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Andrographolide promotes pancreatic duct cells differentiation into insulin-producing cells by targeting PDX-1

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ABSTRACT

Regeneration of β-cells by differentiation of pancreatic progenitor cells has the potential to fundamentally solve the problems of the loss of β-cell function and mass during disease progression in both type 1 or 2 diabetes. Therefore, discovery of novel differentiation inducers to promote islet regeneration is of great significance. Pancreatic and duodenal homeobox1 (PDX-1) is a key transcription factor that promotes the development and maturation of pancreatic β-cells. To screen potential novel small molecules for enhancing differentiation of PNAC-1 cells, a human pancreatic ductal cell lines into insulin-producing cells (IPCs), we developed a high-throughput screening method through fusing the PDX-1 promoter region with a luciferase reporter gene. We screened and identified that andrographolide named C1037 stimulates PDX-1 expression in both mRNA and protein level and significantly promotes PANC-1 cells differentiation into IPCs as compared with that of control cells. The therapeutic effect of C037 in Streptozotocin induced diabetic mouse model through differentiation of pancreatic ductal cells into insulin positive islets was also observed. Our study provides a novel method to screen compounds regulating the differentiation of pancreatic progenitor cells having the potential of enhancing islet regeneration for diabetes therapy.

1. Introduction

Diabetes mellitus is a major chronic disease and affecting millions of people worldwide. According to the report of International Diabetes Federation, the number of people with diabetes in the world reached to 463 million in 2019, and the prevalence of diabetes in adults aged between 20 and 79 in China has reach to 116.4 million people in 2019.

The major reason that causes accumulation of diabetic population is that insulin producing β-cells, which form bulk of islets (65–80%), are targeted for destruction at early stage in type 1 diabetes and in the progression of type 2 diabetes [1]. Therefore, loss of β-cell mass and function is critical step in the development and also for targeted therapy of both type 1 and type 2 diabetes. Currently, efforts are being made to generate insulin-producing cells from stem cells or tissue specific progenitor cells induced by various growth factors or genetic reprograming [1–3], but no reports showed that pancreatic regeneration can be achieved through induction of endogenous pancreatic stem cells/progenitors by herbal extract or compounds. Therefore, identification of novel and safe differentiation inducer is a prime requisite for islet generation and increasing β-cell mass, which could be next generation therapeutics for diabetes.

Pancreatic duodenal homeobox1 (PDX-1), encodes pancreatic duodenal homeoprotein 1, is a member of the ParaHox subfamily of homeotic genes [4]. PDX-1 is a critical insulin transcription factor expressed by pancreatic β-cells, and is crucial in the early stage of pancreas development [5]. It regulates the expression of a variety of genes involved in β-cell function, such as insulin, glucose transporter 2, glucokinase and islet amyloid polypeptide [6]. Other studies have successfully differentiated UCB-MSCs into IPCs by Adenovirus-mediated expression of PDX-1 [3] or PDX-1 mRNA transfection [7]. These studies indicate that PDX-1 is an important factor in regulating pancreatic-endocrine differentiation, especially in the formation of functional β-cells.

From a therapeutic perspective, the introduction by viral vector of foreign genes has raised concerns about genome integration and tumorigenicity [8]. An attractive alternative is to use small molecules that promote high expression of transcription factors to induce endogenous pancreatic progenitor differentiation into mature and functional β-cells. To screen compounds capable of up-regulating the expression of PDX-1, we developed a high-throughput screening assay through fusing the

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PDX-1 promoter region with a luciferase reporter vector and screened compounds using human pancreatic progenitor cell line, PANC-1 ductal carcinoma cells. More than 400 extracted fractions of traditional herb medicines were screened for the PDX-1 promoter activities, and as a consequence, one of them named C754 extracted from *Andrographis paniculata* could up-regulate PDX-1 expression in both mRNA and protein levels, suggesting that C754 might induce PDX-1 expression through either direct or indirect increasing of its promoter activity. Further studies suggested that the compound andrographolide (C1037) is the main active component of C754, and could facilitate PANC-1 cells differentiation into functional insulin-producing cells compared with that of control cells in *in vitro* model and therapeutic effect in STZ induced type 1diabetic mice model through regeneration of insulin positive islets was also observed. Our current study, provide novel idea for developing drug candidate for pancreatic islet regeneration through inducing differentiation of pancreatic progenitor cells into insulin producing cells.

2. Material and methods

2.1. Cell culture and differentiation

Human PANC-1 cells (Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultured in high-glucose DMEM (Hyclone, ThermoFisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL of penicillin, and 100 μg/mL of streptomycin. The medium was changed every alternate day. To induce PANC-1 cells differentiation, basal culture medium was removed. After washing with phosphate buffer (PBS) 3 times, the cells were digested using 0.05% trypsin for 1 or 2 min to loosen the cells but not to detach the cells from their extracellular matrix (ECM) and then cultured in differentiation medium. Differentiation medium was DME/F12 medium (ThermoFisher Scientific, MA, USA) containing 1% BSA and insulin transferrin selenium (Sigma-Aldrich, MO, USA) [8]. Then PANC-1 cells were cultured at 37 °C in a humidified chamber with 5% CO_2. The following criteria of the differentiation were used: 1) islet-like clusters appear post-induction; 2) the islet-like clusters can be stained by DTZ, 3) expression islet specific genes like, insulin, PDX-1, and 4) secretion insulin stimulated by higher glucose.

2.2. Animals and induction of hyperglycemia

All animal studies were approved by the Ethics committee of Xiamen University. Eight weeks-old male Kunming mice, weighing 18–22 g (Beijing Weitong Lihua Experimental Animal Technology, Beijing, China) were housed in Xiamen University Laboratory Animal Center. Mice were raised on a 12 h light/dark cycle at room temperature with enough food and water. Mice were intraperitoneally (i.p.) injected with STZ (150 mg/kg) in 0.1 M citrate buffer (pH 4.2–4.5) to induce hyperglycemia. 3 days after STZ injection, blood glucose level was measured with glucose meter (Roche, IN, USA) and mice with a random blood glucose level up to 16.7 mM were regarded as type 1 diabetic mice (T1D).

2.3. Renal capsular transplantation

Mice were anesthetized with 10% hydration of chloral solution (400 mg/kg). The 200 islets isolated from normal mice or 500 differentiated islet-like cell clusters were handpicked under a dissection microscope to ensure islets of a similar size (~200 μm in diameter) were selected and transplanted. Through the microcatheter, the cells were injected into the renal capsular. The tip of the microcatheter was precisely moved during the implantation procedure and the injected cells was folded and placed in the renal capsular. Blood glucose levels of mice were monitored daily and recorded.

2.4. Treatment of C1037 on type 1 diabetes

The mice either injected with 0.1 M citrate buffer as a blank control or injected STZ (Sigma-Aldrich, MO, USA) (150 mg/kg) in 0.1 M citrate buffer (pH 4.2–4.5) to selectively destroy pancreatic β-cells to induce a condition resembling type 1 diabetes (T1D). The onset of T1D/hyperglycemia was defined as fasting blood glucose levels ≥11.1 mmol/L after 8 h of starvation and random blood glucose level ≥16.7 mmol/L. The animals were randomly divided into 4 groups: 1). Blank control, which is citrate buffer injected mice gavaged with saline solution; 2). Negative control, T1D mice gavaged with saline solution; 3). Positive control group, T1D mice injected with 180 μg/kg of liraglutide (Novo Nordisk, Denmark); and 4). Treatment group, T1D mice gavaged with 50 mg/kg of C1037. All treatments were lasting for 40 days and blood glucose was monitored every ten days.

2.5. Homeostasis model assessment (HOMA-B)

β-cell function was assessed by homeostasis model assessment (HOMA), which was calculated as HOMA-B = 20 × FINS/(FPG-3.5) as we did early[9].

2.6. RNA extraction and real-time RT-PCR (RT-qPCR)

Total RNA was extracted from cells using Trizol (ThermoFisher Scientific, MA, USA). mRNA was reversed transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, MA, USA). cDNA primer sequences for β-actin, insulin, PDX-1, glucagon, and PAX 6 were designed using IDT website (http://sg.idtdna.com/site, CA, USA). The primer sequences were listed in Table S1. Real-time qPCR reactions were performed with TaKaRa Taq™ Hot Start Version (TaKaRa, Hong Kong, R007A).

2.7. Plasmid construction and transfection

The plasmids pENTER/PDX-1 and empty vector were purchased from Vigene Biosciences corporation (Maryland, USA). Approximately 700 bp of human PDX-1 promoter sequence searched in Genecard (http://www.genecards.org), amplified by PCR and excised with *Nhe* I/*Hind* III and inserted into the *Nhe* I/*Hind* III site of pGL4.17 (Promega Biotech, WI, USA) to generate the pPDX-1/luciferase (pPDX-1-Luc) plasmid. The primer sequences for PDX-1 promoter were as follows: forward, 5′-CTAGCTAGCCGAATGCCAGAGTTTCGT-GTGTT-3′, and reverse, 5′-CCCAAGCTTCTCCTCGCCGTTCATGGCT-3′. Transient transfections were conducted with viafect Transfection Reagent (Promega, WI, USA) according to the manufacturer's instructions. Transfection efficacy was reached to approximately 90% as monitored by co-transfection of pEGFP-C1 vector with pENTER/PDX-1. And the transfected cells were expended for Western blotting verification and for differentiation study.

