Pioglitazone/microRNA-141/FOXA2: A novel axis in pancreatic β-cells proliferation and insulin secretion

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Abstract. MicroRNAs (miRs) are considered to be effective, post-transcriptional regulators in the pathophysiology of type 2 diabetes (T2D) and promising treatment targets. However, the function of miR-141 remains to be elucidated. In the present study, upregulation of miR-141 was demonstrated in diabetic mice and elderly diabetic patients. Using reverse transcriptase-quantitative polymerase chain reaction, luciferase reporter assays and western blotting, forkhead box A2 (FOXA2) was identified as a direct target gene of miR-141. The potential role of miRNA-141 or FOXA2 was evaluated by overexpressing or silencing miR-141 or FOXA2, respectively. The increased expression of miR-141 resulted in impaired glucose-stimulated insulin secretion (GSIS) and INS-1 β cell proliferation. In addition, miR-141 silencing in MIN6 pseudoislets or INS-1 β cells led to reduced T2D-associated damage. Furthermore, the expression of miR-141 may be corrected by treatment with pioglitazone, which is widely used for insulin resistance therapy. The present study also demonstrated the mechanism by which miR-141 regulated GSIS and proliferation through FOXA2. Overexpression of FOXA2 in MIN6 pseudoislets increased the effect of the miR-141 inhibitor on GSIS. FOXA2 effectively reversed the effect of miR-141 overexpression on β cell proliferation. In conclusion, the results of the present study indicate that the pioglitazone/miR-141/FOXA2 axis may represent a promising target mechanism for T2D treatment.

Introduction

MicroRNAs (miRs) are groups of highly conserved, small noncoding RNAs that regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of mRNAs (1) to control mRNA stability or degradation at the post-transcriptional level. MiRs are small, but important modulators that are involved in various physiological and pathological processes (2), such as energy homeostasis (3), lipid metabolism, adipogenesis (4) and diabetes. MiRs have been identified as oncogenes or tumor suppressors in cancer (5). Among them, miR-141 has previously been demonstrated to function as a tumor suppressor in various types of cancer including colorectal (6), pancreatic (7), gastric (8) and head and neck squamous cell carcinoma (9).

Diabetes is a metabolic disease characterized by resistance to insulin action in the liver and other metabolic tissues, and by increased blood glucose levels (10-12). Type 1 diabetes (T1D) and type 2 diabetes (T2D) are the most common forms (13,14). T1D occurs due to lack of pancreatic β cell function and autoimmune β cell destruction induced insulin deficiency (15), whereas T2D begins with insulin resistance and defects in insulin sensitivity, and pancreatic β cell dysfunction (16,17). Therefore, understanding the mechanisms underlying pancreatic β cell function may aid the development of novel therapeutic strategies for T2D. However, whether miR-141 is involved in diabetes remains to be elucidated. Pioglitazone is an agonist of peroxisome proliferator-activated receptor-γ and an antidiabetic agent (18). Pioglitazone is used to improve insulin production and increase insulin sensitivity (19,20). However, whether there are other mechanisms by which pioglitazone suppresses diabetes, remains to be determined.

Materials and methods

Reagents and cell culture. The miR-141 mimic, miR-141 inhibitor and the scrambled negative control were purchased from Guangzhou RiboBio Co., Ltd., (Guangzhou, China). The following miRNA sequences were used: miR-141 mimic, 5-UAACACUGUCUGGUAAAGAUGG-3, miR-141 inhibitor, 5-CCAUCUUUACCAGACAGUGUUA-3 scrambled negative control 5-CAGUACUUUUGUGUAGUAC-3. The antibodies for forkhead box A2 (FOXA2 cat. no. ab108422) and β-actin (cat. no. ab8226) were obtained from Abcam (Cambridge, UK). Pioglitazone was purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Pancreatic INS-1 β-cells (American Type Culture Collection Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) at a 37˚C incubator and 5% CO_2 with 100 U/ml penicillin and streptomycin (Chinese Academy of Medical Sciences,

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Beijing, China). MIN-6 pseudoislets (National Infrastructure of Cell Line Resource, Beijing, China) were cultured by plating $6x10^5$ cells into 100-mm Petri dishes and cultured subsequently for 15 days. Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection according to the manufacturer's protocol. A total of 250 pmol miR-141 mimic, miR-141 inhibitor or the scrambled negative control and 2 μl Lipofectamine® 2000 were used per well with a density of $2.5x10^5$ cells (6-well plates). The short hairpin (sh)FOXA2 was purchased from Sigma-Aldrich; Merck KGaA and transfected into the cells using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

Patients. The present study was approved by the Research Ethics Committee of Beijing Tian Tan Hospital, (Beijing, China; reference no. Eth-2014-057). A total of 50 participants (25 male and 25 female) with uncomplicated T2D, aged 60-65 years, were deemed eligible and were enrolled between January 2014 and June 2016. Patients were given written information regarding the objectives of the present study and written informed consent was obtained from all patients. All participants were at a stable weight and did not regularly engage in vigorous physical exercise. A total of 3 ml peripheral venous blood samples for all participants were collected using EDTA-coated tubes. A total of 1 ml serum samples were taken in the morning following a period of overnight fasting. The glucose oxidase method was used for the determination of blood sugar concentration.

