Mangiferin induces islet regeneration in aged mice through regulating p16^{INK4a}

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Abstract. Previous studies by our group on mangiferin demonstrated that it exerts an anti-hyperglycemic effect through the regulation of cell cycle proteins in 3-month-old, partially pancreatectomized (PPx) mice. However, β-cell proliferation is known to become severely restricted with advanced age. Therefore, it is unknown whether mangiferin is able to reverse the diabetic condition and retain β-cell regeneration capability in aged mice. In the present study, 12-month-old C57BL/6J mice that had undergone PPx were subjected to mangiferin treatment (90 mg/kg) for 28 days. Mangiferin-treated aged mice exhibited decreased blood glucose levels and increased glucose tolerance, which was accompanied with higher serum insulin levels when compared with those in untreated PPx control mice. In addition, islet hyperplasia, elevated β-cell proliferation and reduced β-cell apoptosis were also identified in the mice that received mangiferin treatment. Further studies on the mRNA transcript and protein expression levels indicated comparatively increased levels of cyclins D1 and D2 and cyclin-dependent kinase 4 in mangiferin-treated mice, while the levels of p27^{Kip1} and p16^{INK4a} were decreased relative to those in the untreated PPx controls. Of note, mangiferin treatment improved the proliferation rate of islet β-cells in adult mice overexpressing p16^{INK4a}, suggesting that mangiferin induced β-cell proliferation via the regulation of p16^{INK4a}. In addition, the mRNA transcription levels of critical genes associated with insulin secretion, including pancreatic and duodenal homeobox 1, glucose transporter 2 and glucokinase, were observed to be upregulated after mangiferin treatment. Taken together, it was indicated that mangiferin treatment significantly induced β-cell proliferation and inhibited β-cell apoptosis by regulating cell cycle checkpoint proteins. Furthermore, mangiferin was also demonstrated to regulate genes associated with insulin secretion. Collectively these, results suggest the therapeutic potential of mangiferin in the treatment of diabetes in aged individuals.

Introduction

Diabetes, which partly results from either a loss of β-cell mass [type 1 diabetes mellitus (T1DM)] or insulin resistance (T2DM), has a considerably high rate of morbidity worldwide (1,2). For patients with T2DM, although their blood insulin concentrations are presumably high, prolonged disease may ultimately lead to the development of insulin-deficient diabetes, T1DM. Clinical studies on T2DM patients have indicated that the condition is associated with a decreased β-cell mass and increased β-cell apoptosis (3). Of note, in elderly T2DM patients, islet regeneration capability was impaired with age, and thus, such patients have to rely on exogenous insulin injection to maintain blood sugar homeostasis. Although islet transplantation may be performed for severe cases of T1DM, it is limited by the shortage of appropriate organ donors and the long-term prescription of immunosuppressant drugs. Previous studies have demonstrated that in response to physiological and pathophysiological changes, islet β-cells exhibited a compensatory capacity throughout adulthood (4,5). Indeed, after partial pancreatectomy (PPx) in rodents as a classic model of pancreatic regeneration, islet regeneration was observed to occur via the replication of pre-existing differentiated cells, the hypertrophy of β-cells and the differentiation of whole new

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pancreatic lobes (6). Therefore, it appears that the successful induction of insulin secretion in aged mice may be achieved by PPx surgery, despite the acute loss of islet β-cells in the short term. Of note, the study by Tschen *et al* (7), revealed that the β-cell proliferation capability declined with age and that this decline was regulated by the Bmi1/p16^{INK4a} pathway. Consistently, Rankin and Kushner (8) reported that basal β-cell proliferation was severely decreased with advanced age and that in a mouse model, PPx failed to increase β-cell replication in aged mice. p16^{INK4a}, as a negative regulator of the cell cycle, is differentially expressed in aging tissues and has been reported to restrict islet growth (9,10). Therefore, to investigate the mechanisms of mangiferin-induced islet regeneration in aged mice, the present study focused on p16^{INK4a}.

Previous studies on mangiferin, a traditional Chinese medicine isolated from the leaves of *Mangiferina indica* (mango), have identified antitumor (11), antiviral (12), antioxidant (13) and immunomodulatory activities (14). In addition, studies on the anti-diabetic effect of mangiferin revealed that it markedly lowered blood glucose levels in streptozotocin (STZ)-induced diabetic rats (15,16). It was also reported to exert beneficial effects on hyperlipidemia in T2DM (17). Furthermore, mangiferin significantly prevented the progression of diabetic nephropathy and improved renal function (18,19). These anti-diabetic effects of mangiferin may be due to the stimulation of peripheral glucose utilization (20). A previous study by our group on mangiferin clearly indicated that mangiferin treatment markedly induced islet regeneration in young, partially pancreatectomized mice (21). Therefore, to further elucidate the anti-diabetic effect of mangiferin, the present study investigated whether mangiferin induces islet regeneration in aged mice, and evaluated the underlying mechanisms, including the potential regulation of cell cycle regulatory proteins.

Materials and methods

Study design. A total of 90 male C57BL/6J mice (age, 12 months, 26±2 g) were purchased from the Affiliated Animal Institute of Sichuan Academy of Medical Science & Sichuan Provincial People's Hospital (Chengdu, China). Mice were maintained in a 12-h light/dark cycle in an atmosphere of 0.03% CO_2 with free access to water and food. All mice were raised under specific pathogen-free conditions with a controlled temperature (23±2°C). Mice were randomly divided into three groups (n=30 in each group): i) A sham group, in which mice received a sham operation; ii) a PPx control group, in which mice were administered normal saline after PPx surgery; and iii) a mangiferin group, in which mice were intraperitoneally (i.p.) administered 90 mg/kg mangiferin for 28 consecutive days after PPx surgery (17,22). Mangiferin (purity, >98%) was purchased from Desite Co. (Chengdu, China). The animal procedures were approved by the Ethics Committee of Sichuan Academy of Medical Science & Sichuan Provincial People's Hospital (Chengdu, China).

Animal surgery. All animals were fasted for 12 h prior to and 5 h after surgery, after which the animals were given free access to a standard diet and water. Mice were anesthetized by i.p. injection of 50 mg/kg pentobarbital (Beijing Propbs

Biotechnology, Beijing, China), and the spleen and entire splenic portion of the pancreas were then surgically removed, while the mesenteric pancreas between the portal vein and duodenum was left intact. For the sham operation, the spleen was removed, while the pancreas was left intact. All mice were labeled with 0.8 mg/ml 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), continuously provided in drinking water after the PPx surgery.

Biochemical measurements. After the animals were fasted for 8 h, their blood glucose levels were measured with a SureStep Blood Glucose meter (LifeScan, Milpitas, CA, USA). An intravenous glucose tolerance test (IVGTT) was performed by tail vein injection of 1 g/kg D-glucose (Sigma-Aldrich; Merck KGaA) on days 14 and 28. Plasma insulin levels were determined by using an ultrasensitive mouse insulin ELISA kit (cat. no. 80-INSMS-E01; ALPCO, Salem, NH, USA) and plasma glucagon levels were determined by using a glucagon ELISA kit (cat. no. 81518; Crystal Chem Inc., Downers Grove, IL, USA).