2.8. Lenti-PDX-1 construction and infection

2.8.1. Plasmid construction

We created the lentiCRISPRv2-PDX-1 plasmid by inserting gRNA sequence targeting PDX-1 (5′-CGGCGAGGAGCAGTACTACG-3′) into lentiCRISPRv2 vector through the restriction enzyme cutting site BsmBI according to the protocol from ZhangLab [10,11].

2.8.2. Lentivirus packaging and infection

293 T cells were seeded into 6-well plate the day before transfection. The following plasmids (1.5 μg lentiCRISPRv2-PDX-1, 0.75 μg pMDL, 0.45 μg VSV-G and 0.3 μg REV) were mixed in 100 μL OPTI-MEM, and 9 μL VigeneFection (Vigene Biosciences, Shandong, China) was added in another 100 μL OPTI-MEM, incubate for 5 min. Mix the VigeneFection mixture with plasmid mixture and incubate for 30 min.

The culture medium of 293 T cells was changed with serum free medium and then the transfection mixture was added into the wells, the cells were incubated into 37 ℃, 5% CO_2 incubator. Six hours later the culture media was change with completed media and incubate for another 48 h, the virus was harvested and stored at − 80 ℃ for further use.

PANC-1 cells were seeded into 12-well plate and cultured to achieve a confluence of 70%, the culture medium was discard before infection, 1 mL virus and 500 μL fresh medium were added, mix well and cultured in 37 ℃ incubator, Twenty four hours later the medium was changed and cells were cultured in media containing puromycin, 7–10 days later, the cells were trypsinized and re-seeded to 96-well plate, single colon of cells were expended for Western blotting verification and for differentiation study.

2.9. Western blot analysis

Cells were lysed on ice with cell-lysis buffer (20 mM Tris-HCl (pH8.0), 10 mmol/L EDTA, 137 mmol/L NaCl, 1% NP40, 10% glycerol, 100 mmol/L NaF, 1 mM PMSF, 1× Protease inhibitors cocktail, 1× Phosphatase inhibitors cocktail, and 1 mM DTT). The concentration of total protein was determined with BCA protein assay kit (Thermo Scientific, MA, USA). Fifty microgram of total protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane which was then blocked with 5% milk. Antibodies used for Western blotting were rabbit anti-PDX-1 antibody, mouse anti-tubulin antibody (Abcam, MA, USA), goat anti-rabbit antibody and goat anti-mouse antibody (R&D, MN, USA). Blots were developed using WesternBright ECL kit (Advansta, CA, USA) according to the manufacturer's instructions.

2.10. High-throughput screening

PANC-1 cells were plated into 6-well plate and cultured for overnight and both pPDX-1-luc and Renilla luciferase plasmid were transiently co-transfected with viafect Transfection Reagent (Promega, WI, USA) according to the manufacturer's instructions. 24 h post transfection, the cells were treated with compounds at a final concentration of 1.0 μM. Cells were incubated with compounds (Extracted compounds from traditional Chinese herbal medicine, Haifeng Chen's laboratory, Xiamen University, China) for 2 days before the luciferase reporter assay. Cells were then lysed and luciferase activity was assayed using Dual-Luciferase® Reporter Assay System kit (Promega, WI, USA) following the manufacturer's instructions.

2.11. Glucose stimulated insulin secretion assay

After differentiation, islet-like cell clusters were washed with PBS buffer 3 times and then incubated for 1 h in KRB-LG (low glucose, 2.8 mM). 200 μL supernatant was collected and stored at 4 °C. The cells were washed with PBS and treated for another 1 h in KRB-HG (High glucose, 16.7 mM). 200 μL supernatant was collected and stored at 4 °C for insulin concentration assay.

2.12. ELISA

The human insulin Immunoassay Kit (HKU Li Ka Shing Faculty of Medicine, Hong Kong) was used to quantitatively determine insulin concentrations in supernatant according to the manufacturer's instructions. Briefly, 5 μL standards and sample were added to its respective wells and incubated with 100 μL 1× Detection antibody solution at room temperature for 90 min, shaking the plate at 600 rpm. The plate was then washed 4 times with 300 μL 1× wash buffer. 100 μL substrate solution was added to each well and incubated at room temperature for 15 min out of light. After adding 100 μL stop solution to each well, absorbance at 450 nm was measured immediately.

2.13. Homeostasis model assessment for β-cells function (HOMA-B)

HOMA-B assay was performed as we did previously [9]. Briefly, the fasting plasma glucose and insulin levels were used to determine the values of HOMA-B according to the following equations: HOMA-B = (20 × FPI)/(FPG-3.5), where FPI is the fasting plasma insulin concentration (mU/L) and FPG is the fasting plasma glucose level (mmol/L).

2.14. Intraperitoneal glucose tolerance testing (IPGTT)

Mice were single-caged and fasted for 12 h prior to the injections. Glucose (2 mg/g) was injected intraperitoneally. Blood glucose levels were measured at 0, 20, 40, 60, 80, 100 min, and 120 min after glucose injection.

2.15. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, and then permeated with 0.1% Triton at room temperature. Non-specific sites were blocked using 5% BSA for an additional 1 h. Cells were then incubated with rabbit anti-PDX-1 antibody (Abcam, MA, USA) for 1 h. After washing 3 times with PBS buffer, cells were then stained with goat anti-rabbit antibody labeled with FITC for another 1 h out of light. Images were observed using a fluorescent microscope (Zesis, Germany).

2.16. Histology staining/or immunohistochemistry

Pancreatic tissue and kidney tissue were fixed by 4% paraformaldehyde for 24 h, dehydrated, embedded in paraffin and sectioned. After dewaxing in xylene and rehydration in gradient ethanol, sections were stained with hematoxylin and eosin. Or non-specific sites were blocked using 5% BSA for an additional 1 h. Tissues were then incubated with rabbit anti-insulin antibody (Abcam, MA, USA) for overnight. After washing 3 times with PBS buffer, tissues were then stained with goat anti-rabbit antibody labeled with HRP for another 1 h, after washing, the slices were developed in Diaminobenzidine (DAB) solution, and then, the sections were observed by a microscope.

2.17. Statistical analysis

Data were analyzed with one-way ANOVA, using the Graphpad program, and are expressed as mean ± standard error of mean (SEM). Treatment differences were subjected to the Tukey's test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. PANC-1 cells can be differentiated into functional insulin-producing cells (IPCs)

PANC-1 is a cell line obtained from human pancreatic ductal carcinoma, expressing high levels of c-kit and stem cell factor (SCF), which makes PANC-1 the most suitable model for progenitor studies. However, as immortalized carcinoma derivatives, the PANC-1 cells may not accurately reflect the biology of differentiation of normal cells, and therefore, the results we got from PANC-1 were further confirmed *in vivo* animal study. For inducing differentiation, the PANC-1 cells were briefly exposed to trypsin to loosen rather than detach the cells from their extracellular matrix (ECM), cells were then cultured in a serum free medium. Within 24 h, the cell congregation can be observed and the cell aggregation was more intense with the increase of differentiation time. Islet-like cell clusters can be observed within 96 h, and presented crimson red in color if stained by Dithizone (DTZ, Sigma-Aldrich, MO, USA), a zinc-binding substance for staining islet (Fig. 1A). RNA extracted from 6-day islet-like cell clusters demonstrated that

