we have investigated the therapeutic impacts of various doses of our previously developed nanomaterials. The nonviral oral gene delivery system comprises three US Food and Drug Administration (FDA)-approved excipients: protamine sulfate (PS), taurocholic acid (TCA) [a BS] and calcium phosphate (CaP), which selectively deliver the GLP-1 gene (GENE/PTCA). TCA protects the formulation during gastrointestinal tract (GIT) transition and enhances absorption by facilitating ASBT-mediated endocytosis.[14] PS protects the GLP-1 gene from dipeptidyl peptidase-4 degradation and enhances cellular permeability and nuclear import,[15,16] while CaP, a safe and inexpensive transfection agent, enhances transfection efficacy.[17]

The GENE/PTCA treatment is designed for easy large-scale synthesis, translation into clinical settings, and effective treatment at minimal dose. Unlike insulin, it can be taken as an oral suspension, eliminating the need for fasting before medication intake. A single oral dose confirms the secretion of GLP-1 incretin hormone and insulin, which regulate normoglycemia by improving insulin sensitivity and blood sugar tolerance under diabetic conditions. The oral dosage form of the GLP-1 gene works effectively at minimal doses, reducing total blood sugar exposure, normalizing glycosylated hemoglobin (HbA1c) levels, restoring GLP-1 and insulin expressions and secretions, and controlling obesity by reducing total intraperitoneal fat content in the long term. Furthermore, the oral GENE/PTCA formulation exhibits a higher therapeutic window with a minimal amount of gene compared to other GLP-1 receptor agonist formulations reported in recent years.[18−25]

2. RESULTS

2.1. Preparation of Oral Nucleic Acid Formulation

The pBeta-SP-GLP-1-encoded complementary DNA in the furin cleavage site, next to the secretion signal peptide, was isolated and purified from pretransfected*Escherichiacoli*.[26] The sequence of the human GLP-1 gene was matched with the National Center for Biotechnology Information (NCBI) database.[26] The oral gene formulation (GENE/PTCA) preparation process was designed to require a minimum in-process quality control and fewer regulatory processes to manufacture on a larger scale at the most minimal cost (Figure 1A). In brief, PTCA was synthesized by simple conjugation chemistry (Figure 1A, upper panel). The intermediate of BS [4-nitrophenyl chloroformate (4-NPC)] contains an active ester group that reacts readily with the primary amine of PS when activated BS is added to the PS solutions (Figure 1B). The Fourier-transform infrared (FTIR) spectroscopy signals with the C−H stretch of aromatics, C═O stretch of NPC, N−H bending, and C−N stretch confirm the successful conjugation of PS with activated TCA (Figure S1). Subsequently, GENE/PTCA was prepared by adding GLP-1

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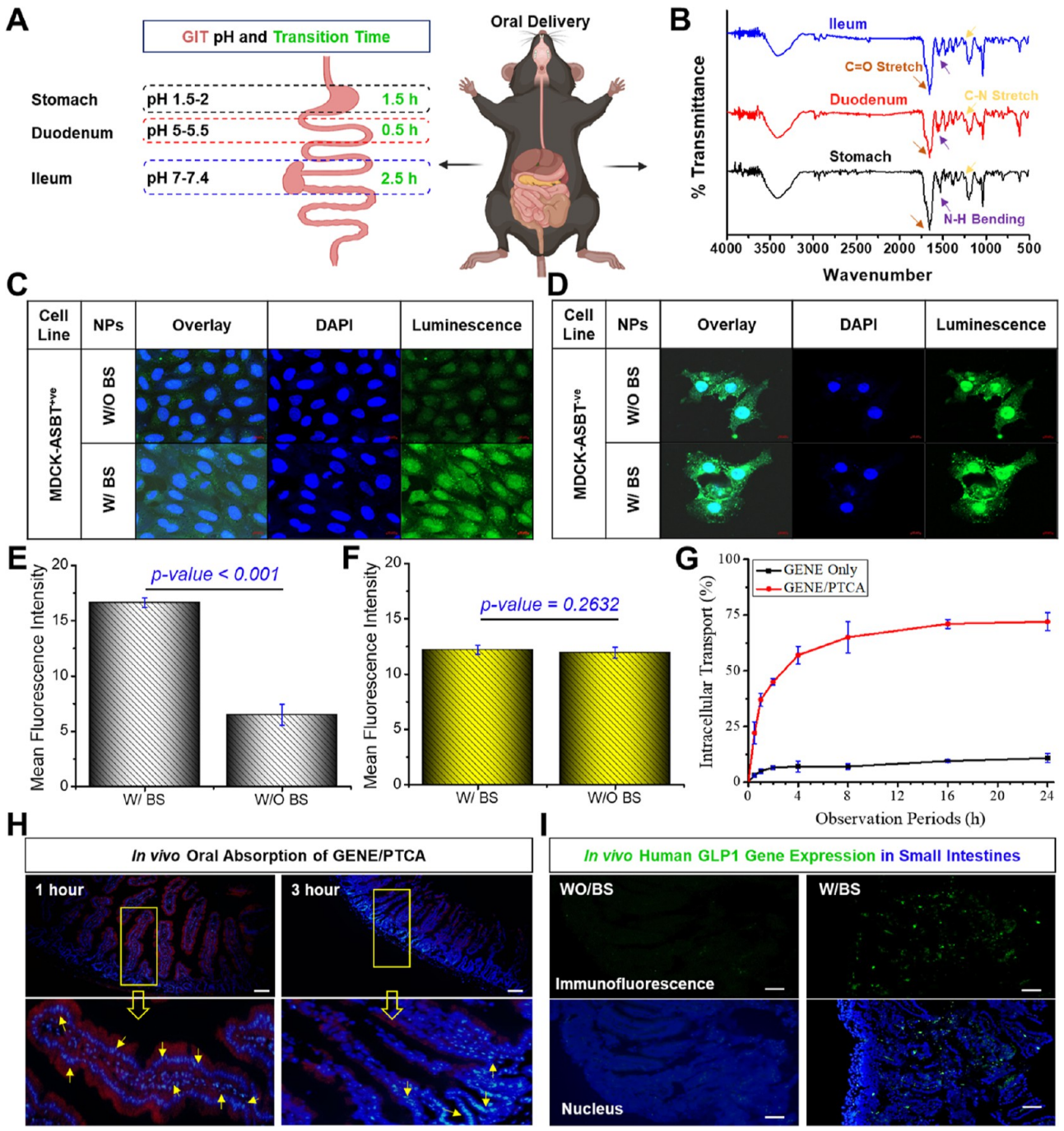