Immunoblotting and cyclin D kinase (Cdk)4 kinase assay. After sacrificing the mice with 150 mg/kg pentobarbital, the remaining pancreas was perfused with collagenase P (Roche, Indianapolis, IN, USA), and the islets were then isolated as previously described (23). The islet tissues were lysed and total protein was quantified as previously described (24), followed by loading onto a NuPage Novex 10% Bis-Tris gel (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for electrophoresis. After electrophoresis, the proteins were transferred onto polyvinylidene fluoride membranes (Pall Corp., Port Washington, NY, USA). The membranes were blocked and subsequently incubated with primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h. Chemiluminescence detection was performed with Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA) and measured directly with a BioSpectrum Imaging System (UVP, Upland, CA, USA). The following primary antibodies were used: Rabbit polyclonal anti-caspase-3 (cat. no. sc-7148; 1:1,000 dilution), rabbit polyclonal anti-B-cell lymphoma 2-associated X protein (Bax; cat. no. sc-493; 1:500 dilution), mouse monoclonal anti-BH3 domain interacting death agonist (Bid; cat. no. sc-135847; 1:1,000 dilution), rabbit polyclonal anti-poly(ADP ribose) polymerase (PARP; cat. no. sc-7150; 1:1,000 dilution), mouse monoclonal anti-cyclin D1 (cat. no. sc-450; 1:1,000 dilution), rabbit polyclonal anti-cyclin D2 (cat. no. sc-450; 1:1,000 dilution), rabbit polyclonal anti-cyclin D3 (cat. no. sc-182; 1:1,000 dilution), rabbit polyclonal anti-cdk4 (cat. no. sc-260; 1:1,000 dilution), mouse monoclonal anti-phospho-signal transducer and activator of transcription 3 (p-STAT3; cat. no. sc-8059; 1:500 dilution), mouse monoclonal anti-STAT3 (cat. no. sc-8019; 1:1,000 dilution), rabbit polyclonal anti-retinoblastoma (Rb; cat. no. sc-7905; 1:1,000 dilution), rabbit polyclonal anti-insulin (cat. no. sc-7953; 1:1,000 dilution), rabbit polyclonal anti-glucokinase (GCK; cat. no. sc-7908; 1:1,000 dilution), rabbit polyclonal anti-insulin promoter factor 1 (PDX-1; cat. no. sc-25403; 1:1,000 dilution) and mouse monoclonal anti-β-actin (cat. no. sc-47778; 1:5,000

dilution) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); rabbit monoclonal anti-p16^{INK4a} (cat. no. ab108349; 1:500 dilution), rabbit monoclonal anti-p27^{Kip1} (cat. no. ab32034; 1:1,000 dilution), rabbit polyclonal anti-phospho-Rb (anti-phospho-S780, cat. no. ab47763; 1:1,000 dilution) and rabbit polyclonal anti-glucose transporter 2 (GLUT-2; cat. no. ab54460; 1:1,000 dilution) antibodies were from Abcam (Cambridge, MA, USA). Rabbit polyclonal anti-cleaved caspase-3 (cat. no. 9661; 1:1,000 dilution) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The HRP-conjugated goat anti-mouse polyclonal immunoglobulin (Ig)G (cat. no. 115-035-003) and HRP-conjugated goat anti-rabbit polyclonal IgG (cat. no. 111-035-003) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and diluted at 1:5,000. β-actin served as the loading control and its levels were used for normalization.

The Cdk4 kinase assay was performed based on a previously described protocol (25). The amount of ^{32}P-labeled glutathione S-transferase-Rb (cat. no. SRP5124, Sigma-Aldrich; Merck KGaA) was evaluated by autoradiography and quantified by analysis with a PhosphorImager and an ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. For analysis of neurogenin (Ngn)3 mRNA abundance, RNA was extracted from the remnant pancreas, and for analysis of other genes, RNA was extracted from the islets. Total RNA from the islets and the remnant pancreas was isolated using an RNeasy micro kit (Qiagen, Valencia, CA, USA) and reverse transcribed as previously described (26). The primer sequences and probes were as previously described (21,23). The RNA and primer were heated to 72°C and slowly cooled prior to reverse transcription at 42°C for 1 h. When cooled to room temperature, the reaction was diluted to 100 μl with RNase-free water. qPCR was performed with SYBR-Green PCR Master mix in a total reaction volume of 20 μl using the following amplification steps: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 15 sec; and then elongation at 55°C for 30 sec. The expression levels were normalized to those of the internal standard GAPDH. Real-time qPCR was performed on an ABI 7900 system using an Applied Biosystems Power SYBR Green PCR Master Mix kit (Thermo Fisher Scientific, Inc.). The copy number was calculated using the $2^{-\Delta\Delta Cq}$ method. The Cq value for GAPDH was used to normalize the samples (27).

Histological staining. Pancreas tissues were fixed in 10% formalin (cat. no. HT-5011; Sigma-Aldrich, Merck KGaA) for 24 h at room temperature, dehydrated, embedded in paraffin (cat. no. A601888; Sangon Biological, Shanghai, China) and cut into 5 μm sections. For immunohistochemical staining, rat monoclonal BrdU antibody (1:100 dilution, cat. no. ab6326; Abcam), rabbit polyclonal Ki67 antibody (1:100 dilution, cat. no. PA1-21520; Thermo Fisher Scientific, Inc.), rabbit polyclonal proliferating cell nuclear antigen (PCNA) antibody (1:100 dilution, cat. no. ab18197; Abcam), and guinea pig polyclonal insulin antibody (1:100 dilution, cat. no. A0564; Dako, Glostrup, Denmark) were used according to a previously described protocol (25). The primary antibodies were detected with corresponding secondary antibodies (1:5,000 dilution;

Jackson Immunoresearch Laboratories Inc.), including horseradish peroxidase (HRP)-conjugated goat anti-rat IgG polyclonal antibody (cat. no. 112-035-003), HRP-conjugated goat anti-rabbit polyclonal IgG (cat. no. 111-035-003), Alkaline Phosphatase (AP)-conjugated goat anti-guinea pig IgG polyclonal antibody (cat. no. 106-055-003), FITC-conjugated goat anti-guinea pig IgG polyclonal antibody (cat. no. 106-095-003), Alex Fluor 647-conjugated goat anti-guinea pig IgG polyclonal antibody (cat. no. 106-005-603), Cy5-conjugated goat anti-rabbit IgG polyclonal antibody (cat. no. 111-175-144). The secondary antibodies were incubated with the section for 2 h at room temperature. For terminal deoxynucleotidyl transferase deoxyuridinetriphosphate nick end labeling (TUNEL) staining, a Promega Fluorometric DeadEnd kit (Promega Corp., Madison, WI, USA) was used following the manufacturer's protocols.

Images of the islets were captured with a Nikon 80i microscope (Nikon Corp., Tokyo, Japan). All of the microscopy imaging and quantification procedures were performed by two technicians who were blinded to the experimental conditions of each sample. The numbers of β-cells, BrdU(+) β-cells, Ki67(+) β-cells, PCNA(+) β-cells and TUNEL(+) β-cells were manually counted and checked with Image-Pro Plus 6.3 software (Media Cybernetics, Silver Spring, MD, USA). At least 10 islets containing at least 1,000 β-cells were counted per mouse, and at least 10 consecutive sections from eight mice per group were stained. The 1st and 10th section were selected at a 50-μm distance. Islet diameters were determined with an intraocular calibrated grid. Islet size was also measured in images of insulin-stained islets converted to gray scale at a magnification of x400.

Analysis of relative β-cell volume was performed via point counting morphometry using a 56-point grid. An average of 10,000 points/mouse were counted. The islet β-cell mass was calculated by multiplying the relative β-cell volume by the total weight of the remnant pancreas.