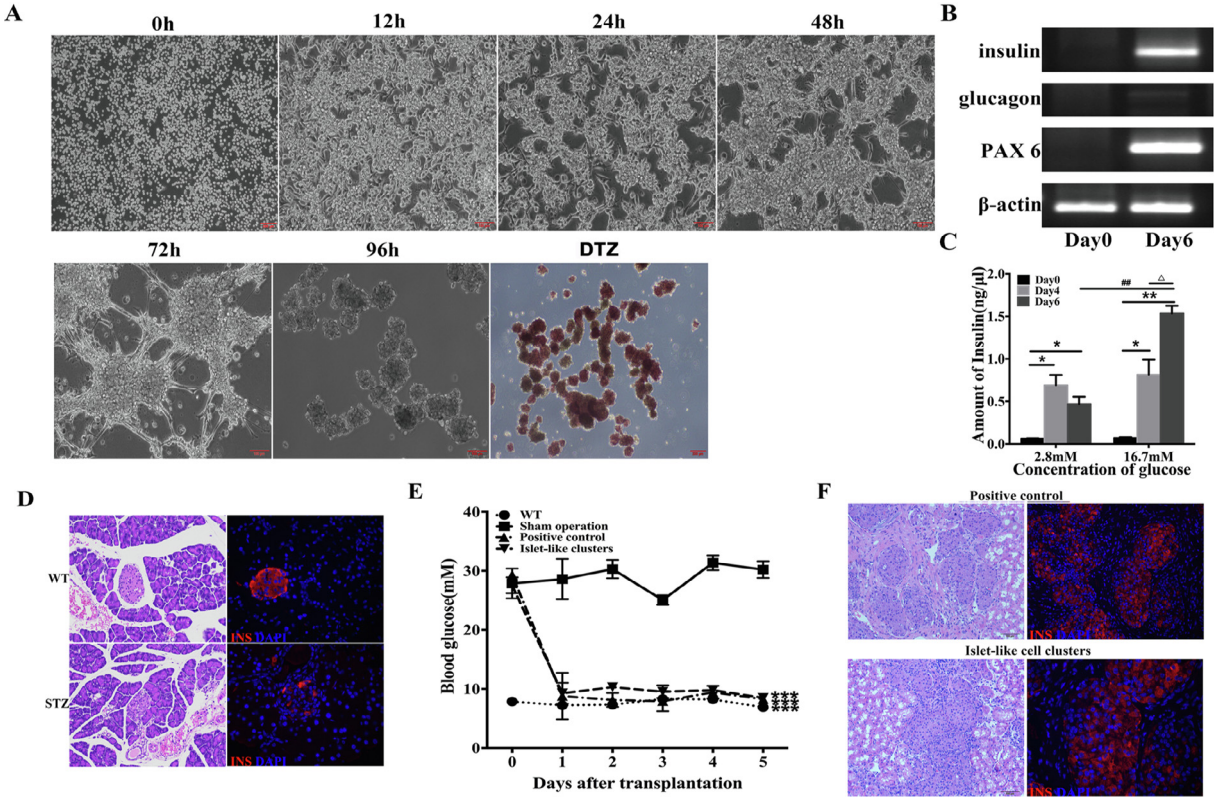

Fig. 1. PANC-1 cells can be differentiated into functional insulin producing cells. PANC-1 cells were differentiated in defined media, and (A) Representative bright field image of cells under differentiation for 0 h, 12 h, 24 h, 48 h, 72 h, 96 h and observed at 10× magnification, dithizone stained cell clusters on day 4th and observed at 5× magnification (n = 3); (B) Representative results shown that at day 6 of exposure to SFM/ITS, expression of the transcripts for insulin, glucagon, and Pax-6 in differentiated cells (n = 3); (C) Concentrations of insulin on day 0, day 4 and day 6 induced either 2.8 mM or 16.7 mM glucose respectively. *p < 0.05, **p < 0.01 *vs* control; $^{\#\#}$p < 0.01 16.7 mM compared to 2.8 mM of glucose; $^{\Delta}$p < 0.05 Day 6 compared to Day 4. Results were expressed as mean ± SD of three independent experiments; (D) for transplantation of insulin producing cells (IPCs) differentiated from PANC-1 cells, the animals were separated into four groups: WT (litter mate mice, without any treatment), Sham operation (WT + sham operation), Positive control (Diabetes + Islet isolated from WT mice), Islet-like clusters (Diabetes + IPCs). Representative histology and immunofluorescence image of pancreases from wild type and STZ-induced mice, respectively (n = 5) were shown; (E) Random blood glucose level post transplantation. ***p < 0.01 *vs* sham operation. Results were expressed as Mean ± SEM of five animals in each group; (F) Representative histology and immunofluorescence image of renal capsular of mice transplanted with normal mouse islets (positive control) or insulin-producing cells differentiated from PANC-1 cells (n = 5) were shown.

insulin, glucagon and PAX6 were expressed at the transcription level (Fig. 1B). To detect insulin secretion, cells were treated with either 2.8 mM or 16.7 mM of glucose and insulin in the supernatant was detected by ELISA analysis. The results showed that insulin can be detected from differentiated cells at both day 4 and day 6 in the supernatant, however, the insulin secreted by cells at day 4 under the stimulation of two different glucose concentrations showed no significant difference, implying functionally immature although morphologically like normal islets, while insulin level produced in the supernatant from differentiated cells at day 6 stimulated with 16.7 mM of glucose was significantly higher than those for cells with 2.8 mM glucose, confirming a functional similarity to normal human islets (Fig. 1C). For proving conceptually, whether IPCs, differentiated from PANC-1 cells, survive and regulate blood glucose homeostasis *in vivo,* STZ induced type 1 diabetic Kunming mice model, which is well accepted diabetic model, was established. As shown in Fig. 1D, the pancreatic islet from wild type mice were evenly dispersed and mostly oval or round, the marginal lines of the islets were clear with no obvious structural change as examined by H&E staining, strong and positive insulin stain also can be found in immunofluorescence staining (Fig. 1D), however, the islets in STZ treated mice were sparse, atrophic, blurred and some of the islets were in a hollow state with inflammatory cell infiltration. Immunofluorescence results showed that the insulin expression in STZ treated mice decreased significantly with only a few insulin particles were observed as compared with wild type mice. These results demonstrated that i.p. injection of STZ caused damage of pancreatic islets, a typical phenomenon of type 1 diabetes. Transplantation either the normal islet cells (Positive Control) isolated from pancreas of normal mice or IPCs differentiated from PANC-1 cells under the renal capsule, the blood glucose levels were reduced significantly at day one after transplantation and the blood glucose was maintained in a normal range in the next few days (Fig. 1E). Mice in positive control group and IPCs group were sacrificed 5 days after transplantation, H&E staining and immunofluorescence staining were carried out to observe the state of the IPCs under the renal capsule. IPCs were evenly distributed in the kidney tissue and produced insulin (Fig. 1F), which demonstrated that IPCs can survive and were functional to produce insulin *in vivo* to maintain the homostasis of blood glucose level. These results demonstrated that the method for differentiation PANC-1 cells into functional insulin producing cells has been established.

3.2. PDX-1 plays critical role in PANC-1 differentiation into insulin-producing cells

To investigate the functional role of PDX-1 during the differentiation of PANC-1 into IPCs, immunoblot study was carried out from 0 h to 96 h in clusters of differentiating PANC-1 cells. The results showed that PDX-1 expression was increased about 32% within 12 h of differentiation and decreased continuously as time prolonged (Fig. 2A, B). Furthermore, PDX-1 was translocated from the cytoplasm to the nucleus