Figure 2. Structural stability, ASBT-mediated cellular uptake and transport, and *in vivo* oral absorption and gene expression of GENE/PTCA. (A) The schematic represents the presence of different pH values in different parts of the GIT. (B) Structural integrity of the tested NPs during gastrointestinal transitions. Orange (Accent 2) arrows indicate C═O stretch; gold (Accent 4) arrows represent C−N stretch; purple arrows show N−H bending. (C,D) Proof-of-principal study of ASBT-mediated cellular uptake of GENE/PTCA in the small intestine. (E,F) Quantitative measurement of Figure 2C,D, respectively. (G) Intracellular transport of GENE/PTCA in an ASBT-expressed cell line. (H) Cross-sectional morphology of the mouse intestine after oral administration of GENE/PTCA. The yellow arrows indicate the presence of test NPs inside the intestinal epithelium. (I) *In vivo* gene expression of GENE/PTCA in the intestine of a genetically engineered diabetes mouse ($Lepr^{DB}/Lepr^{DB}$). Mean ± SD, N = 7, and scale = 100 μm.

gene solutions to the PTCA solution (Figure 1A, lower panel). The spherical surface morphology of GENE/PTCA has been confirmed *via* transmission electron microscopy (Figure S2A). Also, the gene encapsulation efficiency has been confirmed using a gel electrophoresis assay with a 1:1 (w/w) ratio of PTCA and the GLP-1 gene (Figures S2B,C). The resulting oral nanoformulation exhibits a uniform molecular mass distribution with a heterogeneity index measurement of 0.247 ± 0.02 (Figure 1C). Observing the dynamic light scattering (DLS) and zeta-potential analysis, we concluded that PTCA formed slightly negatively charged nanoparticles (−5.1 ± 1.1 mV) with the GLP-1 gene through electrostatic interaction at an average hydrodynamic size of 232.22 ± 4.12 nm (Figure 1D,E). The negative surface charge of GENE/

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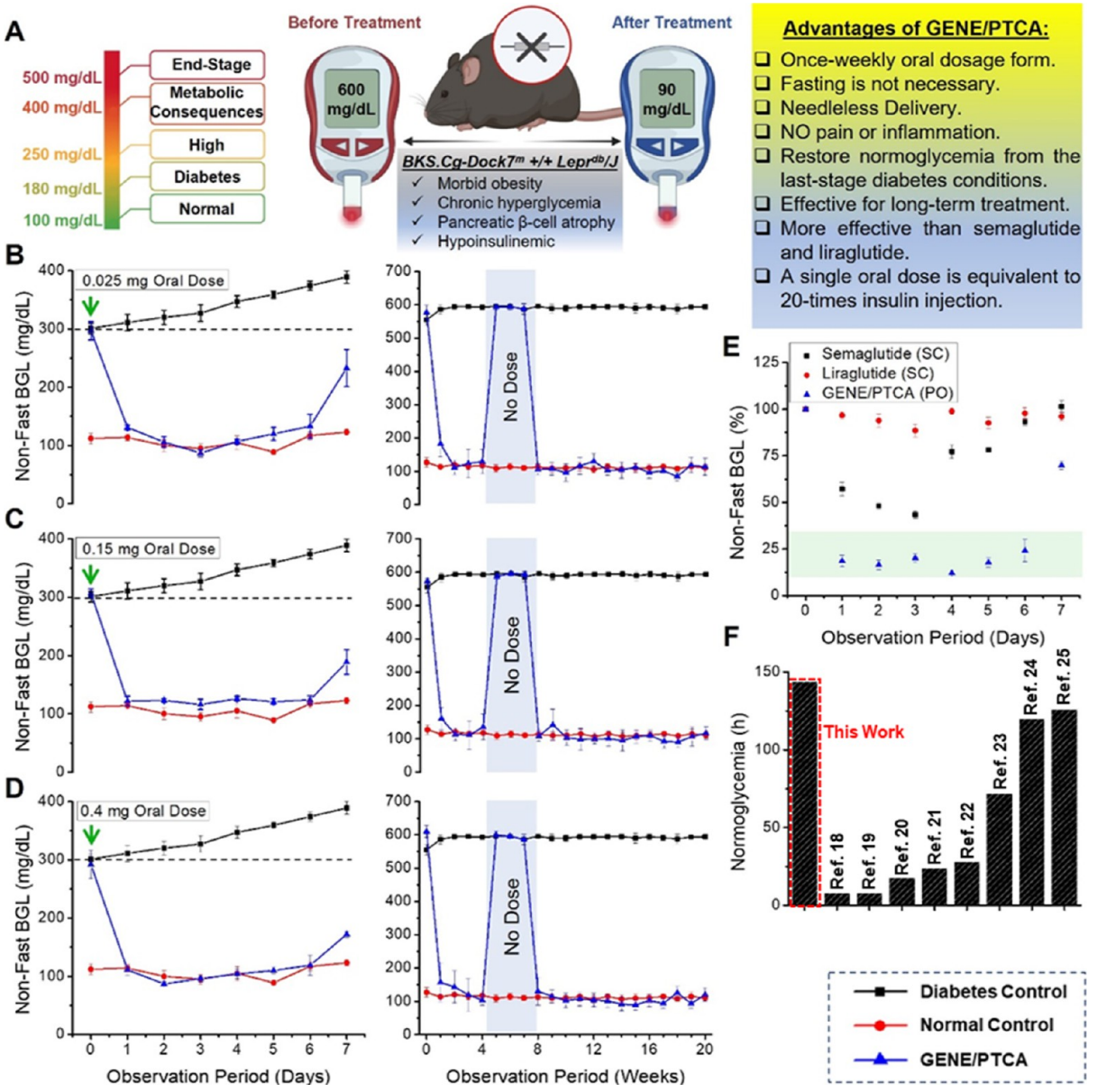