Islet isolation and primary culture of islet β-cells. Male adult (age, 3 months, 22-24 g, n=20) and aged (age, 12 months, 26-28 g, n=90) mice were anesthetized and sacrificed by i.p. injection of 150 mg/kg pentobarbital. The pancreas was subsequently perfused with 1 ml collagenase P (1.0 mg/ml) and digested at 37°C for 15 min. Isolated cells were then centrifuged at 250 x g for 2 min at 4°C. The supernatant was discarded and the cells were re-suspended in 10 ml Hank's balanced salt solution. The intact re-suspended islets were handpicked using a pipette under a dissection scope, and primary β-cell cultures were prepared from the handpicked islets. Subsequently, 400-500 islets were incubated in 1 ml 1X accutase solution (cat. no. A6964; Sigma-Aldrich; Merck KGaA) for 15 min at 37°C. The digestion was stopped by adding cell culture medium, and the cells were collected by centrifugation at 300 x g for 5 min at 4°C. Subsequently, the islet cells were cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Inc.), 50 μM β-mercaptoethanol (Sigma-Aldrich; Merck KGaA), 1 mM sodium pyruvate (Sigma-Aldrich; Merck KGaA), 2 mM L-glutamine (Sigma-Aldrich; Merck KGaA) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

(Sigma-Aldrich; Merck KGaA) at 37˚C in a humidified atmosphere containing 5% CO_2.

p16^{INK4a} overexpression and knockdown. The PCR product of the p16^{INK4a} gene was cloned into pcDNA3 plasmids (Addgene, Cambridge, MA, USA) under the control of a cytomegalovirus promoter, and the plasmids were transfected into the primary cultured islet cells in the presence of Lipofectamine LTX (Thermo Fisher Scientific, Inc.). Small interfering RNA (siRNA) specific for p16^{INK4a} pool (cat. no. 12578) and non-targeting siRNA controls were obtained from GE Dharmacon (Lafayette, CO, USA). Transfection with the siRNAs was performed using Dharmafect (GE Dharmacon).

In vitro proliferation assay. Islet cells at the logarithmic growth phase were seeded in a 24-well plate ($2x10^5$ cells per well) and incubated at 37˚C for 24 h. Mangiferin was then added and the cells were incubated for another 24 h. Subsequently, 10 μl of a 5 mg/ml solution of MTT was added to each well, followed by incubation at 37˚C for 4 h. Subsequently, the medium was removed and the plates were thoroughly agitated for 1 h. Finally, termination buffer was added to each well, and the absorbance at 570 nm was measured with a spectrophotometer (Model 3550 Microplate Reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The proliferation rates were calculated from the optical density (OD) according to the following formula: Cell proliferation (%) = [OD 570 nm (drug)/OD 570 nm (control)] x 100%.

Statistical analysis. Values are expressed as the mean ± standard error of the mean from at least three independent experiments. Statistical significance between multiple groups was determined by analysis of variance followed by a Bonferroni post hoc test, and between 2 groups using Student's t-test using GraphPad Prism (version 5.0 for windows) statistical software package (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference between groups.

Results

Mangiferin promotes homeostasis in aged mice. According to a previous study by our group, treatment with mangiferin (90 mg/kg) maintained glucose homeostasis in adult mice by islet regeneration induced by mangiferin (21). Therefore, the present study investigated the regulatory role of mangiferin in aged mice. As presented in Fig. 1A, compared with the mice in the sham group, the PPx control mice exhibited significantly increased blood glucose levels. In turn, mangiferin treatment markedly reduced the blood glucose levels in PPx mice. Although the mice that received mangiferin treatment did not maintain normal blood glucose to the same extent as the sham-operated mice, a time-dependent decrease in blood glucose levels was observed from day 3 to day 10. Subsequently, the body weight of the mice in each group was assessed, revealing that the body weight of the mangiferin-treated mice were higher when compared with that of the PPx control mice (Fig. 1B). Further analysis by IVGTT clearly substantiated the hypothesis that islet regeneration was induced by mangiferin. According to the IVGTT data on days 14 (Fig. 1C) and

28 (Fig. 1D), mangiferin partially recovered the impaired glucose tolerance of the PPx mice on day 14 and maintained the partially recovered glucose tolerance on day 28. Furthermore, the serum insulin levels (Fig. 1E) and glucagon levels (Fig. 1F) were measured, and it was observed that mangiferin-treated aged mice had elevated levels of insulin secretion and had no effect on glucagon secretion compared with those in the PPx group. These experiments illustrated that mangiferin treatment maintained homeostasis in aged mice after PPx.

Mangiferin induces islet β-cell proliferation and hyperplasia in aged mice. To further explore the mechanisms via which mangiferin promotes homeostasis, the remaining pancreas tissues were stained with specific antibodies for proliferating cells, and the proliferation rates were subsequently quantified. Representative immunohistochemical staining images of the islet β-cells and proliferating cells are provided in Fig. 2A. It was evident that the islets of mangiferin-treated mice had more proliferating cells (indicated by arrows) on day 14 than those of the PPx mice. Of note, with prolonged treatment with mangiferin for 28 days, an increased quantity of BrdU-labeled proliferating cells was present, which provided evidence that mangiferin induces islet regeneration in a time-dependent manner. To further validate this hypothesis, cell proliferation was assessed with two endogenous markers, PCNA and Ki67. According to the levels of proliferating cells indicated by BrdU-labeling (Fig. 2B), Ki67 labeling (Fig. 2C) and PCNA labeling (Fig. 2D), mangiferin induced robust cell proliferation in the islets of aged mice. Although it has been reported that pancreatic duct epithelial cells may be considered as progenitor cells that contribute to neogenesis, no proliferation of the duct cells was observed (data not shown). Therefore, it appears that mangiferin induced islet regeneration in aged mice mainly via inducing the proliferation of β-cells. However, whether those proliferated cells resulted in islet hyperplasia remains elusive. To investigate this, the remaining pancreas was subsequently weighed (Fig. 2E) to calculate the relative β-cell volume (Fig. 2F) and β-cell mass (Fig. 2G). It was revealed that the relative β-cell volume and β-cell mass of mangiferin-treated mice were significantly increased relative to those in PPx control mice, which is consistent with the results on β-cell proliferation.