Animals. The animal experiment was approved by the Institutional Animal Care and Use Committee of Beijing Tian Tan Hospital. A total of 40 C57BLKS/J db/db mice or 40 C57BL/6 mice (8 weeks old; 20-25 g; male) were purchased from The Jackson Laboratory (Bar Harbor, ME USA). All animals were housed on a 12-h light-dark cycle, at 21±1˚C with a humidity of 55-65% and free access to food and water. C57BL/6J mice were fed a standard feed (D01060501; 10% kcal from fat) or a high-fat diet (HFD; D01060502, 58% kcal from fat; Research Diets, animal center of Beijing Tian Tan Hospital) for 12 weeks. Pioglitazone (10 mg/kg/day) was mixed with the food and orally fed to db/db mice or HFD mice for 10 days. The pancreatic islets were isolated following 12 weeks of the diet and the RNA was isolated. Blood was collected to quantify the glucose levels once a week during treatment.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For miR extraction, $2x10^5$ cells or 2 μg tissues samples were lysed in RNAiso for miRNA (miRCURY™ RNA Isolation kit, Takara Biotechnology Co., Ltd., Dalian, China). Next, 2 μg total RNA or miRs in each group were used for RT to obtain the first strand cDNA by using the PrimeScript Reverse Transcriptase (Takara Biotechnology Co., Ltd.). The following temperature protocol was used for reverse transcription: 25˚C for 10 min, 42˚C for 30 min and 85˚C for 3 min.

The reactions were performed on an ABI Prism Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR-Green (LightCycler® 480 SYBR-Green I Master, Product No. 04707516001; Roche Diagnostics GmbH, Mannheim, German). The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ (21) and the expression of endogenous GAPDH mRNA or U6 was used to quantify the amplification. The experiments were repeated at least 3 times, independently. The primers used were as follows: GAPDH forward (F), 5'-GAGAAGTATGACAACAGCCTC-3' and reverse (R), 5'-ATGGACTGTGGTCATGAGTC-3'; FOXA2 F, 5'-CACCATCAGCCCCACAAAAT-3' and R, 5'-GGGTAG TGCATGACCTGTTCG-3'; U6 F, 5'-CTCGCTTCGGCAGCA CA-3' and R, 5'-AACGCTTCACGAATTTGCGT-3'; -miR-141 F, 5'-CGCTAACACTGTCTGGTAAAG-3' and R, 5'-GTG CAGGGTCCGAGGT-3'. Cycling parameters were as follows: 95˚C for 5 min; 40 cycles of 95˚C for 15 sec and 60˚C for 1 min.

Western blot analysis. Total protein was extracted from the cells and lysed in 0.5 ml cell lysis buffer (Total Protein Extraction kit; ProMab Biotechnologies, Inc., Richmond, CA, USA) at 4˚C for 45 min. Following centrifugation at 13,000 x g for 15 min at 4˚C, the concentration of the supernatant was determined using a bicinchoninic acid protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). An equal amount of protein (25 μg/lane) was resolved on 10% SDS-PAGE gels and then transferred onto polyvinylidene fluoride membranes. Following blocking with 5% non-fat milk for 30 min at room temperature, the membranes were incubated with the primary antibodies overnight at 4˚C: FOXA2 (1:1,000) and β-actin (1:2,000). Horseradish peroxidase (HRP)-conjugated antibodies goat anti-mouse immunoglobulin (Ig)G-HRP, (cat. no. sc-2005; 1:3,000; goat) or anti-rabbit IgG-HRP (cat. no. sc-2004; 1:3,000; goat) at room temperature were used as secondary antibodies. An enhanced chemiluminescence detection kit (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used to for signal detection. The films were quantified using ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA). At least three independent repeats of the experiments were performed.