Figure 3. (A) Range of the blood glucose level (BGL) indicating the severity of the disease (left). In this study, we have used a genetically engineered diabetes mouse model (BKS.Cg-Dock7^{m} +/+ Lepr^{DB}/J) as Lepr^{DB}/Lepr^{DB} to demonstrate the therapeutic efficacy of GENE/PTCA. Schematic showing the blood glucose meter reading before and after treatment with GENE/PTCA (middle). The summary of Figure 3 presents the advantages of oral GENE/PTCA therapy (right). (B–D) Nonfast BGL of mice treated with a single oral dose of GENE/PTCA containing 0.025, 0.15, and 0.4 mg of GLP-1 gene, respectively (left panel). The dashed lines represent the baseline of BGL. The right panel represents the nonfast BGL data of mice repeatedly administered with a once-weekly oral dose of GENE/PTCA containing 0.025, 0.15, or 0.4 mg of GLP-1 gene per mouse for 90 days. The shaded regions indicate when mice were stopped given doses to prove that the therapeutic outcomes came from GENE/PTCA. Phosphate-buffered saline (PBS)-treated Lepr^{DB}/Lepr^{DB} and C57BL/6J mice were used as the diabetes and normal controls, respectively. (E) Blood glucose response of mice treated with a single dose of semaglutide (SC), liraglutide (SC), or GENE/PTCA (PO). The green-shaded region indicates the normoglycemic range. (F) Comparison study of therapeutic efficacy between GENE/PTCA and recently reported other GLP-1RA. Mean ± SD, N = 7.

PTCA and mucin in the intestinal epithelium prevents them from attaching through physical repulsion, which will not impede the particles from reaching their target site.[27]

2.2. Structural Stability, Cellular Uptake, and Intracellular Transport

Maintaining the structural integrity of PTCA to protect the therapeutic gene in harsh gastric conditions during GIT transition after oral administration (Figure 2A) is crucial. FTIR spectroscopy analysis confirms the robust structural stability of PTCA during gastric emptying and the intestinal digestive processes (Figure 2B). No differences in the PTCA structure have been found at different pHs, satisfying the critical requirements of oral delivery vehicles, such as resistance to GIT pH.[28] In addition, Figure S3 supports the protective effects of PTCA on genes in different biological barriers of the oral route.

GENE/PTCA was intended to target the ASBT of small intestinal cells. To ensure the ASBT-mediated oral absorption of GENE/PTCA, we first examined the cellular uptake of fluorescent-labeled GENE/PTCA (W/BS) in ASBT-trans-

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fected *Madin−Darby canine kidney* (MDCK) cells (MDCK-ASBT^{+ve}) and then compared it with the nontargeted nanoparticles (NPs) (W/O BS). The confocal laser scanning microscopy images showed stronger intracellular green fluorescence in MDCK-ASBT^{+ve} cells after treating cells with GENE/PTCA containing a BS ligand on their surface than the gene formulations lacking a BS ligand (GENE/PS or W/O BS) (Figure 2C,E). When BS is present on the surface of NPs, it leads to their strong localization in the nucleus. This localization might be facilitated by the role of PS in transporting nucleic acids from the cytoplasm to the nucleus.[15] Both NPs contain PS, but without BS, the NPs cannot enter the cytoplasm. As a result, the localization of NPs without BS is not observed, despite the presence of PS. To further verify the affinity of the BS to ASBT, which is involved in intracellular uptake and absorption of GENE/PTCA, ASBT-knockout MDCK cells (MDCK-ASBT^{-ve}) were treated with Gene/PS or GENE/PTCA. Both NPs showed an almost similar intracellular fluorescence intensity in the ASBT^{-ve} cell line (Figure 2D). Interestingly, no significant difference in the cellular uptake profiles has been found in the MDCK-ASBT^{-ve} cells between the targeted and nontargeted oral nanoformulations, demonstrating the affinity of the BS ligand in cellular interaction with the ASBT (Figure 2F).

2.3. Oral Absorption and *In Vivo* Gene Expression Kinetics

Oral absorption of therapeutic molecules is associated with the membrane transport of active pharmaceutical ingredients with excipients from the apical layer to the basolateral side before they enter the bloodstream. Figure 2G shows that the functionalization of the NP with the BS ligand could guide ASBT-directed cellular uptake of therapeutic genes and their successful intracellular transport across cells. The results further demonstrate that the BS transporter significantly enhances the NP's transport when NPs are tagged with a BS. However, the intracellular transportation of naked genes is very poor.

Furthermore, the visualized microscopic images of the GENE/PTCA inside the ileal epithelium suggest that the therapeutic gene can be endocytosed and transported through intestinal cells after oral delivery (Figure 2H). After 1 h of oral delivery, most of the intestinal cells could intake NPs tagged with a BS ligand. However, 3 h after oral delivery, we did not observe many NPs outside of cells. Interestingly, the number of NPs inside and outside the cells decreased over time. This study suggests that GENE/PTCA remained intact during the gastrointestinal transition *in vivo* after oral administration and reached the intestinal epithelium over time. Within 1 h of oral delivery, most of the intestinal cells start to uptake and transport GENE/PTCA *via* ASBT, and within 3 h, NPs can be taken up by intestinal epithelium cells. We next investigated the ASBT-guided *in vivo* gene expression kinetics of test NPs in leptin receptor-deficient diabetic mice administered orally with or without BS-functionalization of the test NPs encoding the GLP-1 gene. The expression of the GLP-1 gene in the small intestine indicates that PTCA could be a promising nonviral oral gene delivery vehicle (Figure 2I). This result also suggests that the BS ligand could protect the therapeutic gene from degradation, enhance oral absorption, and promote gene expression after oral gene delivery.[29]

2.4. Therapeutic Efficacy

The insulin demands in the blood change rapidly depending on the patient's diet, lifestyle, and metabolism.[30] Also, the blood glucose level (BGL) as well as the severity of the disease vary from patient to patient (Figure 3A, left). Therefore, the rapid insulin administration may not be the solution to control hyperglycemia. To understand the therapeutic effect of GENE/PTCA in diabetes management, we have used a well-accepted genetically modified hyperglycemic animal model to recapitulate human hyperglycemia characterized by morbid obesity, pancreatic-cell atrophy, hypoinsulinemic effects, and uncontrolled BGLs (Figure 3A, middle).[31] A single oral dose of GENE/PTCA containing 0.025, 0.035, 0.05, 0.15, 0.25, and 0.4 mg of the therapeutic gene per mouse showed a significant reduction in nonfast BGLs compared to the PBS-treated group (left panels of Figures 3B−D and S4A−C). The normoglycemia was maintained up to 1 week after a single oral administration, regardless of dose, unlike release kinetics-based action. However, in the dose−response study, mice did not respond to 0.0025 or 0.0035 mg of the oral dose. A single oral dose of 0.0025 or 0.0035 mg GENE/PTCA reduced nonfast BGLs by 15.04 ± 1.23 or 17.41 ± 2.16%, respectively. These results suggest that oral protein−peptide replacement therapy requires a specific quantity of therapeutic genes to transfect the target cells.