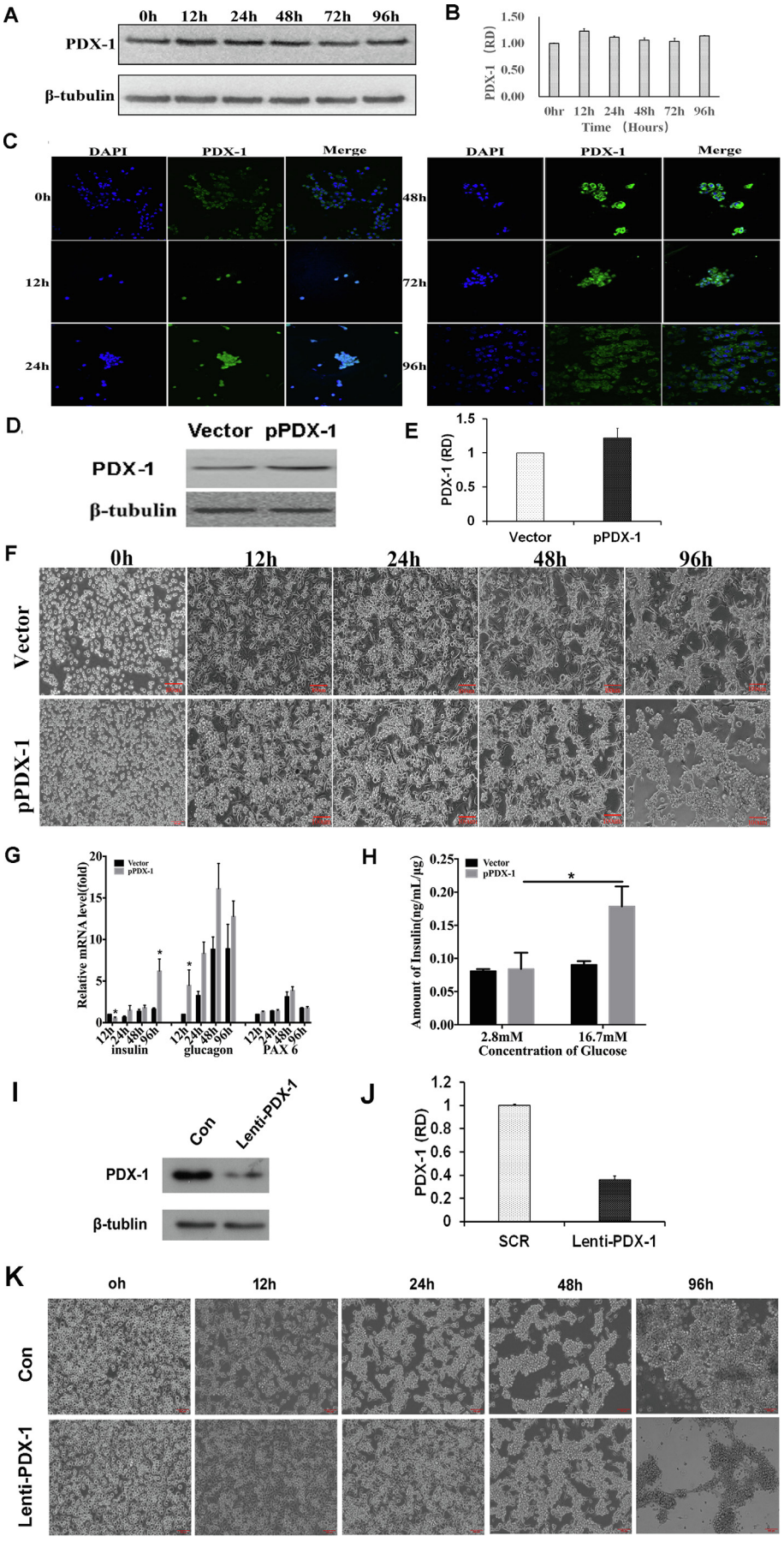

Fig. 2. Important role of PDX-1 in PANC-1 cells differentiation into IPCs. (A, B) Representative Western blot images and protein expression of PDX-1 in induced cell clusters at 0 h, 12 h, 24 h, 48 h, 72 h, 96 h respectively (n = 3 per time point), RD: Relative Density *vs* β-tubulin; (C) Representative immunofluorescent images showing the localization of PDX-1 in cell clusters under differentiation for 0 h, 12 h, 24 h, 48 h, 72 h, 96 h (n = 3 per time point); (D, E) Representative Western blot images and protein expression of PDX-1 in PANC-1 cells transfected with either empty vector (Vector) or pPDX-1, RD: Relative Density *vs* β-tubulin; (F) Representative bright field images showing the morphology of differentiated PANC-1 cells at 0 h, 12 h, 24 h, 48 h, 96 h (n = 3 per time point); (G) RT-qPCR analysis of pancreas for the expression of relevant genes (insulin, glucagon, PAX 6) in different time point post-differentiation. *p < 0.05 pPDX-1 group *vs* Vector group. Results were expressed as mean ± SD of three independent experiments; (H) Concentrations of insulin in vector or pPDX-1 group on day 0, and day 4 induced with 2.8 mM and 16.7 mM glucose respectively. *p < 0.05 16.7 mM of glucose *vs* 2.8 mM of glucose in PDX-1 over-expressed groups. Results were expressed as mean ± SEM of three independent experiments; (I, J) Representative Western blot images and protein expression of PDX-1 in PANC-1 cells infected either with control (Con) or Lenti-PDX-1 (n = 3 per group), RD: Relative Density *vs* β-tubulin; (K) Representative bright field images shown the morphology of differentiated PANC-1 cells at 0 h, 12 h, 24 h, 48 h, 96 h (n = 3 per time point).

in the early stages of differentiation and subsequently translocated from the nucleus back into the cytoplasm as shown in immunofluorescence stain (Fig. 2C), indicating that PDX-1 is likely involved in initiating PANC-1 differentiation. To further evaluate the efficacy of PDX-1 on PANC-1 differentiation into IPCs, PANC-1 cells were transfected either with pcDNA3.1-PDX-1 or pcDNA3.1 plasmid (Vector control). Compared with vector control, relative higher expression of PDX-1 was observed in PDX-1 transfected cells (Fig. 2D, E). PANC-1 cells transfected with either empty vector or PDX-1 were cultured in complete media at day 0 and then subjected to differentiation using SFM/ITS. The morphology of differentiated PANC-1 cells in both groups were observed at 0 h, 12 h, 24 h, 48 h and 96 h, and results showed that, compared to vector control, more islet-like cell clusters in PDX-1 overexpressed groups were observed (Fig. 2F). Differences in expression of insulin, glucagon, PAX6 between the groups in successive time points were tested by RT-qPCR (Fig. 2G). In comparison with the vector group, cells in the pPDX-1 expressing group showed higher expression of insulin and glucagon, with no significant change in PAX 6 expression. To further determine the efficiency of differentiation, differentiated cells at 96 h in both groups were stimulated with 2.8 mM or 16.7 mM of glucose. At both concentrations, cells induced in either groups produced insulin in the supernatant. However, the amount of insulin secreted by cells in the vector group showed no significant difference between the two glucose conditions, presuming that cells in vector group did not fully differentiate into mature islet-like cell clusters within 96 h, while the insulin concentrations produced by cells in pPDX-1 expressed group were higher than those cells in the vector group upon stimulation by 16.7 mM of glucose compared to 2.8 mM (Fig. 2H). To further confirm whether down-modulation expression of PDX-1 affect PANC-1 cells differentiates into IPCs, the PANC-1 cells were infected by lentivirus carried PDX-1 gRNA. Knock-out efficacy was evaluated by Western blot, as shown in Fig. 2I, J, Lenti-PDX-1 significantly down-regulated expression of PDX-1, and as a consequence, the differentiation of PANC-1 cells into IPCs was abolished completely at 96 h (Fig. 2K). The results from the gain-of-function as well as loss-of-function experiments indicate that PDX-1 plays a critical role in the differentiation of PNAC-1 into IPCs.

3.3. Designing a high-throughput (HTP) screen model and compounds screening.

As PDX-1 plays critical role in the differentiation of PANC-1 cells into IPCs. Therefore, we wish to screen compounds that accelerate the differentiation of PANC-1 cells through up-regulating of PDX-1 expression. Therefore, we developed a high-throughput screen assay by fusing the PDX-1 promoter region with a firefly luciferase reporter vector named pGL3.14 to generate pPDX-1-Luc vector (Fig. 3A). Fluorescence value, which represents the ability of compounds in increasing PDX-1 promoter activity, from PANC-1 cells extracts induced by different compounds was measured. More than 400 extracted fractions of herbal medicines were screened and the enhancement ratio by each compound was calculated based on the fluorescence value (Fig. 3B). Eleven fractions (Fig. 3B, red cycle) with high enhancement ratios were selected to further validate their activity on PDX-1 expression. After 24 h being treated with compounds, RNA was isolated from PANC-1 cells to assess the ability of compounds to upregulate endogenous PDX-1 expression. Results showed that C421, C582, C713, C754, C807 could slightly increase *PDX-1* gene expression (Fig. 3C). In addition, the effects of C421, C582, C713, C754 and C807 on PDX-1 protein were assessed, and results shown that C582, C713, C754, C807 significantly increased the expression of PDX-1 at protein level (Fig. 3D, E). Since it has been reported that *Andrographis paniculata* a traditional Chinese herb medicine has anti-diabetic effect [12,13], thus, C754, a extracted fraction of *Andrographis paniculata* was selected for further study.

3.4. C1037 is the active ingredient of C754.

To screen out the active ingredients of C754, C754 was analyzed by HPLC-HR-MS. The total ion chromatogram (TIC) of C754 gave the characteristic pseudo-molecular ion peak at t_R = 8.7, 701.4251 ([2 M + H]$^+$) and 351.2161 ([M + H]$^+$) in positive mode (Fig. 4A, B, D), which was in accordance with the retention time and molecular formula $C_{20}H_{30}O_5$ of reference standard andrographolide (C1037) (Fig. 4C, E). Thus, C1037, as one of the main components of C754, was further investigated for its regulation capbility of PDX-1 promoter activities.

Consistent with C754, the expression of PDX-1 in cells treated with different concentrations of C1037 (1.25 μM, 2.5 μM, 5.0 μM) was significantly upregulated in a dose-dependent manner as shown in luciferase assay (Fig. 5A). In accordance with the luciferase results, the PDX-1 mRNA (Fig. 5B) and protein levels (Fig. 5C, D) were also induced by C1037 in a dose-dependent manner.

3.5. C1037 accelerates PANC-1 cells differentiation into IPCs

To test whether C1037 could promote differentiation of PANC-1 cells into insulin-producing cells, PANC-1 cells were subjected to SFM/ITS with or without C1037 and microscopically observations were recorded in time-dependent manner. In both groups, PANC-1 cells form cell clusters that resembled islets after 96 h of culture. However, different condensation patterns were noticed between two groups, with cells in C1037 treatment triggering earlier cell cluster formation (Fig. 6A). By detecting the islet-relevant gene (insulin, glucagon, PAX 6) expression, we found that cells induced with C1037 showed higher expression of insulin and lower expression of glucagon, with no significant change in PAX 6 expression (Fig. 6B), indicating that the proportion of β-cells in differentiated cells with C1037 treatment was more robust and the proportion of α-cells was less than that of control cells. Glucose stimulated insulin secretion results showed that at both concentrations of glucose, cells in two groups produced insulin in the supernatant. However, the amount of insulin secreted by cells in control group showed no significant difference between the two concentrations of glucose. The insulin concentrations expressed by cells in C1037 induced group were higher than those for cells in control group, and secreted more insulin stimulated by 16.7 mM glucose compared with 2.8 mM glucose treatment (Fig. 6C). Therefore C1037 promotes the PANC-1 differentiation into matured insulin producing cells.

3.6. C1037 treatment restores glucose homeostasis in type 1 diabetic mice

In order to test whether C1037 has therapeutic effect in diabetic animal model, type 1 diabetic (T1D) mice induced by STZ were treated with saline, Liraglutide or C1037, citrate buffer injected mice were used as blank control. Results showing that compared with blank control, the blood glucose level in saline group is significantly higher ($^{\#\#}p < 0.01$). While compared with saline group, the blood glucose levels both in liraglutide treated group ($^{**}p < 0.01$) and C1037 treated group ($^{*}p < 0.05$) decreased significantly starting from day 10 and lasting till the end of experiments (Fig. 7A). The body weights were similar among different groups of mice except significantly lower in saline group compared with blank control on day 20 ($^{\#}p < 0.05$), 30 ($^{\#\#}p < 0.01$), and 40 ($^{\#\#}p < 0.01$) (Fig. 7B). To determine the underlying cause of reducing blood glucose by C1037, the mice were then performed with intraperitoneal glucose tolerance test (IPGTT) test, at about 20 min after a glucose injection (2 g/kg) into peritoneal cavity of mice that were fasted for 16 h, the blood glucose levels were increased in both liraglutide and C1037 treated group, and then decreased, the courses were comparable with blank control, but significantly lower compared to saline group at 40 ($^{*}p < 0.05$), 60 ($^{*}p < 0.05$), 80 ($^{*}p < 0.05$) and 120 min ($^{**}p < 0.01$) in liraglutide treated group and at 80 ($^{*}p < 0.05$), 100($^{*}p < 0.05$), 120 min ($^{*}p < 0.05$) in