Mangiferin inhibits β-cell apoptosis in aged mice. Previous clinical studies on T2DM patients clearly demonstrated that their β-cell mass was decreased due to increased β-cell apoptosis (3,28). Therefore, therapeutic approaches comprising the inhibition of β-cell apoptosis may not only palliate hyperglycemia, but reverse and prevent the progression of the disease, and thus, the present study further focused on the effect of mangiferin on β-cell apoptosis. A previous study by our group illustrated that mangiferin inhibited β-cell apoptosis in young mice (21). In the present study, the remnant pancreas was stained using an *in situ* TUNEL apoptosis detection kit. Of note, the islets of PPx mice lacking mangiferin treatment exhibited more apoptotic cells (green cells indicated by arrows; Fig. 3A) than those of mice treated with mangiferin. Consistent with these qualitative observations, calculation of the percentages of apoptotic cells (TUNEL-positive islet β-cell percentage) revealed that mangiferin treatment markedly
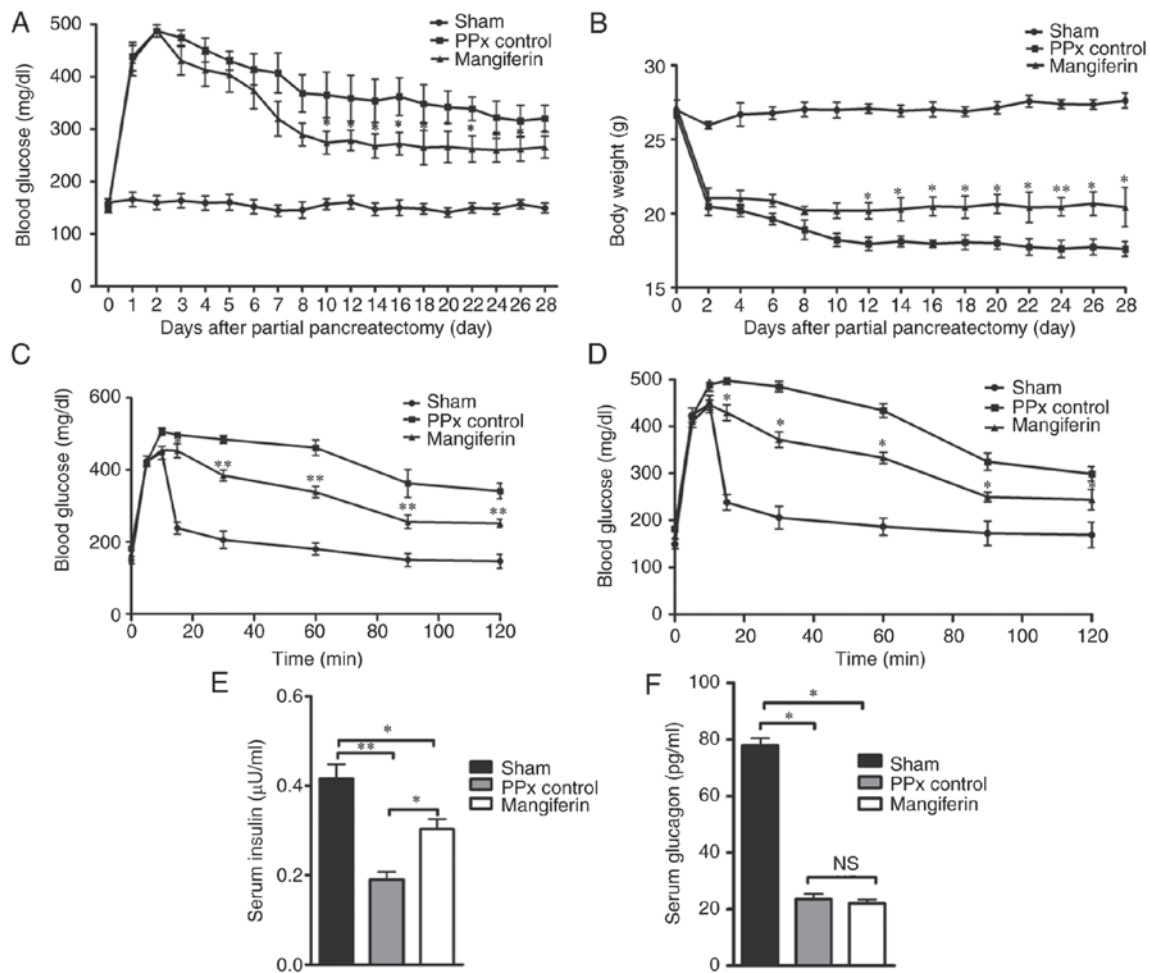
Figure 1. Mangiferin (90 mg/kg) induces metabolic changes in aged mice. (A) Comparison of fasting blood glucose levels (P<0.05 by ANOVA with Bonferroni post hoc test between all groups at the same time-point). (B) Comparison of body weights (P<0.05 and P<0.01 by ANOVA with Bonferroni post hoc test between all groups at the same time-point). (C) IVGTT on day 14 (P<0.05 and P<0.01 by ANOVA with Bonferroni post hoc test between all groups at the same time-point). (D) IVGTT on day 28 (P<0.05 by ANOVA with Bonferroni post hoc test between all groups at the same time-point). (E) Comparison of serum insulin levels. (F) Comparison of serum glucagon levels. Values are expressed as the mean ± standard error of the mean (n=10 in each group). *P<0.05; **P<0.01 vs. PPx control group at the same time-point. NS, not significant; IVGTT, intravenous glucose tolerance test; PPx, partial pancreatectomy; ANOVA, analysis of variance.

inhibited apoptosis in the aged mice (Fig. 3B). To further validate that mangiferin inhibited cell apoptosis in the islets of the aged mice, key proteins in the caspase pathway were analyzed (Fig. 3C). Activation of caspases has a central role in apoptosis, and caspase-3 serves as the convergence point of different apoptotic signaling pathways (29). As presented in Fig. 3C, caspase-3 was de-activated upon mangiferin treatment with increased pro-caspase-3. Bid induces a conformational change of Bax (30), and Bax induces the release of cytochrome c from the mitochondria during apoptosis (31). The present results suggested that mangiferin-treated cells had lower expression levels of Bax and Bid compared with those in the control, suggesting that more apoptotic events were inhibited by mangiferin. Furthermore, decreased cleavage of PARP was identified in the mangiferin-treated group. Therefore, changes in the apoptotic tendency of β-cells, along with enhanced proliferation capability, likely contributed to the increased levels of insulin secretion *in vivo*.

Mangiferin inhibits islet β-cell senescence in aged mice. A study by Krishnamurthy *et al* (9) indicated that p16[INK4a]

restricted proliferation and regeneration in the islets of aged mice, while mice lacking p16[INK4a] exhibited enhanced islet proliferation rates. To assess whether mangiferin treatment prevents β-cell senescence, *in situ* fluorescence staining of p16[INK4a] was performed. Representative images of p16[INK4a] immunofluorescent staining are presented in Fig. 4A. Quantification of the staining clearly indicated that >50% of β-cells were labeled with p16[INK4a] after PPx and also in the sham operated group, indicating that PPx did not induce marked senescence compared with the sham group. In contrast to the PPx controls, the islets of mangiferin-treated mice exhibited positive staining for active p16[INK4a] at a rate of ~35%, indicating that mangiferin markedly inhibits β-cell senescence (Fig. 4B). It was therefore concluded that mangiferin treatment inhibits β-cell senescence in aged mice.