Interestingly, all treatment groups showed nonsignificant BGLs with normal mice, indicating transgenesis-based therapeutic effects of GENE/PTCA. However, the BGLs in the Lepr^{DB}/Lepr^{DB} mice exceeded normoglycemia after 1 week, as did the untreated control (Figure 3B−D, left panel). The right panel of Figure 3B−D shows that GENE/PTCA can restore normoglycemia from end-stage diabetes conditions. The GENE/PTCA-treated genetically engineered diabetes mice showed excellent glycemic control compared with the untreated PBS control in long-term treatment and maintained normoglycemic levels for an extended period (Figure S6A−C). However, the interruption in the weekly dose results in restoring hyperglycemia. These observations concluded that the achievement of normal BGLs in the diabetic mice model indeed comes from the treatment with GENE/PTCA, and the normoglycemic effect can be maintained from the continuous treatment of GENE/PTCA to meet a critical requirement of the long-term treatment for diabetic condition. No therapeutic difference was found among the various doses of GENE/PTCA in terms of bioactivity. Since this is an oral dosage formulation, there are no issues related to pain and inflammation.

An oral administration of GENE/PTCA with the highest dose (0.4 mg/mouse) to the normal mice did not reduce the BGLs (Figure S5), suggesting that the antihyperglycemic effects of GENE/PTCA depend on the glucose concentration in the blood. GENE/PTCA can work only when blood glucose exceeds the normal range (Figures 3B−D and S5). In contrast, daily multiple insulin injections sometimes lead to hypoglycemia and hyperinsulinemia, which are harmful and even fatal for people with diabetes.[32] Over 20 weeks, no hypoglycemia was observed during the entire study period in any mouse treated with various doses of GENE/PTCA due to the glucose-dependent therapeutic effect of GENE/PTCA (Figures 3B−D and S5). Next, to estimate the acceptability of GENE/PTCA in clinical settings, we compared its antihyperglycemic effect with semaglutide, liraglutide, and insulin (Figures 3E and S6). In these comparative studies, semaglutide, liraglutide, or insulin was injected into diabetic mice through the recommended subcutaneous (SC) route. A single insulin injection exhibited only a suboptimal reduction of BGLs for a

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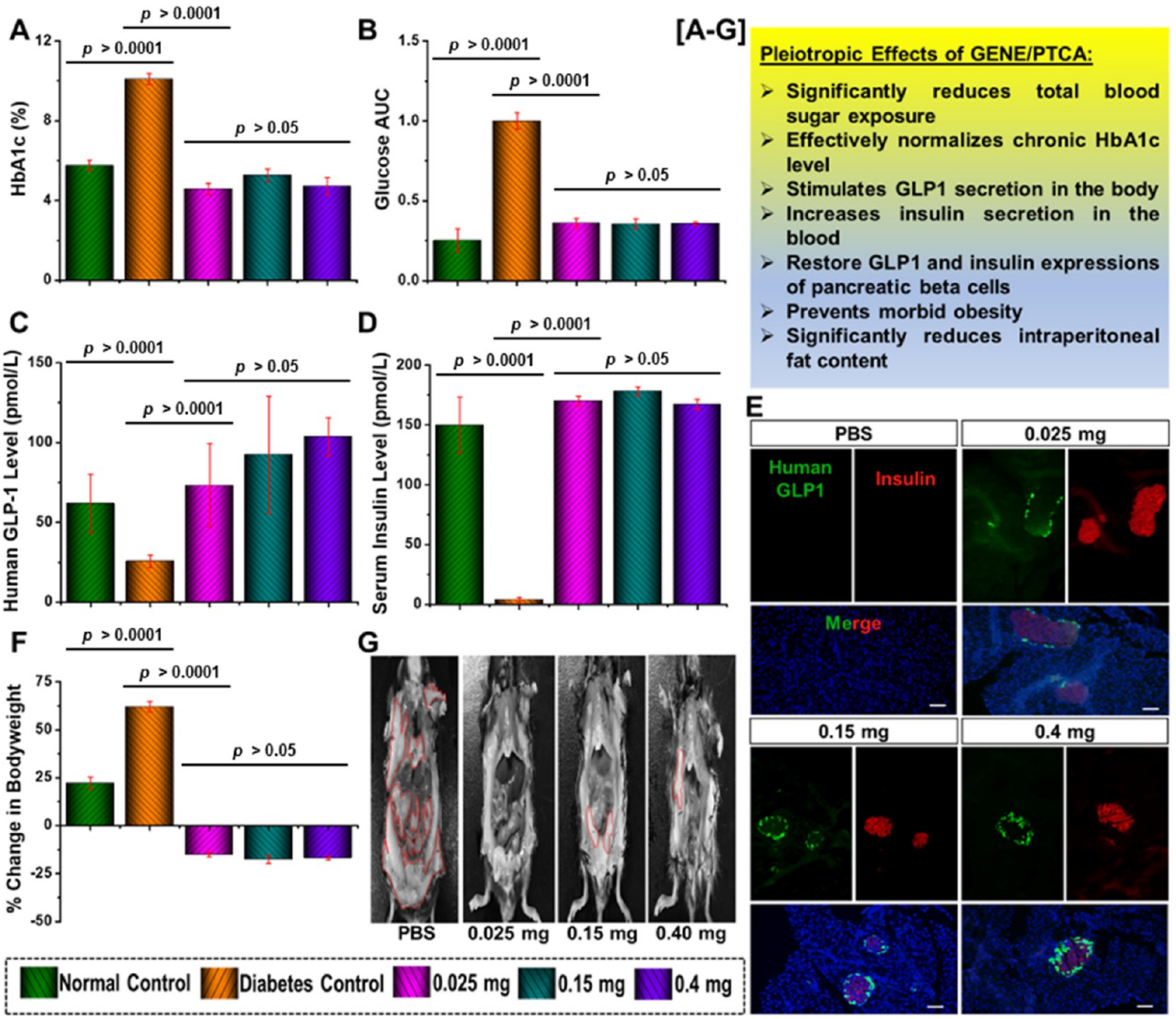