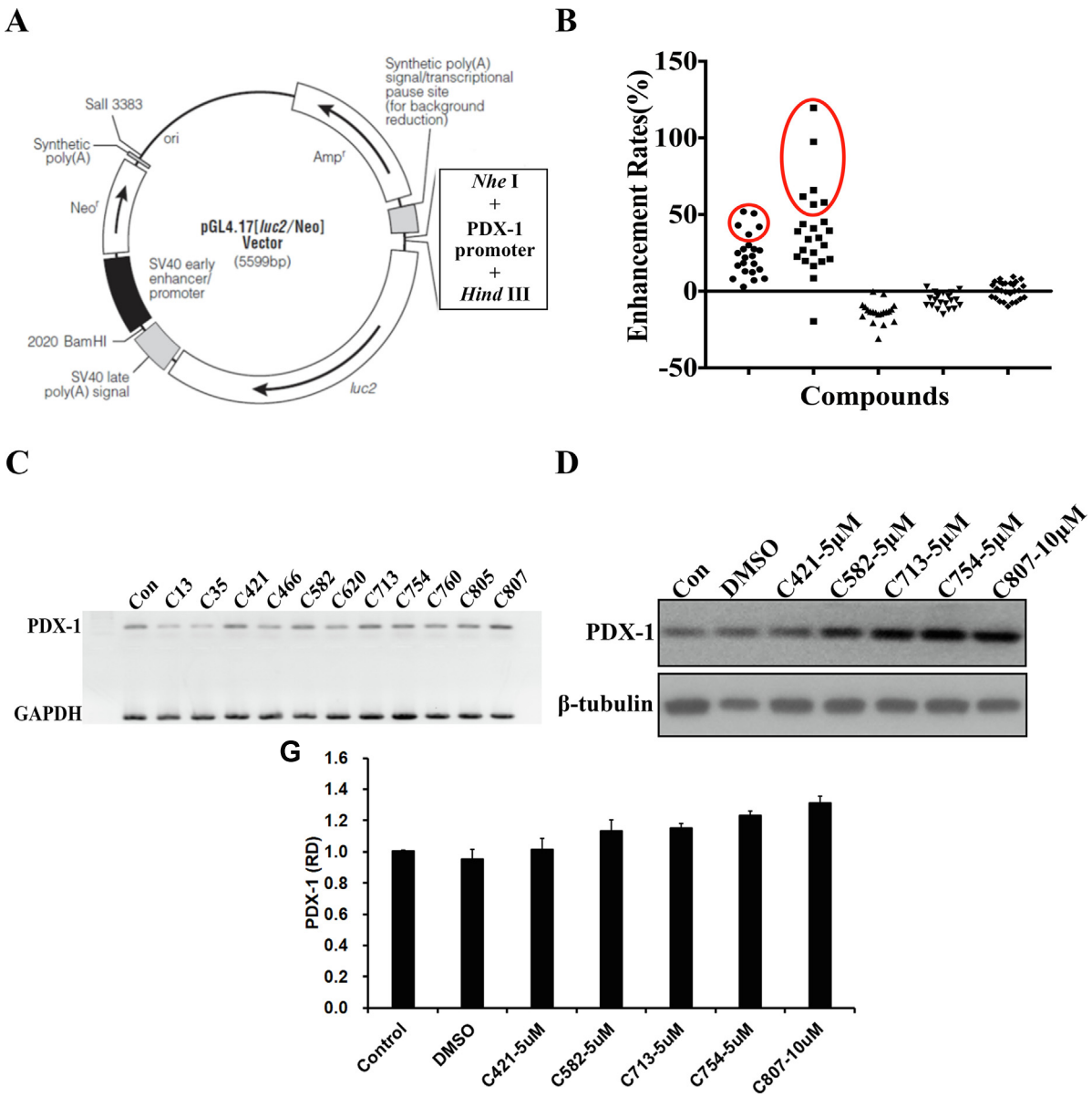

Fig. 3. C754 induces PDX-1 gene and protein expression. (A) Establishment of HTP screening model through fusing the PDX-1 promoter region with a luciferase reporter vector; (B) The enhancement ratio of each compound, scored relative to control cells treated with DMSO; (C) RT-PCR analysis of PDX-1 expression in PANC-1cells induced by selected compounds for 24 h. GAPDH was used as a loading control; (D, E) Representative Western blot images and protein expression of PDX-1 in PANC-1 cells treated with selected compounds for 48 h. Tubulin was used as an internal loading control. Results were expressed as mean ± SD of three independent experiments, RD: Relative Density *vs* β-tubulin.

C1037 treated group (Fig. 7C), indicating that glucose homostasis was restored in both liraglutide and C1037 treated group. To further determine whether this phenomena was related with restored β-cell function, the HOMA-B was assessed, compared to blank control, the HOMA-B score was significantly lower in saline group ($^{\#}p < 0.05$), while compared to saline group, the HOMA-B score was significantly increased in both liraglutide and C1037 treated groups (Fig. 7D, $^{*}p < 0.05$). These results demonstrating β-cell function is abnormal in sanline group, while restored in both liraglutide and C1037 treated groups.

3.7. Treatment with C1037 enhances PDX-1 positive islets, restores morphology of pancreas, and increases insulin positive islets in type 1 diabetic mice

Since treatment of C1037 significantly decreases blood glucose level and increase HOMA-B score, we wondered if this is related to enhanced expression of PDX-1 and restored insulin positive islets morphology in T1D mice, the immunofluorescent staining, H & E stain and immunohistochemistry analysis were performed. Results shown that compared with control, less and disrupted islets were found in H & E stain of 20× or 200× magnification in saline group, while compared with saline group, the number and morphology of islet in both liraglutide or C1037 treated group were restored (Fig. 9A). It was further confirmed by immunohistochemistry stain that, compared with control, less and destruction insulin positive islets were observed in saline group, whereas the number of PDX-1 positive islets (Fig. 8) and insulin positive islets are increased in both liraglutide or C1037 treated groups, compared with saline group (Fig. 9B).

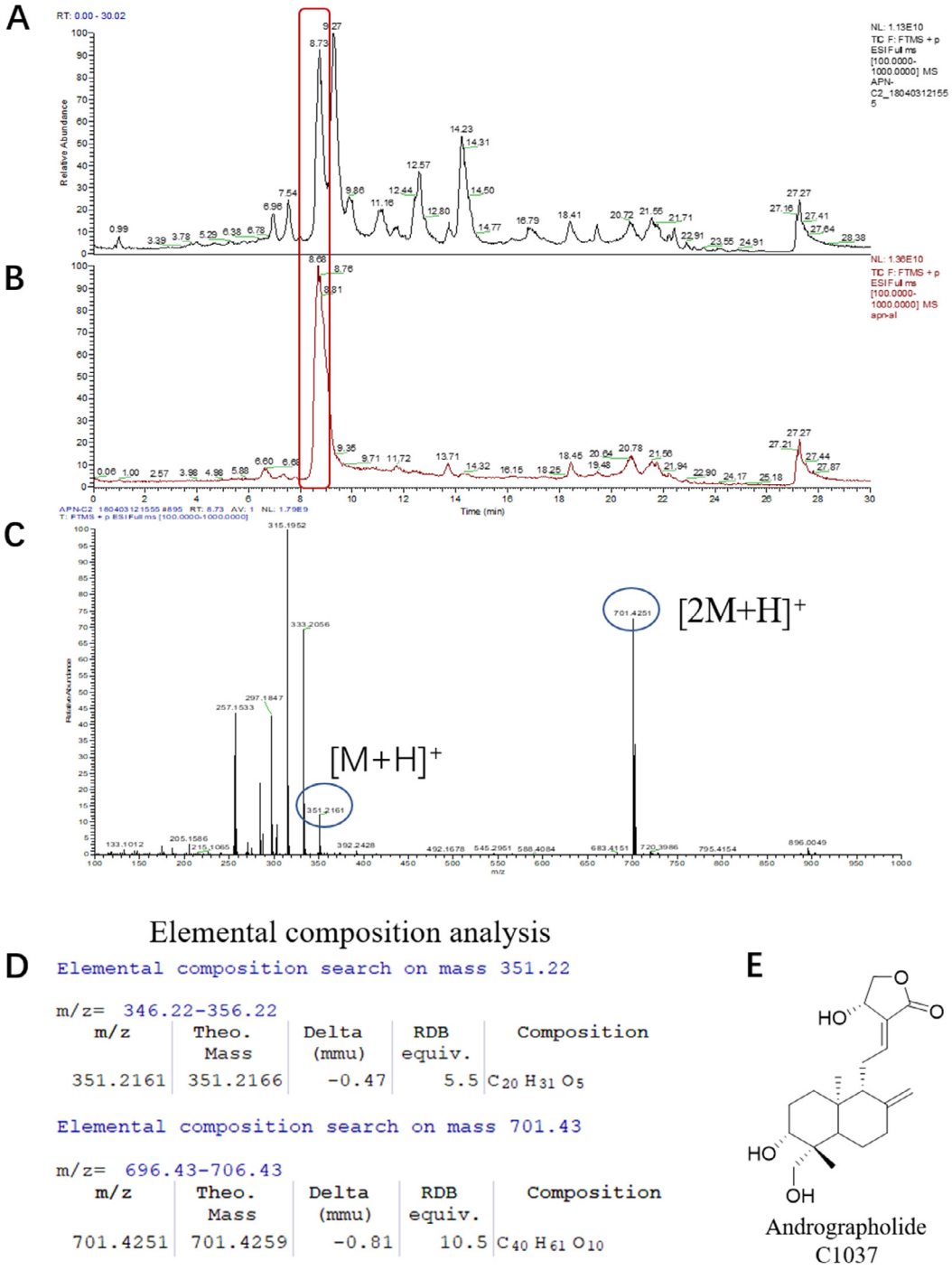

Fig. 4. HPLC-HR-MS analysis of C754 and C1037. (A) Total ion chromatogram (TIC) of C754 in positive mode; (B) Total ion chromatogram (TIC) of C1037 in positive mode; (C) The MS spectrum of C1037; (D) The elemental composition analysis of the peak at t_R = 8.7 min; (E) The chemical structure of C1037.