Mangiferin regulates cell cycle proteins. A plethora of literature suggests that cyclin D/Cdk4 complexes have a critical role in the cell cycle regeneration of β-cells (32-34), and a previous study by our group indicated that mangiferin induced β-cell regeneration via the regulation of cell cycle complexes (21).
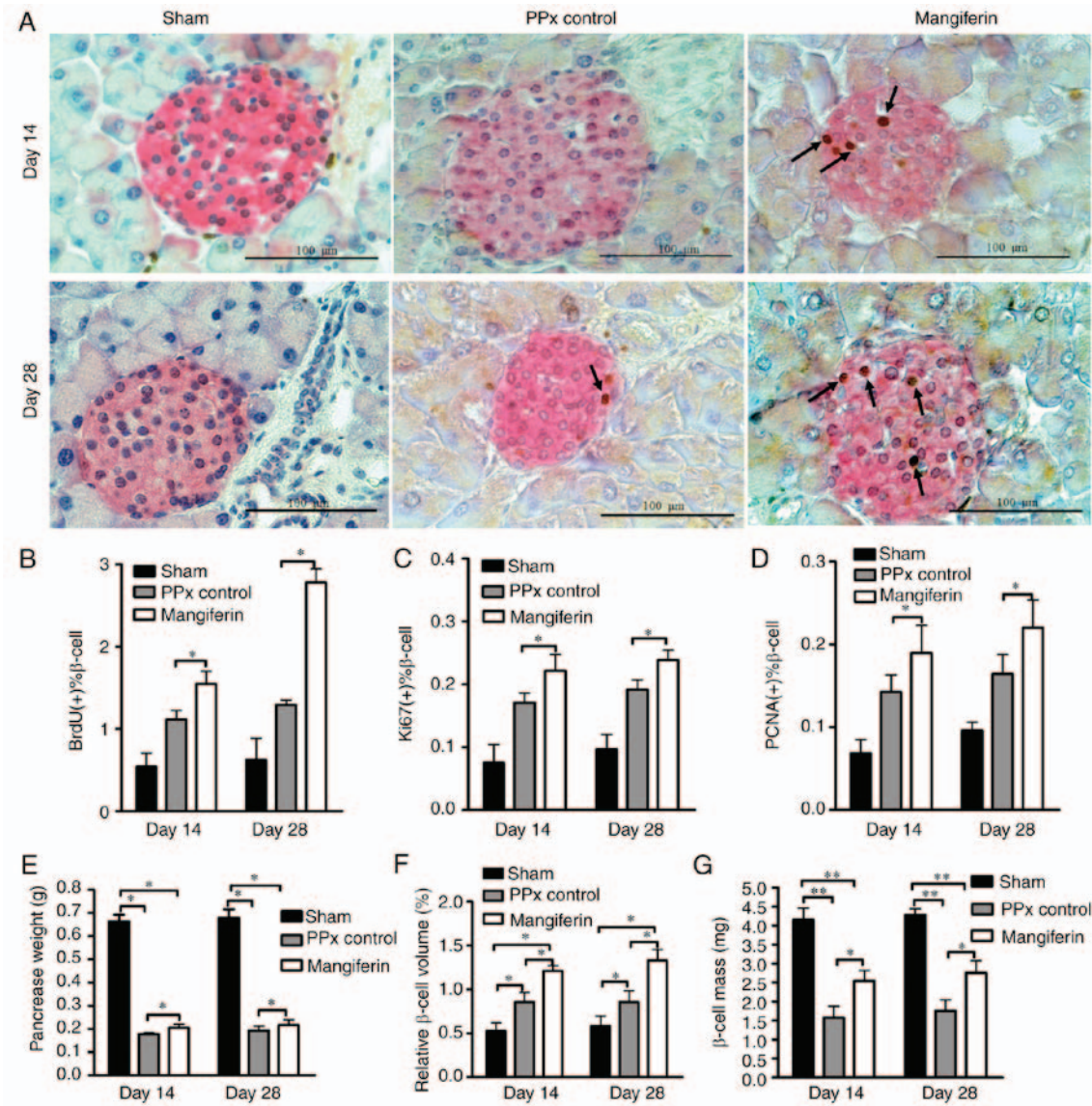
Figure 2. Mangiferin regulates islet regeneration. (A) Representative immunohistochemical images of insulin-positive cells (red) and BrdU-labeled cells (brown) in the different groups. Arrows indicate the BrdU-labeled cells (scale bar, 100 μm). (B-D) Percentages of (B) BrdU-positive, (C) Ki67-positive and (D) PCNA-positive among insulin-positive β-cells in the different groups were calculated. At least 10 islets containing at least 1,000 β-cells were counted per mouse (n=6). (E) Comparison of remnant pancreas weights. (F) Relative β-cell volume determined by point counting. (G) β-cell mass calculated by multiplying the relative β-cell volume by the total weight of remnant pancreas. Two or three slides (200 μm apart) from the broadest pancreatic sections were subjected to β-cell mass measurement (n=8 for each group). Values are expressed as the mean ± standard error of the mean. *P<0.05 and **P<0.01. BrdU, bromodeoxyuridine; PPx, partial pancreatectomy; PCNA, proliferating cell nuclear antigen.

Therefore, to investigate whether cyclin D-Cdk4 complexes may be regulated by mangiferin in aged mice, the expression of cyclin D1, -D2 and -D3, as well as Cdk4, p16^{INK4a} and p27^{Kip1} was assessed at the protein (Fig. 5A) and mRNA (Fig. 5B) level. As expected, mangiferin greatly increased the transcription and translation of cyclin D1 and -D2, as well as Cdk4. Furthermore, the expression levels of p16^{INK4a} and p27^{Kip1} were significantly reduced by mangiferin in the aged mice. Quantification of the western blot results indicated that the expression and phosphorylation levels of STAT3 were markedly elevated in the mangiferin-treated mice relative to those in the untreated PPx controls, and the total STAT3 protein levels of PPx control was also significantly higher than those of the sham group. Notably, PPx could induce the increased expression of STAT3, and mangiferin

treatment could induce the phosphorylation and activation of STAT3 (Fig. 5C and D).

To confirm the effects of mangiferin on β-cell proliferation via the regulation of p16^{INK4a}, islet cells of adult and aged mice were isolated and cultured in the presence of mangiferin or vehicle (dimethyl sulfoxide) (Fig. 6A); furthermore, the p16^{INK4a} gene was knocked down in the isolated islet cells of the aged mice (Fig. 6B) and p16^{INK4a} was overexpressed in the isolated islet cells of the adult mice (Fig. 6C). Of note, mangiferin induced the proliferation of the islet cells from the adult and the aged mice (Fig. 6D). Furthermore, p16^{INK4a} silencing in islet cells from aged mice led to elevated proliferation rates, regardless of whether the islets were treated with or without mangiferin (Fig. 6E). In addition, mangiferin treatment of the isolated islet cells of the adult mice with over-
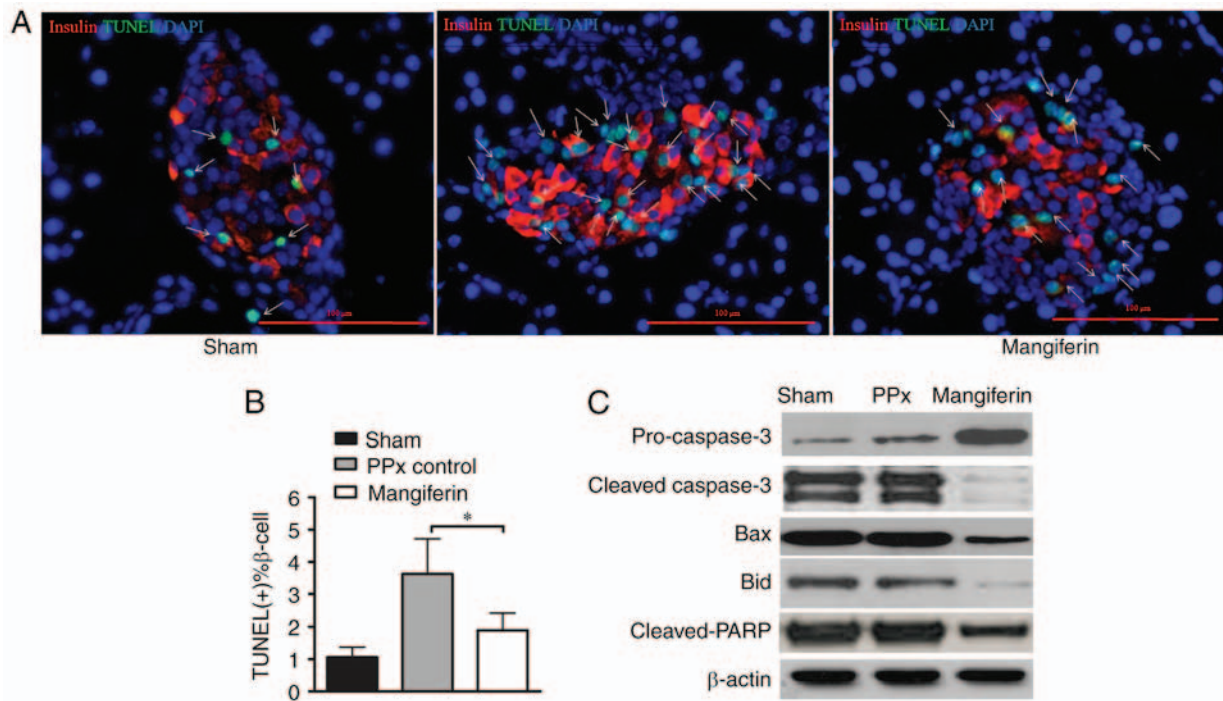
Figure 3. Mangiferin inhibits apoptosis. (A) Representative immunofluorescence images of TUNEL-positive cells (green) in the different groups. White arrows indicate the TUNEL-positive cells (scale bar, 100 μm). (B) The percentage of TUNEL-positive cells in the different groups was calculated. Values are expressed as the mean ± standard error of the mean. [*]P<0.05. (C) Analysis of apoptosis-associated proteins by western blot analysis. TUNEL, terminal deoxynucleotidyl transferase deoxyuridinetriphosphate nick end labeling; PPx, partial pancreatectomy; PARP, poly(ADP ribose) polymerase; Bax, B-cell lymphoma 2-associated X protein; Bid, BH3 interacting-domain death agonist.
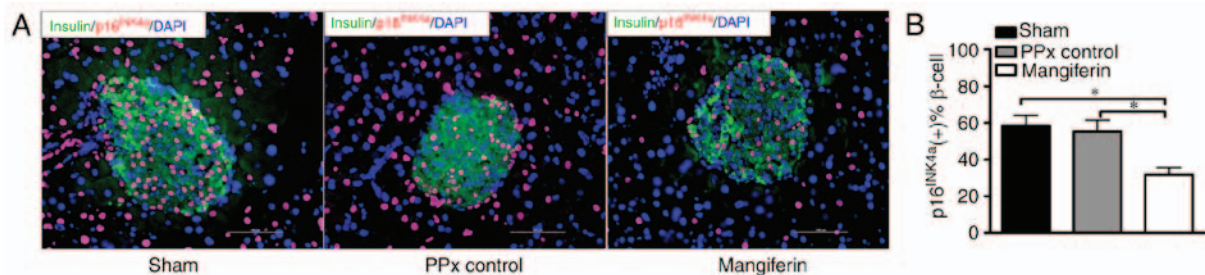