Figure 4. (A) The HbA1c test suggests that the average BGL of mice during the entire study period was maintained at a normal level. (B) After once-weekly oral delivery of GENE/PTCA, the total blood glucose exposure was calculated in all treatment groups. The glucose area under the curve (AUC) of GENE/PTCA-treated mice was normalized to that of the PBS-treated diabetes mice. (C,D) GLP-1 and insulin levels (C,D, respectively) significantly increased after treatment, suggesting a transgenesis-based therapeutic efficacy of GENE/PTCA. (E) The human GLP-1 and insulin expression in the diabetes pancreas dramatically improved after oral gene therapy, indicating that GENE/PTCA can efficiently enhance protein replacement therapy. (F) The percentage changes in the body weight of diabetic mice treated with different doses of the test NP dose independently controlled the morbid obesity compared to that of the PBS-treated group. (G) The macro-photograph of intraperitoneal fat contents of mice treated once-weekly for 90 days suggests that GENE/PTCA reduces and controls morbid obesity. (A–G) Summary of Figure 4. Mean ± SD, $N = 7$, and scale = 100 μm.

few hours due to no meeting of requirement about multiple daily injections to achieve therapeutic efficacy (Figure S6). However, an SC injection of semaglutide was not adequate for a few days in the $Lepr^{DB}/Lepr^{DB}$ mice.

Next, we compared the therapeutic efficacy of the lowest dose of GENE/PTCA with the most cited or promising GLP-1 receptor agonist formulations reported in recent years.[18−25] As shown in Table S1 and Figure 3F, other oral GLP-1 formulations can only maintain normoglycemia for a few hours compared to GENE/PTCA.[18−20] Although GLP1-pCB shows a nearly similar therapeutic profile (126 h glycemic control) with the oral GENE/PTCA formulation that facilitates 144 h of glycemic control, GLP1-pCB requires a higher dose to be injected subcutaneously (Table S1).[25]

2.5. Dose-Independent Pleiotropic Effects

The HbA1C level reflects an integrated view of average blood glucose over 3−4 months.[33] After 90 days of repeated treatment, all groups treated with GENE/PTCA experienced a significant reduction of HbA1C (%) from high to normal HbA1C levels, comparable to the standard control (Figures 4A and S7A). Notably, consistent normoglycemic effects of the GENE/PTCA in measuring total glucose exposures in the diabetic treatment groups irrespective of doses were also observed, reflecting an integrated view of average blood glucose over long-term treatment periods in all treatment groups (Figures 4B and S7B).

Besides the evident improvement of normoglycemia in diabetic mice from the long-term treatment of GENE/PTCA, high plasma GLP-1 and insulin levels expressed by GENE/

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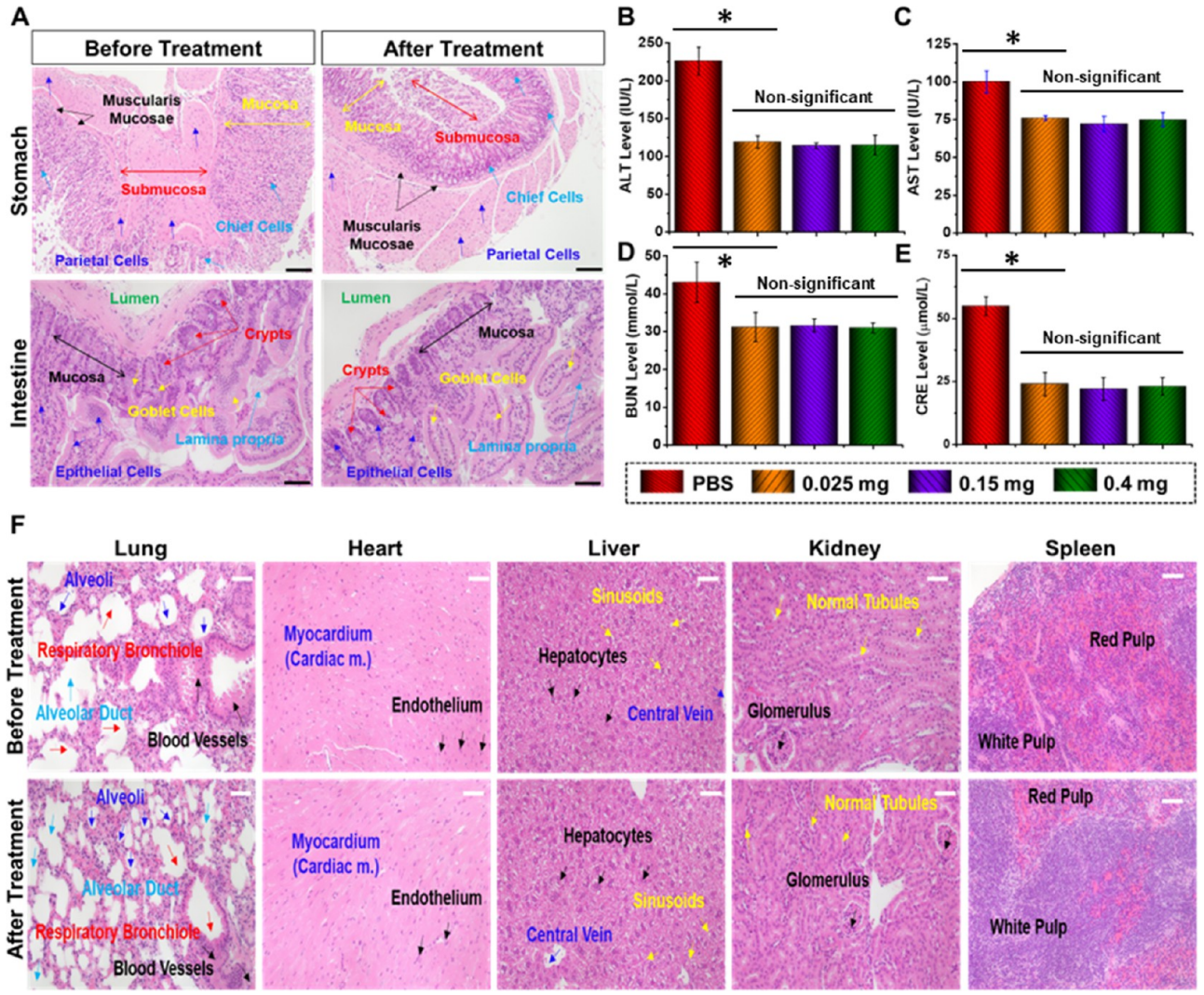