4. Discussion

Diabetes is a non-infective chronic disease, affecting about 463 million populations worldwide and 116.4 million people in China in 2019 [14]. The loss of β-cell mass and function is critical in the progression of both type 1 and advanced type 2 diabetes. Currently, various strategies have been developed to promote recovery of the β-cell mass, like bioartificial pancreas [15], nanotechnology to encapsulation incretin to protecting and regeneration of β-cell [16], alpha-cell to beta-cell transdifferentiation [17,18], and differentiation of embryonic stem cells or multipotent stem cells into insulin-producing cells, providing novel ideas for cell replacement and treatment of diabetes [19].

The stem cells that have the potential to differentiate into insulin-secreting cells include embryonic stem cells, and adult stem cells or mesenchymal stem cells derived from various tissues such as pancreas, liver, central nervous system, bone marrow, adipose tissue, etc. [20,21]. Recently, methods for differentiation non-β-cells into insulin producing cells through induction or genetic reprograming provide a novel idea for diabetes treatment [22–25], and many transcription factors related with pancreas development were overexpressed to regulate β-cells differentiation (mainly PDX-1).

PDX-1, also known as insulin promoter 1, is an important transcription factor for normal pancreatic development and islet β-cell differentiation and maturation. In fact, in addition to the absence of

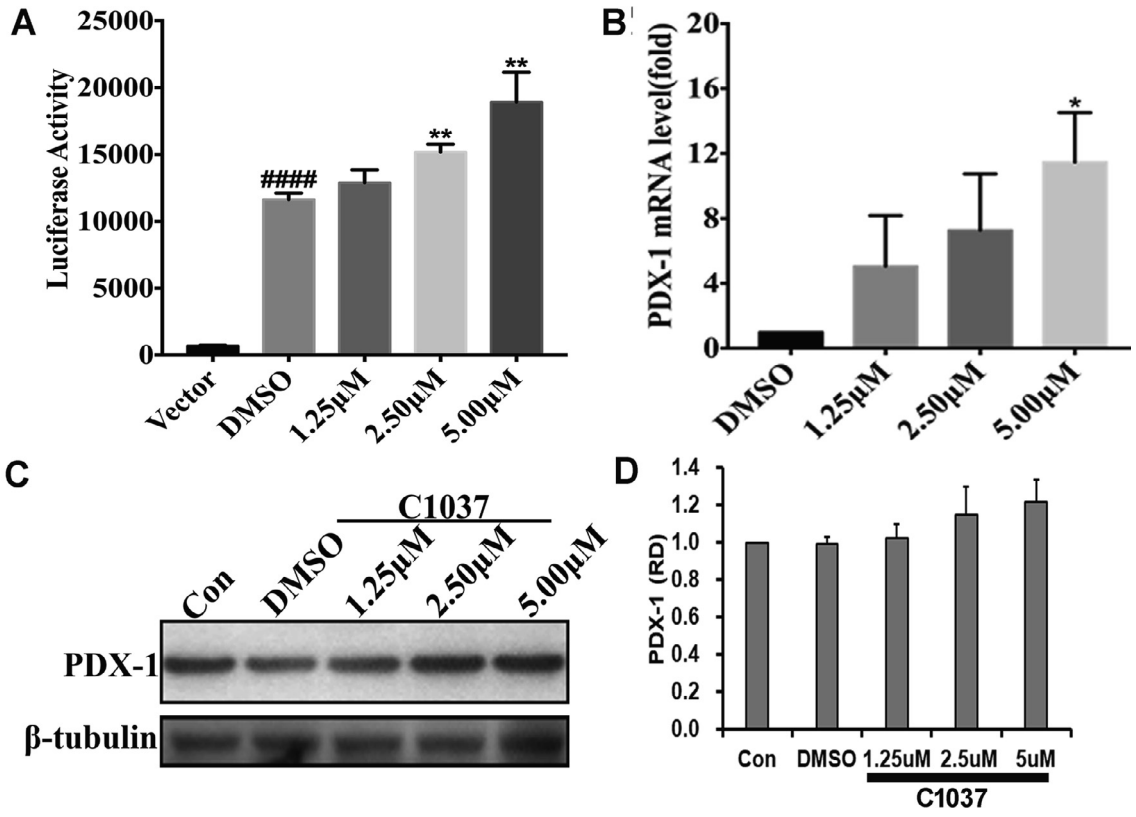

Fig. 5. C1037 up-regulates expression of PDX-1. (A) Luciferase activity in PANC-1 cells treated with C1037 in indicated concentration. $^{####}p < 0.0001$ *vs* vector control; $^{**}p < 0.01$ *vs* DMSO; (B) RT-qPCR analysis of PDX-1 expression in PANC-1 cells induced by C1037 for 96 h. The fold change was scored relative to control cells treated with DMSO. $^{*}p < 0.05$ *vs* DMSO; (C, D) Representative Western blot images and protein expression of PDX-1 in PANC-1 cells treated with C1037 for 48 h. Tubulin was used as an internal loading control. Results were expressed as mean ± SD of three independent experiments. RD: Relative Density *vs* β-tubulin.

Bruner's glands and the lack of intestinal endocrine differentiation in the stomach and duodenum, heterozygous $Pdx1^{+/-}$ mice exhibited an age-dependent deterioration of glucose tolerance, reduced glucose-stimulated insulin secretion and higher apoptosis sensitivity [6]. Additionally, $PDX1^{-/-}$ knockout mice also showed loss of pancreatic formation [5].

The expression of PDX-1 plays an important role in inducing differentiation of stem cells derived from different tissue sources into insulin producing cells (IPCs). Studies showed that after removing most of the pancreas, new islets were found in the surrounding of remaining cells and pancreatic duct epithelial cells, and the newly born islets were mainly derived from the differentiation of pancreatic ductal epithelial stem cells under the action of PDX1 [26]. This process is similar to that of embryonic development. During embryonic development, the liver and pancreas can be trans-differentiated in certain conditions from pancreatic endocrine precursor cells in the liver, through expression of insulin transcriptional factors related to the development of pancreatic endocrine cells, such as HNF, PDX-1, and Ngn3, and so on [27]. Additionally, PDX-1 has been used as a reprogramming factor to trigger various sources of mesenchymal stem cells and embryonic stem cells to differentiate into endocrine pancreatic cells [28–32]. PDX-1 overexpression induces liver stem cells to differentiate into IPCs [27]. Yang et al. [33] confirmed that the fusion gene PDX1-VP16 induced the liver stem cell line (WB-1 cells) into cells that produce and secrete insulin. Consistent with these studies, we also observed that PDX-1 plays critical role in the differentiation of PANC-1 cells into IPCs (Fig. 1).

Therefore, we aimed to identify small molecules capable of promoting pancreatic duct cells differentiation into insulin producing cells through enhancing the expression of the transcription factor PDX1. First, we observed that expression level and pattern of PDX-1 is changed during the differentiation of PANC-1 cell. Transfection of PANC-1 cells with PDX-1, followed by SFM/ITS culture, accelerates phenotypic changes towards insulin producing cells (IPCs, Fig. 2D, E, F, G, H), while down-modulation of PDX-1 expression abolished differentiation of PANC-1 into IPCs (Fig. 2I, J, K), which is consistent with previous report on the critical role of PDX-1 in pancreatic β-cell development [31,32].

We then establishment a High-Throughput Screening (HTS) assay by fusing PDX-1 promoter region to a luciferase reporter vector and performed compounds screening to identify compounds capable of up-regulating PDX-1 expression. From 400 herbal extracted fractions of herbal medicines, C754, an extracted fraction from *Andrographis paniculata*, which could significantly increase the PDX-1 expression, was identified and analysed (Fig. 3). Then HPLC-MS were performed to identify the active ingredients within C754. Results showed that the molecular weight of the main components in C754 was 350 Da, consistent with that of our predicted compound, andrographolide. After analysis in the same chromatographic condition, the molecule marked with Peak 1 and andrographolide standard have same retention time, indicating that the molecule marked with Peak 1 is andrographolide (C1037) (Fig. 4).