Figure 4. Mangiferin inhibits senescence. (A) Representative immunofluorescence images of p16[INK4a] staining (magenta) in the islets (green) for the different groups (scale bar, 100 μm). (B) Percentages of p16[INK4a]-positive cells in the different groups were determined. Values are expressed as the mean ± standard error of the mean. [*]P<0.05. PPx, partial pancreatectomy.

expression of p16[INK4a] significantly increased the proliferation rate (Fig. 6F).

Furthermore, to address whether the activity of Cdk4 may be regulated by mangiferin, a Cdk4 kinase assay was performed. Previous studies demonstrated that Rb is an important substrate of the cyclin D1/Cdk4 complex, and that its activation is closely associated with senescence (35). In the Cdk4 assay, increased radioactive labeling of the substrate is considered to indicate greater enzymatic activity of Cdk4. As presented in Fig. 7A and B, the *in vitro* kinase assay indicated lower levels of labeled phosphorylated Rb in the mangiferin-treated group when compared with that in the control group. In addition, *in vivo* analysis of Rb phosphorylation on serine 780 revealed that mangiferin treatment greatly reduced the phosphorylation of Rb, suggesting that the enhanced Cdk4 activity resulted in decreased Rb activity (Fig. 7C). Collectively, these results indicate that

mangiferin may induce β-cell proliferation through the regulation of cell cycle proteins.

Mangiferin regulates insulin secretion-associated genes. To elucidate the mechanism underlying mangiferin-induced insulin secretion in aged mice, it was assessed whether mangiferin regulates β-cell-specific genes, including insulin, GCK, GLUT-2 and PDX-1, as critical genes for β-cell function. As expected, a significant increase in the protein expression levels of insulin, GLUT-2, GCK and PDX-1 was detected in the islets of the mangiferin-treated mice (Fig. 8A). Accordingly, the mRNA levels of these genes were also elevated (Fig. 8B). Notably, mangiferin treatment led to upregulation of the expression of PDX-1, the upstream gene of insulin. However, no changes in the transcription or translation levels of Ngn3 were observed in mangiferin-treated mice when compared with those in the controls (Fig. 8), suggesting
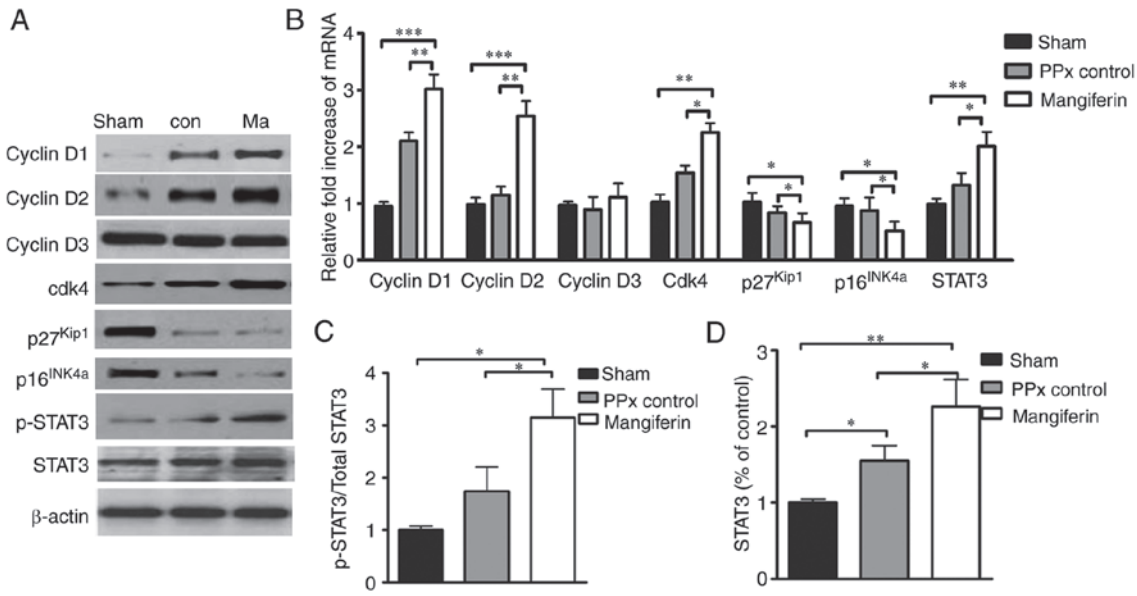
Figure 5. Mangiferin regulates cell cycle-regulatory proteins. (A) Representative western blots of cyclin D1, D2 and D3, as well as Cdk4, p16INK4a, p27Kip1, STAT3 and phospho-STAT3. (B) Reverse transcription-quantitative polymerase chain reaction analysis of cell cycle regulators. (C and D) Quantified levels of (C) phospho-STAT3 vs. total STAT3 ratio and (D) total STAT3 determined by grey-value scan of the blots. Values are expressed as the mean ± standard error of the mean of at least three independent experiments. *P<0.05, **P<0.01 and ***P<0.001. STAT3, signal transducer and activator of transcription; Cdk, cyclin D kinase; PPx, partial pancreatectomy; con, control; Ma, mangiferin-treated group.
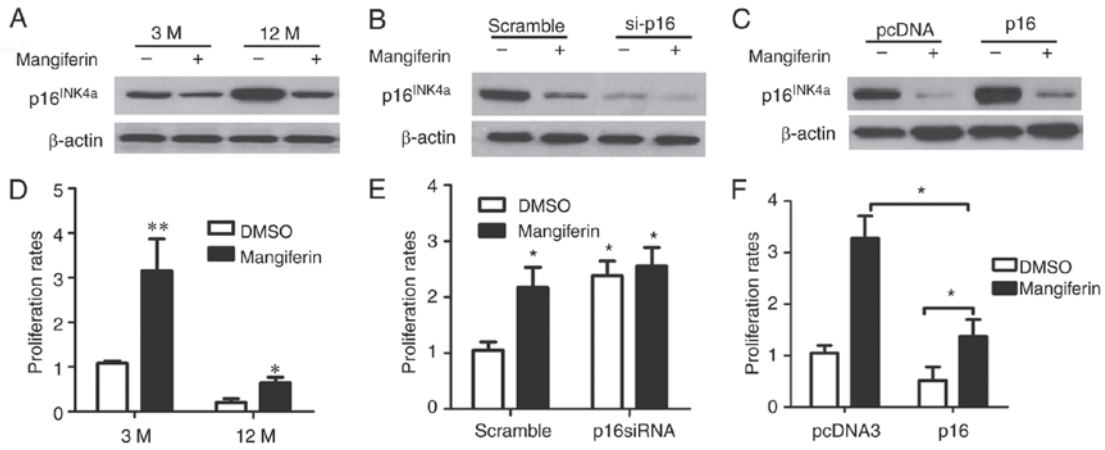