Figure 5. Before and after treatment, a histopathology screening of stomach and intestinal tissues indicates no dose-related toxicity. Mice were treated with the highest dose of GENE/PTCA for 20 weeks. (B,C) The enzymatic activity of alanine transaminase (ALT) and aspartate aminotransferase (AST) activity significantly decreased in all treatment groups after treatment, indicating pleiotropic therapeutic effects of GENE/PTCA, respectively. (D,E) The blood urea nitrogen (BUN) and creatinine (CRE) blood tests suggest that diabetes subjects' kidney function significantly improved after treatment, respectively. (F) No toxicity has been found in any major organs after treatment with GENE/PTCA. Mean ± SD, $N = 7$, $^*p < 0.001$, nonsignificant >0.05, and scale = 100 μm.

PTCA were also observed (Figure 4C,D). All treatment groups showed at least a 40-fold enhancement in plasma insulin compared to the PBS control, indicating that sustained blood glucose control comes from GLP-1-induced normal insulin levels (Figure S8A,B). There was no statistical significance among various therapeutic groups, indicating that a minimum GLP-1 gene concentration is required for successful gene therapy. Once it achieves this, oral GLP-1 gene therapies display similar therapeutic effects irrespective of doses using the PTCA system (Figure S8A,B). These results are consistent with those in Figures 3B–D and 4A,B, which show that the therapeutic outcomes come from the secretion of GLP-1 after oral gene therapy. An increased expression of GLP-1 following the high insulin level was observed in the pancreatic islets of $Lepr^{DB}/Lepr^{DB}$ mice treated with different doses of GENE/PTCA, suggesting the therapeutic effects of the persistent expression of GLP-1 and insulin (Figure 4E). To further monitor the additional weight control effect of GLP-1 besides blood glucose control, we checked the changes in body weight and intraperitoneal fat content after a three month treatment of GENE/PTCA in the diabetic $Lepr^{DB}/Lepr^{DB}$ mice. Figures 4F,G and S9 show that GENE/PTCA could significantly control morbid obesity by reducing the endogenous fats in $Lepr^{DB}/Lepr^{DB}$ mice. However, the normal mice increased their weight by 21% due to unrestricted access to excess food, which is normal. No adverse effects of oral GENE/PTCA associated with GIT were observed throughout treatment.

2.6. Toxicological Profile

The *in vivo* toxicity study of GENE/PTCA has been evaluated after long-term treatment, with the highest dose in $Lepr^{OB}/Lepr^{OB}$ mice after repeated oral administration for 20 weeks. We previously assessed the safety of this system in $Lepr^{DB}/Lepr^{DB}$ mice in a prior study.[13] We did not observe any organ damage or enlargement after long-term treatment, indicating that oral gene therapy with GENE/PTCA did not induce acute toxicity in obese mice (Figure S10). After oral delivery, the first destination of any therapeutics is the stomach, and GENE/PTCA was designed in such a way that it could be absorbed by

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the ASBT of the intestine. Hence, analysis of the toxicity of these two organs is crucial. We performed histopathology analysis of these tissue sections to evaluate whether repeated gene therapy for an extended period causes toxicity or damage to the stomach and intestine. Figure 5A (upper panel) shows no significant changes in these tissue sections. In brief, the stomach mucosa and submucosa regions show no damage after treatment. In addition, the muscularis mucosae are clearly visible as found before treatment. The parietal and chief cells are appropriately organized in the entire section. In intestinal tissue sections, no changes in the histological pattern of the mucosa, crypts, or lamina propria have been detected (Figure 5A, lower panel). Also, the intestinal epithelium was uniformly distributed in the entire cross-sectional area with no changes in nuclear shape. The presence of goblet cells also suggests that GENE/PTCA does not disturb the intestinal lining in the brush borders.

It has been reported that the dysfunction of the liver and kidneys is closely related to the disease progression. To evaluate how oral GENE/PTCA therapy interacts with the biochemical biomarkers of blood, we first checked the enzyme activities of ALT and AST (Figures 5B,C and S11A,B). A high level of ALT and AST indicates a higher progression of diabetes and a loss of liver function. The PBS-treated group increased ALT and AST levels due to the severity of diabetes, but the treatment group exhibited a significantly lower level of these two enzymes. This observation let us conclude that long-term treatment of diabetes and obesity with oral GENE/PTCA therapy could improve liver function. The BUN and CRE levels in blood further demonstrate the function of the GLP-1 gene in restoring the function of the kidney (Figures 5D,E and S11C,D). Apart from the changes in these blood biomarkers (Figures 5B,E and S11A,D), we did not observe any pathological changes in the lung or heart (Figure 5F, lung and heart). No abnormalities in the histological observations of sinusoids, central veins, and hepatocytes were found in the liver (Figure 5F, liver). In addition, the glomerulus looks normal in the *hematoxylin and eosin (H&E)* staining data after treatment (Figure 5F, kidney). No changes in the histology of spleen tissue sections between treated and untreated groups indicate that GENE/PTCA inactivates the immunoneutralization (Figure 5F, spleen). Therefore, these data suggest that oral gene therapy with GENE/PTCA is safe in *in vivo* and *ex vivo* studies.