We then further confirmed that C1037 induces PDX-1 expression both at transcript and protein levels in PANC-1 cells in a dose-dependent manner (Fig. 5). Treatment with C1037 enhanced differentiation and maturation of PANC-1 into IPCs, as indicated by inducing expressions of several genes related to β-cells (PDX-1, insulin, Pax6), and could produce more insulin in the supernatant stimulated by glucose (Fig. 6). To further assess whether C1037 has therapeutic effect on type 1 diabetic (T1D) mice, single higher doses of β-cell toxin streptozotocin (STZ, at 150 mg/kg) was intraperitoneal (i.p.) injected into mice to induced T1D mice model [34,35]. The blood glucose level was elevated above 16.7 mM (Fig. 7A), function of β-cells was lost (Fig. 7D) and

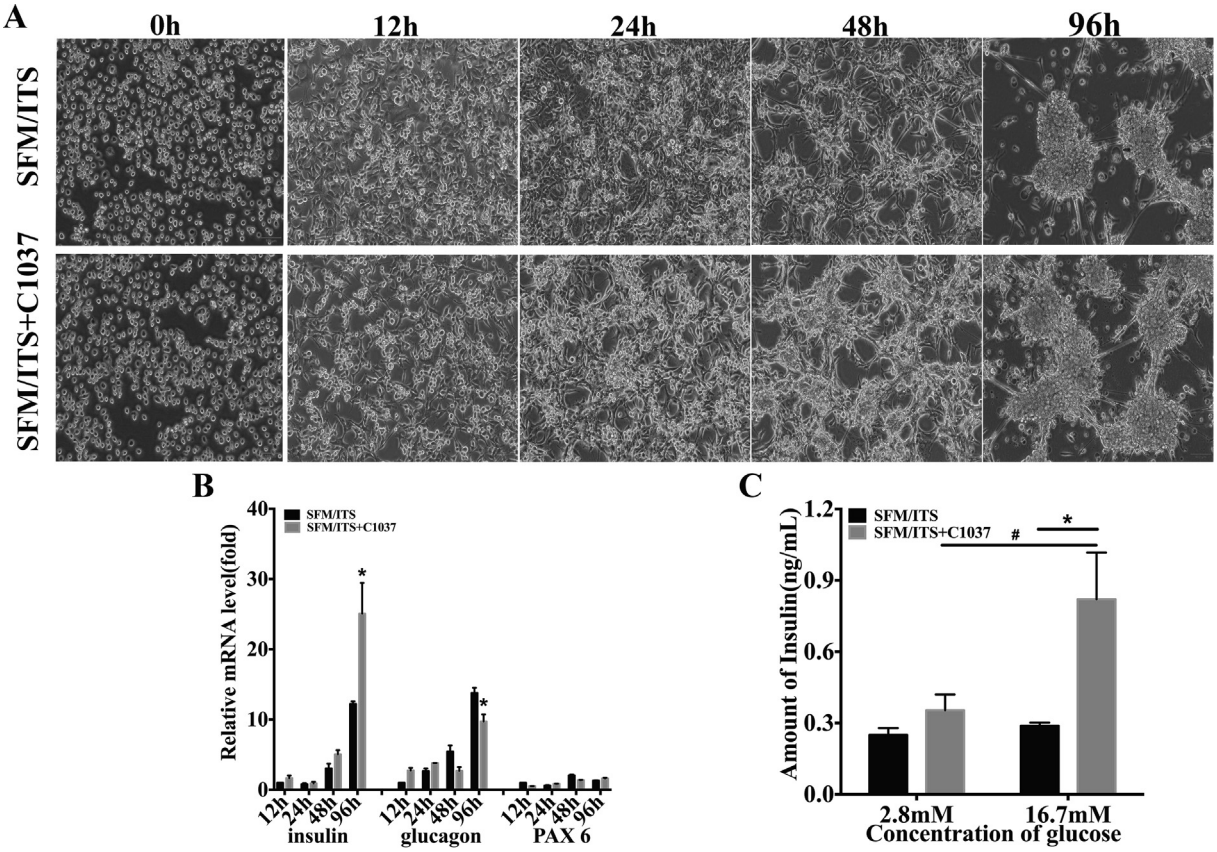

Fig. 6. C1037 induces PANC-1 cells differentiation into functional insulin-producing cells. (A) Representative bright field image of cells induced with or without C1037 treatment under differentiation for 12 h, 24 h, 48 h, 72 h, 96 h at 10× magnification; (B) RT-qPCR analysis of pancreas related genes (insulin, glucagon, PAX 6) expression in a time-dependent manner. *p < 0.05, C1037 treated group compared to non-treated group. Results were expressed as mean ± SD of three independent experiments; (C) Concentrations of insulin in cells with or without C1037 treatment on day 4 induced with 2.8 mM and 16.7 mM glucose respectively. *p < 0.05, C1037 treated group compared to non-treated group, #p < 0.05, 16.7 mM of glucose compared to 2.8 mM of glucose treated group. Results were expressed as mean ± SD of three independent experiments.

existing pancreatic islet was destructed (Fig. 9A). Because liraglutide is the only drug capable of improving glycaemic control [36,37] and inducing neogenesis of β-cells as the clinical anti-diabetic drug [38,39], it was thus used as a positive control, and as evidenced from our experiments, compared to saline group, significantly decreased blood glucose level (Fig. 7A) in T1D mice treated with 180 μg/kg of liraglutide was observed 10 days after treatment and lasting till 40 days with increased β-cell function (Fig. 7D), restored islet morphology (Fig. 9A),

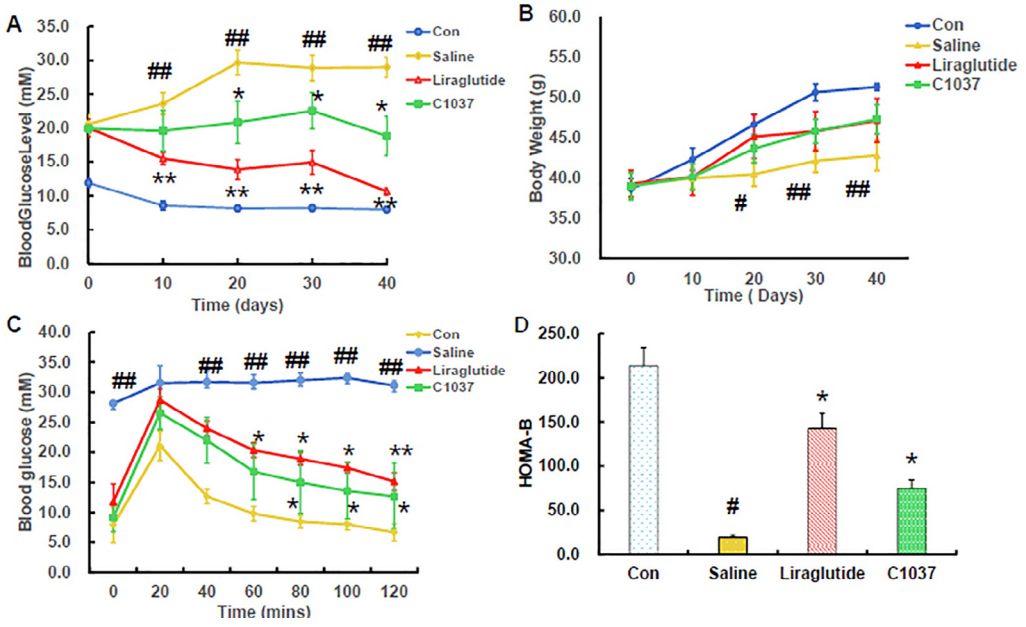

Fig. 7. Therapeutic effect of C1037 on type 1 Diabetes. STZ induced type 1 diabetes mice were separated into three groups: saline (Diabetes + saline); Liraglutide (diabetes + liraglutide), and C1037 (diabetes + C1037), and untreated litter mate mice were used as control (non diabetes), Control and saline groups of mice were treated with saline, and 180 μg/kg of liraglutide (i.p) or 50 mg/Kg of C1037 treated to the mice for 40 days and blood glucose level (A), body weight (B) were monitored. At end of treatment, intraperitoneal glucose tolerance test (IPGTT) test (C), and HOMA-B (D) were performed. #p < 0.05 compared to blank control, *p < 0.05, and **p < 0.01 compared to saline group. Results were expressed as mean ± SEM of six animals in each groups.