Figure 6. Mangiferin mediates the inhibition of p16INK4a. Effect of mangiferin treatment on the expression levels of p16INK4a in islet cells from (A) young and aged mice, (B) from aged mice subjected to siRNA-mediated knockdown of p16INK4a and (C) from young mice subjected to overexpression of p16INK4a. (D-F) Proliferation rates of islet cells from (D) young and aged mice, (E) aged mice with si-p16 or scrambled siRNA transfection and (F) young mice with p16 overexpression or empty vector transfection in the presence or absence of mangiferin. Values are expressed as the mean ± standard error of the mean of at least three independent experiments. *P<0.05 and **P<0.01. 3M, 3 months; si-p16, siRNA targeting p16; siRNA, small interfering RNA; DMSO, dimethylsulfoxide.
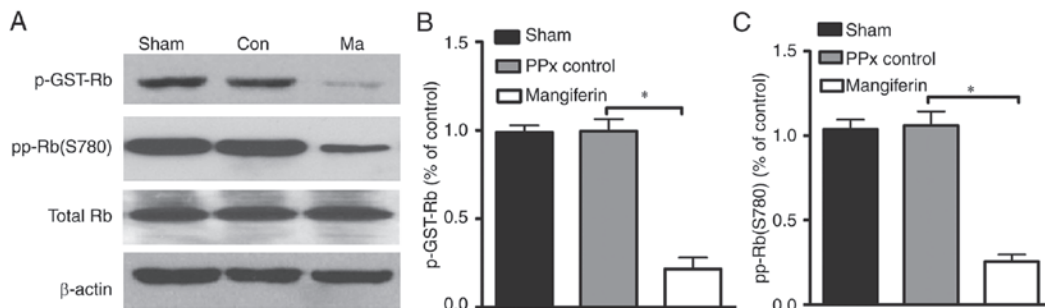

Figure 7. Mangiferin increases Cdk4 activity. In this assay, the substrate of Cdk4, the labeled p-GST-Rb, was assessed. (A) *In vitro* Cdk4 kinase activity and *in vivo* Rb phosphorylation at serine 780 was assessed. (B) Quantification of the *in vitro* (B) phosphorylated GST-Rb and (C) phosphorylated Rb at serine 780 vs. total Rb was determined by grey value scan. Values are expressed as the mean ± standard error of the mean of at least three independent experiments. *P<0.05. Cdk, cyclin D kinase; con, control; Ma, mangiferin-treated group; p-Rb, phosphorylated retinoblastoma protein; PPx, partial pancreatectomy; GST, glutathione S-transferase.
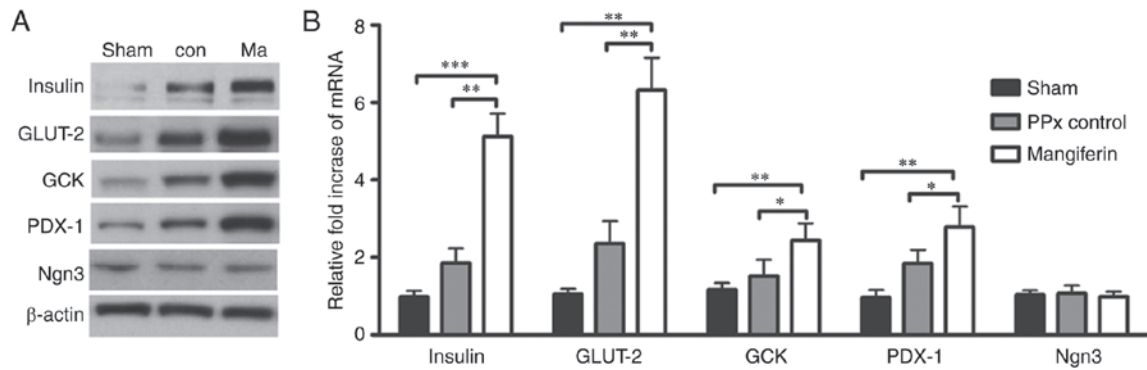
Figure 8. Mangiferin upregulates β-cell-specific genes. (A) Insulin, GLUT-2, GCK and PDX-1 expression levels were assessed by western blot analysis. (B) Insulin, GLUT-2, GCK and PDX-1 mRNA levels were assessed by reverse transcription-quantitative polymerase chain reaction analysis. Values are expressed as the mean ± standard error of the mean. *P<0.05, **P<0.01 and ***P<0.001. PDX-1, insulin promoter factor 1; GLUT-2, glucose transporter 2; GCK, glucokinase.

that no neogenesis occurred in the aged mice. Therefore, it was concluded that mangiferin treatment markedly contributes to insulin secretion through the regulation of insulin-associated genes.

Discussion

To the best of our knowledge, the present study was the first to report that islet regeneration was present in mangiferin-treated aged mice. Furthermore, along with the increased proliferation of islet β-cells, comparatively reduced blood glucose levels, enhanced glucose tolerance and slightly but gradually increased body weight were identified in the mangiferin-treated aged mice. The islet cells were labelled with the proliferation markers BrdU, Ki67 and PCNA, and staining was quantified to evaluate the rate of islet regeneration. All three different markers were increased in the mangiferin-treated group, indicating that the drug induced the proliferation of islet β-cells. Furthermore, as negative regulators of the cell cycle have a critical and fundamental role in the re-entry of islet β-cells into the cell cycle, cell cycle-associated genes and proteins were analyzed in the present study, and the results indicated that mangiferin induced the activation of cyclin D/Cdk4 complexes and inhibited the expression of p16[Ink4a] in aged mice following PPx. In addition, genes associated with β-cell function were significantly upregulated by mangiferin. Taken together, these results clearly indicated that mangiferin treatment induced islet regeneration and maintained homeostasis in the aged mice with PPx by modulating cell cycle regulators and insulin secretion-associated genes.