3. DISCUSSION

Oral gene delivery for protein replacement therapy is currently exciting and one of the most difficult jobs. Recently, many approaches have been proposed for the oral delivery of proteins, peptides, or genes that are in the early stages of development.[6,8,34−36] Undoubtedly, those delivery technologies are promising, but the development of a standard oral dosage on a therapeutically relevant mg scale remains to be demonstrated in most cases.[6] Here, we have demonstrated an oral dosage form of the therapeutic gene in a microgram scale (25 μg or 0.025 mg) that would be less than ∼2.5 mg for a 60 kg diabetes woman.[37]

GENE/PTCA is effective for up to 1 week in a genetically engineered diabetes animal model. Our study suggests that an oral dose of GENE/PTCA is equivalent to at least 20 SC injections of insulin and more potent than other GLP-1 agonists, such as semaglutide and liraglutide in preclinical studies (Figure 3E). The therapeutic goal of PTCA-mediated oral gene therapy highly depends on the delivery strategy and transfection efficiency of the gene and not on the amount of therapeutics (Figures 3B−D and S4). The nonsignificance outcomes within the different treatment groups will allow the production of GENE/PTCA using minimal amounts of genes; therefore, oral dosage forms of GENE/PTCA will be an affordable and long-term treatment option.

Therefore, we conclude that GENE/PTCA facilitates a large-scale production ability in the pharmaceutical industry due to its easy synthesis protocol (Figure 1A,B), which is highly stable in GIT and can be absorbed *via* ASBT-mediated transcytosis by intestinal enterocytes (Figures 2 and S3). Upon absorption, GENE/PTCA transfects the intestinal cells (Figure 2I) and secretes biologically active GLP-1 in response to BGLs, and thereby, the blood concentration of GLP-1 goes up (Figure 4C).[6,19,38,39] Finally, the insulin levels increased with an elevated level of GLP-1 in the blood (Figure 4D). A higher expression of GLP-1 and insulin in the diabetic pancreas and previous report support this statement (Figure 4E).[19] In this case, diabetic mice produce and secrete GLP-1 after being transfected by GENE/PTCA. Hence, the therapeutic effects depend on whether they are getting transfected, not on the therapeutic dose. Since the body secretes GLP-1 and insulin, not GENE/PTCA, all doses exhibit similar effects (Figures 3B−D, 4, S4, S7, and S8). The results also suggest that GENE/PTCA could control obesity by reducing the body's endogenous fat content, which is one of the most common side effects of diabetes (Figures 4F,G, and S9). This means GENE/PTCA can also be taken as an antiobesity drug because it can significantly reduce abnormal weight gain but not cause hypoglycemia in nondiabetes subjects (Figure S7).[8] These results suggest that GENE/PTCA could be highly suitable for patients with diabetes and obesity as a cost-effective treatment.

4. CONCLUSIONS

This work studied the therapeutic efficacy of different doses of GENE/PTCA oral gene therapy over an extended period. GENE/PTCA can be easily synthesized in a larger scale in the pharmaceutical industry due to its easy synthesis protocol requiring one-step conjugation chemistry. This oral gene therapy approach is more effective than the subcutaneous dosage forms of semaglutide, liraglutide, or insulin. Oral GENE/PTCA significantly enhances the secretion of GLP-1 and insulin from the body in response to high blood glucose level. It is highly safe for long-term treatment and does not cause hypoglycemia due to glucose-dependent therapeutic actions. Our study suggests that the oral GENE/PTCA formulation could be a promising alternative to injection-based therapeutics for diabetics.

5. EXPERIMENTAL SECTION

5.1. Materials

Sigma-Aldrich (USA) provided the following substances: protamine sulfate (a salt derived from salmon and classified as grade X, amorphous powder), fluorescein isothiocyanate, 4′,6-diamidine-2′-phenylindole dihydrochloride, 4-nitrophenyl chloroformate, Triton X-100, thiazolyl blue tetrazolium bromide, calcium phosphate, and HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) (sodium salt, 99%). ACROS (Belgium) provided taurocholic acid (TCA, sodium salt hydrate 98%, MW: 515.7058 g/mol). The Korean Cell Line Bank supplied MDCK and MDCK-ASBT cells. Gibco by Life Technologies (USA) supplied Dulbecco's modified Eagle's medium, minimum essential medium, RPMI medium 1640, fetal bovine serum,

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penicillin, and 0.05% trypsin-ethylenediaminetetraacetic acid. Spectrum Laboratories (USA) provided Dialysis Tubing molecular weight cutoff (MWCO) 2 kDa. BioNieer (Daejeon, Korea) supplied PBS. The plasmid purification kit NucleoBond Xtra Maxi Plus was obtained from MACHEREY-NAGEL (Germany).

5.2. Extraction and Purification of GLP-1 from the Host Strains

Prof. Minhyung Lee from Hanyang University, Seoul 04763, South Korea, provided 800 μL of bacterial growth and 200 μL of 50% glycerol containing the GLP-1 plasmid (Figure S1) transfected *E. coli*. The DH5alpha cells were cultured and purified in our lab to produce GLP-1 plasmid on a large scale. The GLP-1 plasmid was propagated by adding an appropriate selective antibiotic to autoclaved bacterial growth medium containing agar, Luria Broth (LB), and distilled water. The GLP-1 plasmid-transfected *E. coli* bacteria were collected from LB agar plates and inoculated into sterile LB medium. OD_{600} was measured for maximum standardized bacterial growth, and a large culture was prepared, which was centrifuged and purified by using the NucleoBond Xtra Maxi Plus EF kit assay. The human GLP-1 gene sequence was confirmed to match the NCBI database,[26] and the purity concentration of the GLP-1 plasmid was determined using a spectrophotometer. The hydrodynamic radius and surface potential of the GLP-1 plasmid were measured using a Zetasizer (Nano-s90, Malvern, UK).