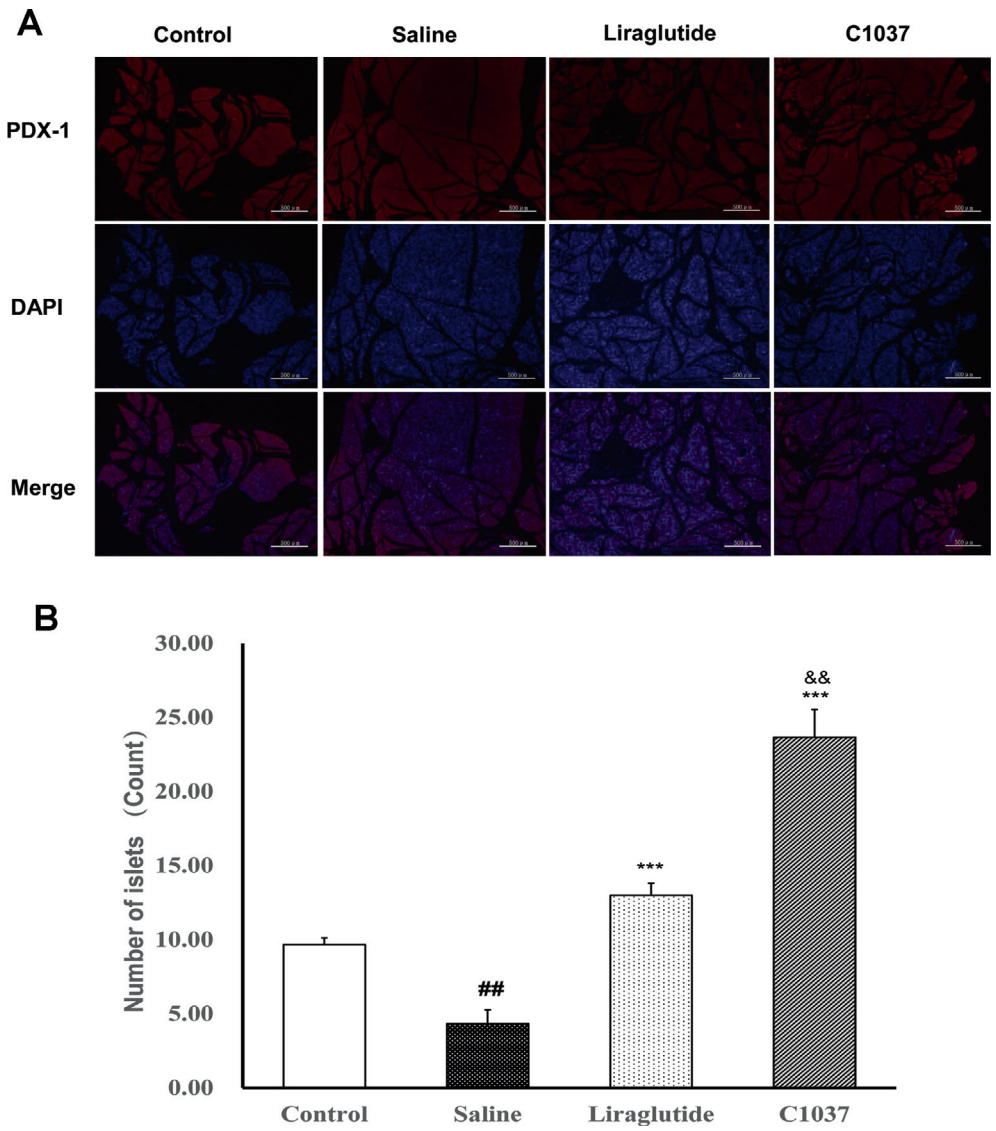

Fig. 8. Treatment with C1037 increases number of PDX-1 positive islets in type 1 diabetic mice. The pancreas of the different group of mice from Fig. 7 were analysed by immunofluorescent stain, and representative immunofluorescent image in the pancreas from different groups were shown (A, and the number of PDX-1 positive islets were counted and analyzed (B). $^{\#\#}p < 0.01$ *vs* Control; ***$p < 0.001$ *vs* Saline group; $^{\&\&}p < 0.01$ *vs* Liraglutide group (n = 3 per group).

and increased number of insulin producing cells (Fig. 9B). Similarly, compared with saline group, lower blood glucose level in T1D mice (Fig. 7A), and decreased blood glucose level in IPGTT test (Fig. 7C) was also observed in C1037 treated group. We further tested weather observed lower blood glucose effect by C1037 is related with increased number of PDX-1 positive islet and recovery of β-cell function, immunofluorescent staining was performed in the pancreas from different group. Results shown that the number of PDX-1 positive islets were significantly increased in C1037 treated group compared either saline group or liraglutide group (Fig. 8). As compared to saline group, the function of β-cells in C1037 treated group were restored as indicated by increasing HOMA-B score (Fig. 7D). We further observed that, as compared with saline group, in which the morphology of islet was disrupted (Fig. 9A), the number of islets (Fig. 9A, B, 20× magnification) and insulin positive stains were decreased (Fig. 9B), increased number of islets (Fig. 9A, B, 20× magnification), restored islet morphology (Fig. 9A, 200× magnification), and increased insulin positive islets (Fig. 9B, 200× magnification) were observed in T1D mice treated with C1037, indicating that regeneration of β-cells were induced by C1037 treatment *in vivo*, which is constant with early report that andrographolide prevents development of autoimmune diabetes in NOD mice [12] and hypoglycemic and protects β-cells from H_2O_2 and other reactive oxidative species-induced damage [13]. However, since regeneration of pancreatic islets and β-cells could be achieved by proliferation of existing β-cells [40], by neogenesis from α-cells [41,42], or by regeneration from endogenous progenitors [43] or duct-progenitors [44,45], we are unable to identify exactly where the newly generated islets or insulin positive cells came from in our current study, but considering the data from in vitro study, PANC-1 cell, a human pancreatic ductal progenitors, can be enhanced to differentiate into functional insulin producing cells (Fig. 6) by C1037, therefore, more likely, the regenerated islets or insulin positive cells in animal study may come from differentiation of pancreatic ductal progenitors, which will need to be experimentally validated by future study.

Overall, for the first time, our current study developed a new method for screening compounds that regulate the expression of PDX-1 as a surrogate to identify differentiation inducers to promote islet regeneration. Simultaneously, we identified and functionally characterized compound C1037, which could accelerate PANC-1 cells differentiation through up-regulating the expression of PDX-1 in vitro, and inducing expression of PDX-1 in pancreas of T1D mice, showing a promising for diabetes treatment, especially for type 1 diabetes.

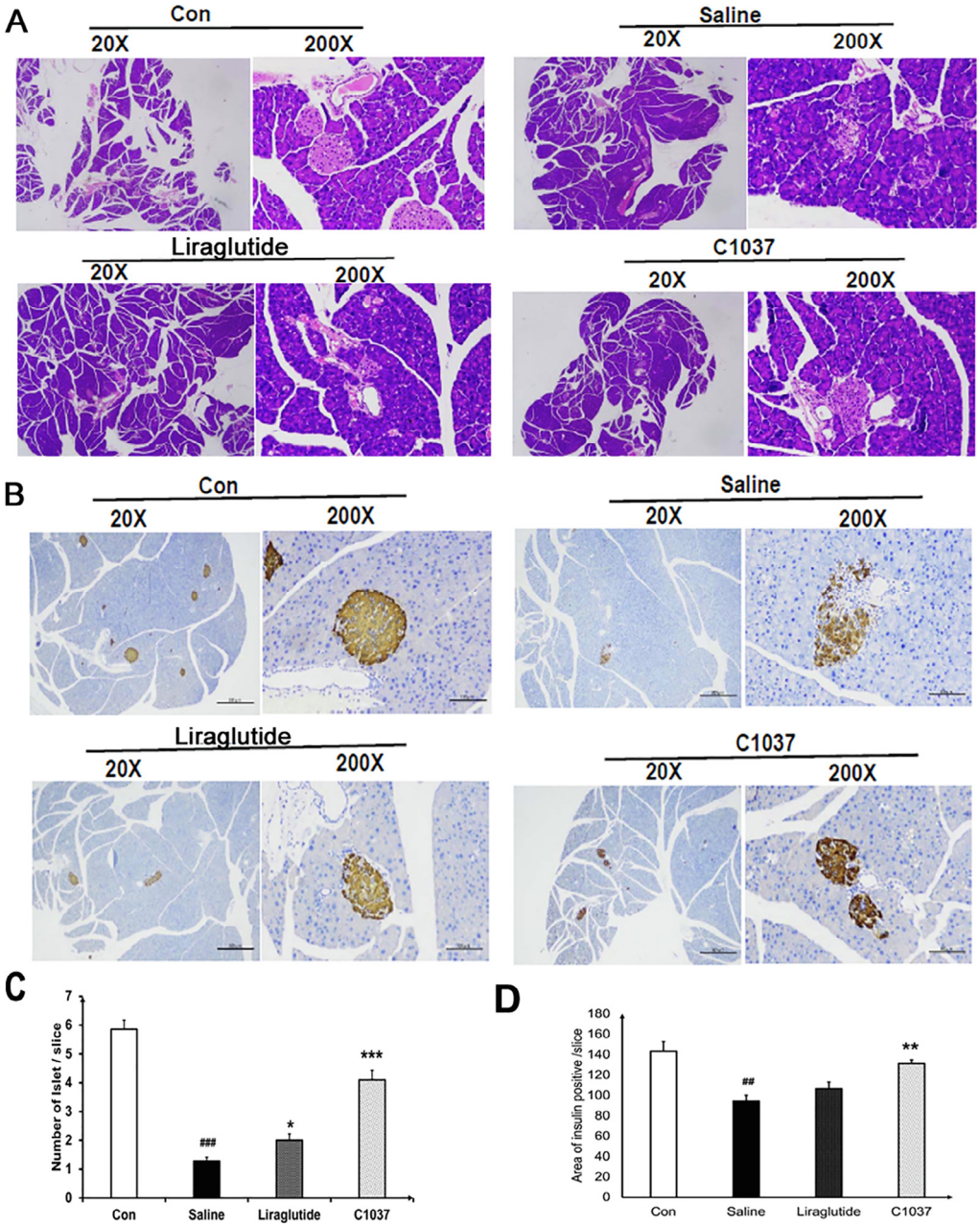

Fig. 9. Treatment with C1037 increases insulin positive islets in type 1 Diabetic mice. H & E (A) and immunohistochemistry stain (B) were performed in the pancreas of the different group of mice from Fig. 7, and representative H & E stain (A) and immunohistochemistry (B) image were shown in pancreas of mice (n = 3 per group).

However, these anti-diabetic effects of C1037 are still preliminary, and future pre-clinical and clinical examinations should be needed to ensure the safety and efficacy of C1037 for development as a drug lead candidate.

Author contributions

S.M.Z, F.R. H, W.J.T., and J.S.L., designed, conducted the experiments, and drafted the manuscript. L.X.Q, researched data, W.J.H, H.F.C, and L.L.C. conceived the project, designed the experiments, reviewed the results and wrote the manuscript.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcp.2019.113785.

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