Previous clinical studies demonstrated that, due to the increased rate of β-cell apoptosis, the β-cell mass was decreased in T2DM patients (3). Thus, therapeutic approaches that inhibit apoptosis and increase the β-cell mass may be effective novel strategies for the treatment of T2DM, particularly for patients of advanced age. In the present study, a gradual control of homeostasis was observed in the mice treated with mangiferin, suggesting that the mice still possessed islet regeneration capacity, which led to the gradual recovery of blood glucose levels. By genetic lineage tracing, Dor et al (36) determined that terminally differentiated β-cells retained a significant proliferative capacity in vivo. Their

study also suggested that pre-existing β-cells, rather than pluripotent stem cells, were the major source of new β-cells during adult life and after PPx in mice (36). Teta et al (37) also demonstrated that the growth and regeneration of adult β-cells did not involve any specialized progenitors. Thus, the present study evaluated the proliferation rates of islet β-cells in the different groups of mice, and the results indicated that the mice treated with mangiferin possessed a higher islet regeneration capability. As mentioned above, adaptive β-cell proliferation is severely restricted with advanced age; therefore, β-cell proliferation was observed and quantified using three different proliferation markers, BrdU, Ki67 and PCNA according to previously reported procedures (38,39). By immunohistochemical staining for BrdU, Ki67 and PCNA, the proliferated cells were labeled to visualize islet regeneration that was stimulated by mangiferin in the aged mice after PPx. It is widely acknowledged that cell proliferation depends on various cell cycle check point proteins (40). To elucidate the mechanism of mangiferin-induced regeneration of islet cells, it was assessed whether mangiferin regulates cell cycle proteins. From the results, it was obvious that various cell cycle proteins were regulated by mangiferin, and as the proliferation of islet cells is controlled by cell cycle proteins, it was suggested that mangiferin treatment induced islet regeneration in aged mice by modulating cell cycle regulators and also insulin secretion-associated genes. The results of the labeling with the three different markers of cell proliferation clearly indicated that mangiferin treatment was able to ameliorate the diabetic condition by inducing islet β-cell proliferation. This also implied that, for human patients of advanced age who have little regenerative capacity with regard to β-cell mass, mangiferin may be a promising therapeutic. As a decrease in β-cell mass is a primary pathogenic factor in T2DM, the present study also assessed the β-cell mass in the mice by determining the remnant pancreas weight, relative β-cell volume and relative β-cell mass. As expected, the β-cell mass of the mangiferin-treated mice exhibited a comparative increase relative to that in the untreated PPx control group, indicating that mangiferin induced hyperplasia of the pancreas. In studies on T2DM patients, Butler et al (3, 41) reported that a major defect leading to the decrease in β-cell mass was an increased rate of β-cell apoptosis. Therefore, therapeutic approaches designed

to inhibit apoptosis may ameliorate of halt the progression of T2DM, and the present results regarding apoptosis strongly suggest that mangiferin inhibits β-cell apoptosis.

The present study addressed the important issue of whether islets in elderly individuals still possess proliferation capacity using a mouse model. In previous studies on rodents, the plasticity of the β-cell mass was correlated with β-cell proliferation (42). The results of the present study clearly indicated the proliferation potential of islets in aged mice. However, the mechanisms underlying the proliferation of β-cells during islet regeneration require further investigation. Studies by Krishnamurthy *et al* (9,10) suggested that β-cell mass expansion may be regulated by p16^{Ink4a}, as a negative regulator of cyclin D/Cdk4. In addition, in transgenic mice overexpressing p16^{Ink4a}, reduced islet cell proliferation and a reduction in the regenerative capacity of islets was observed following STZ-induced degeneration of the islet β-cell mass (8). Senescence and apoptosis rely on telomere shortening and p16^{INK4a} activation (43). Based on the results of a previous study by our group, indicating that mangiferin regulates cell cycle proteins (21), the hypothesis that mangiferin administration induces islet regeneration in aged mice through the regulation of cell cycle proteins was proposed. In the present study, p16^{INK4a}, a marker of aging, was demonstrated to be downregulated in mangiferin-treated mice, suggesting that the anti-aging effect of mangiferin occurred via the regulation of p16^{Ink4a}. Compared with the sham group, PPx did not induce a marked increase of senescence. However, mangiferin could inhibit senescence of aged mice. It has been reported that senescence requires activation of Rb and the expression of their regulators, most prominently p16^{INK4a}. As p16^{INK4a} targets Rb, an *in vivo* phosphorylation assay of Rb was performed, and decreased activation of Rb was identified in the mangiferin-treated mice. These pre-clinical data on mangiferin therapy may aid in elucidating the regulation of β-cell proliferation and β-cell mass in T2DM.

It has been widely acknowledged that apoptosis, necrosis and autophagy are three types of programmed cell death (PCD) (44). Fehsel *et al* (45) demonstrated that islet cells undergo necrosis instead of apoptosis after the injection of STZ or nitric oxide (NO). Hoorens *et al* (46) indicated that interleukin-1 also mediated islet necrosis. These effects have been attributed to the induction of NO synthase in β-cells and subsequent generation of toxic NO levels. These studies provided evidence that necrosis occurs in islets. However, an overwhelming amount of studies have indicated that apoptosis is the major pathway for islet cell death, particularly for islets of aged mice (7,8) and p16^{INK4a} has been indicated to induce senescence of islets in aged mice (9). Furthermore, numerous studies reported that hyperglycemia induces apoptosis of islets (8,41,47,48). Based on these previous studies, with regard to PCD of islets, apoptosis may be the predominant type of cell death, particularly for aged islets. In the study by Hoorens *et al* (46), Hoechst 33342 and PI were used to stain the cells, and the double-positive cells were regarded as necrotic cells. A limitation of the present study is that no Hoechst 3342 plus PI staining, sorting of the cells by flow cytometry and quantification of the RIP1 expression levels was performed. However, according to previous studies on aged islets, the major cell death pathway is apoptosis and in addition, the

western blot results of the present study on pro-caspase-3, cleaved caspase-3, Bax, Bid and cleaved PARP indicated the activation of the caspase-dependent apoptotic pathway in aged islets. However, in the present study, mangiferin increased the expression levels of pro-caspase-3 and decreased the cleavage of caspase-3 and PARP. In addition, mangiferin treatment could also inhibit the expression of Bax and Bid. Therefore, the conclusion is drawn that mangiferin inhibits islet cell apoptosis as one of the mechanisms for the decreased blood glucose levels in PPx mice after mangiferin treatment.

A study by Xu *et al* (49) demonstrated that during islet regeneration after injury, Ngn3, a basic helix-loop-helix transcription factor, was activated in duct-associated stem or progenitor cells of islet β-cells. It was therefore suggested that Ngn3 is critical for the development of endocrine cells in the islets and for islet neogenesis. In addition, this previous study demonstrated that β-cell neogenesis was activated when the β-cell mass was reduced as a compensatory mechanism (49). A previous study by our group reported that mangiferin activates Ngn3 in young PPx mice, and subsequently induces neogenesis in pancreatic duct cells/progenitor cells (21). However, in the present study, no proliferating cells were observed in the duct. Of note, no change in the expression levels of Ngn3 was identified in the islets of the mangiferin-treated mice when compared with those in the controls. This indicated that with age, not only the islet regeneration capacity had declined, but that the neogenic ability of islets was also lost.

Taken together, the results of the present study were consistent with the role of mangiferin in modulating β-cell proliferation and stimulating insulin secretion; the mangiferin-treated aged mice exhibited reduced hyperglycemia and glucose intolerance, increased serum insulin levels, and an expanded β-cell mass attributed to increased β-cell proliferation and decreased apoptosis. Investigation into the mechanisms of mangiferin-induced islet regeneration revealed that mangiferin modulated cell cycle regulators and insulin secretion-associated genes. However, these experiments provided preliminary data based on aged rodents, and thus, the applicability to humans remains elusive. However, mangiferin may be a promising potential novel therapeutic for the treatment of diabetes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

HW, XH and TL performed the partial pancreatectomy operation, islet isolation and culture and the PCR experiments. YL and GH performed the western blotting and MS analyzed the data. SD and HY revised the manuscript. RT and YW designed the experiment and wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of Sichuan Academy of Medical Science & Sichuan Provincial People's Hospital (Chengdu, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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