5.3. Preparation and Characterization of GENE/PTCA

PTCA was synthesized by chemically linking 4-NPC to TCA. After harvesting TCA-NPC, a liquid−liquid phase separation method was used to extract and separate TCA-NPC. The production yield was nearly 80%. The activated TCA was added to the PS solution, stirred for 24 h, and dialyzed using an MWCO membrane for 48 h. The lyophilized sample was stored at 4 °C. FT-IR and Zetasizer spectroscopy confirmed successful synthesis. The yield of PTCA was nearly 90%. The regioisomeric composition resulting from the use of *p*-nitrophenyl chloroformate was not determined in this study, but ongoing research is dedicated to addressing this aspect for future considerations. To prepare GENE/PTCA, the GLP-1 gene was first dissolved with 2 M $CaCl_2$ and then mixed with PTCA in 2x HEPES buffered saline and incubated at room temperature for 10 min. Encapsulation efficiency was measured by gel electrophoresis and NanoDrop. The stability of GENE/PTCA was tested in simulated stomach, gastrointestinal, and serum conditions using FT-IR analysis. Transition times in the stomach, duodenum, and ileum were determined based on previous reports.

5.4. ASBT-Guided Absorption Study

We utilized fluorescein isothiocyanate (FITC)-conjugated GENE/PTCA to investigate the role of BS in cellular uptake and translocation in ASBT-expressing or ASBT-knockout MDCK cells. MDCK-ASBT cells are commonly used to study drug absorption or distribution mediated by ASBT. We followed a previously established protocol and confirmed the absence of cell contamination using a light microscope. The treated cells were incubated at 37 °C with 5% CO_2 for 4 h and stained with *4′,6-diamidino-2-phenylindole* (DAPI)-blue dye to label cell nuclei. The fluorescence of FITC was measured to quantify the cellular uptake of FITC-conjugated NPs.

5.5. Animal Study

Animal experiments were conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) regulations of Hanyang University (Seoul 04763, Republic of Korea) for the ethical treatment of laboratory animals. To evaluate the therapeutic efficacy of the treatment in advanced and spontaneous diabetic strains, male 6 week old $Lepr^{DB}/Lepr^{DB}$ and $Lepr^{OB}/Lepr^{OB}$ mice, deficient in leptin receptor and leptin protein, respectively, were procured from Gempharmatech in Nanjing, China. The standard nondiabetic model was established using normal C57BL/6J mice. The mice were housed in a controlled photocycle (12 h light and 12 h dark) room and provided ad libitum access to food and water. Experimental groups were created based on the study design.

5.6. *In Vivo* Gene Expression Kinetics of GENE/PTCA

We investigated the impact of oral GENE/PTCA treatment on GLP-1 gene expression in leptin receptor-deficient $Lepr^{DB}/Lepr^{DB}$ mice. Following treatment, we excised the small intestine from the mice and processed 5 μm-thick tissue sections. The sections were deparaffinized using xylene and rehydrated through a gradient of ethanol. After antigen retrieval, we blocked the tissues with 20% goat serum and incubated them with rabbit polyclonal anti-GLP-1 immunoglobulin overnight at ice-cold temperatures. The tissues were subsequently treated with secondary antirabbit antibodies in the dark. After washing with PBS, we counterstained the tissues with DAPI medium. Finally, we visualized the tissue expression kinetics of the NPs by using a high-resolution fluorescence microscope.

5.7. Long-Term Therapeutic Efficacy

We assessed the effectiveness of GENE/PTCA in long-term treatment using male $Lepr^{DB}/Lepr^{DB}$ mice aged 12 weeks. GENE/PTCA was administered orally once a week for 90 days at doses based on the mouse body weight. BGLs and body weight changes were monitored daily, and blood glucose AUC was measured and compared with the diabetic control group. HbA1C levels were measured at the end of the study by using enzyme-linked immunoassay (ELISA) kits. PBS-treated $Lepr^{DB}/Lepr^{DB}$ mice and untreated C57BL/6 mice served as control groups. The oral dose of GENE/PTCA ranged from according to the body weight, as mentioned before. The mice were closely monitored throughout the study. The study concluded that GENE/PTCA significantly reduced BGLs and improved body weight changes compared with the diabetic control group. HbA1C levels were also significantly reduced in GENE/PTCA-treated mice.

5.8. Measurement of Serum GLP-1 and Insulin

Following 90 days of repeated weekly oral administration of GENE/PTCA to $Lepr^{DB}/Lepr^{DB}$ mice, blood samples were collected from the tail vein and stored in VACUETTE blood collection tubes (Greiner Bio-One, Germany). The level of GLP-1 and insulin in the serum was measured using ELISA kits and following the manufacturer's protocols.

5.9. Immunohistochemistry (IHC) Study

To perform IHC, pancreases were harvested from $Lepr^{DB}/Lepr^{DB}$ mice treated with a single oral dose of GENE/PTCA. Tissue sections were deparaffinized, treated with primary antibodies against insulin and GLP-1, and then incubated with secondary antibodies. After counter-staining with DAPI, the expressions of insulin and GLP-1 in the pancreas were assessed using a high-resolution fluorescence microscope.

5.10. Tissue Histology

Toxicity studies were conducted on $Lepr^{OB}/Lepr^{OB}$ mice, an obese model, using a weekly dose of GENE/PTCA containing different amounts of the GLP-1 gene over 20 weeks. Major organs, including the heart, lung, liver, kidney, spleen, stomach, and small intestine, were subjected to tissue histopathological analysis using the previously reported methodology. The tissues were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned into 5 μm-thick parts, and stained with H&E. A pathological assessment of each tissue section was performed using a microscope at the end of the study.

5.11. Statistical Analysis

The data in this study are presented as mean ± standard deviation (SD). Statistical analysis was conducted using analysis of variance (ANOVA) in GraphRobot for comparison between two or more groups. A *p*-value of less than 0.05 was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnanoscienceau.3c00035.

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Additional details and resources can be accessed through the Web site or by contacting the CORRESPONDING author directly. FT-IR analysis of all compounds; TEM images; information on GLP-1 loading and encapsulation efficiency; particle stability; dose−response study; impact of insulin on blood glucose levels; post-treatment HbA1c levels; glucose AUC; GLP-1 and insulin levels; data on changes in body weight following treatment; assessments of *in vivo* organ toxicity; measurements of ALT, AST, BUN, and CRE levels; and therapeutic effects observed at the preclinical level for various GLP-1 formulations developed in contrast to GENE/PTCA (PDF)

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Notes

The authors declare no competing financial interest.

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