MTT assay. Cells were seeded at density of $3x10^5$ cells/well into 6-well plates and cultured, with or without pioglitazone (0.5 μM). The control cells well treated with PBS. The MTT kit was purchased from Invitrogen; Thermo Fisher Scientific, Inc. Cells were incubated with 0.5 mg/ml MTT at 37˚C and cultured for an additional 4 h, and then 50 μl dimethyl sulfoxide was added into each well to stop the reaction. The absorbance was measured at 540 nm using a Synergy HT microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Luciferase reporter assay. TargetScan Human version 7.0 (www.targetscan.org) predicted that FOXA2 was a potential target of miR-141. The wild-type (WT) or mutant (MUT; without miR-141 binding site) human FOXA2 3'UTR sequences were synthesized using Quik Change Multi Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA) and separately cloned into the pGL-3 luciferase reporter plasmid (Promega Corporation, Madison, WI, USA). The recombinant plasmids were termed pGL3-FOXA2-WT and pGL3-FOXA2-MUT. These plasmids were co-transfected with 50 nm miR-141 mimic or inhibitor or their negative control using Lipofectamine® 2000. Cell lysates were prepared and luciferase assays were performed 48 h after transfection. Luciferase activity was normalized to *Renilla* luciferase activity.

Fasting blood glucose (FBG) quantification. The mice were fasted for 6 h and 0.5 ml blood samples were collected from

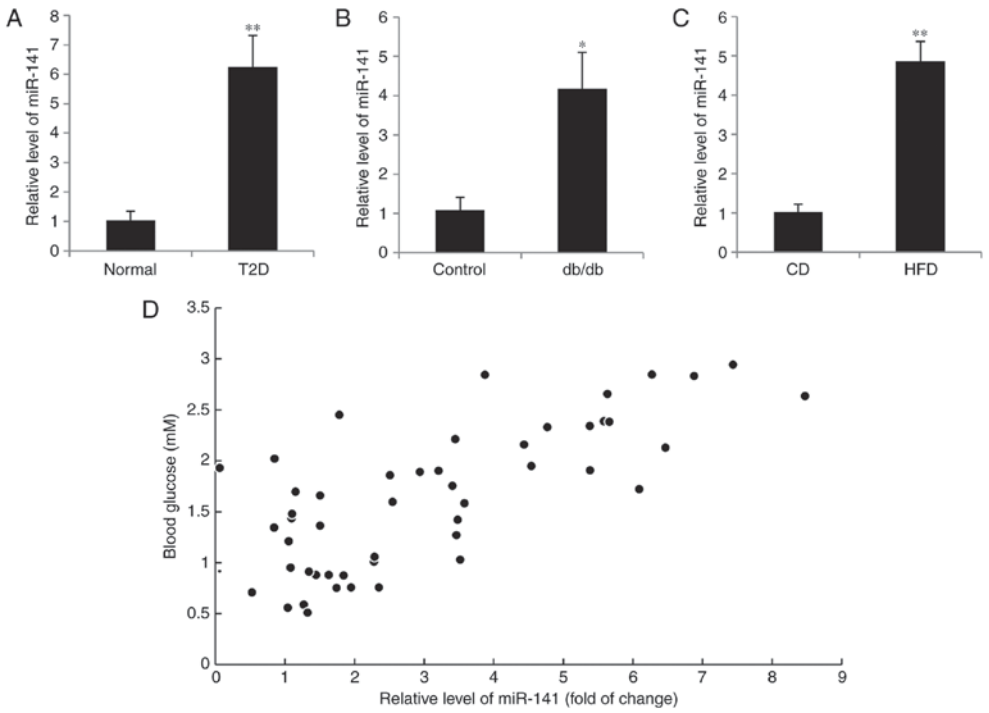

Figure 1. Expression of miR-141 was upregulated in diabetic mice and humans. RT-qPCR was used to detect the miR-141 expression level in the (A) T2D patient blood samples and the normal subjects, (B) pancreatic islets of db/db mice and control mice and (C) pancreatic islets of C57BL/6 mice fed CD or HFD. Data are presented as the mean ± standard deviation. (D) Using Pearson's correlation analysis, a positive correlation was observed between miR-141 expression and blood glucose concentration in T2D patients. *P<0.05, **P<0.01 vs. control. RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction; miR, microRNA; HFD, high fat diet; T2D, type 2 diabetes; CD, control diet.

the orbital venous plexus. FBG concentration was immediately quantified using a blood glucose meter and strips (Roche Accu-Check; Roche Diagnostics, Basel, Switzerland).

Statistical analysis. SPSS version 19.0 (IBM Corporation, Armonk, NY, USA) was used to perform the statistical analysis. Data were expressed as the mean ± standard deviation. The difference between groups was performed by analysis of variance followed by the Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-141 is upregulated in diabetic mice and humans. Due to the suppressive function of miR-141 in hepatocarcinogenesis (22-24), it was hypothesized that miR-141 may also have a role in T2D. RT-qPCR was used to examine the miR-141 plasma level in 50 patients with diabetes. When compared with the blood of normal subjects, miR-141 expression was significantly increased in the diabetic patients (P<0.01; Fig. 1A). It is of note that the expression of miR-141 was also upregulated in the pancreatic islets of db/db mice compared with the control mice (P<0.05; Fig. 1B). C57BL/6 mice were fed with a HFD for 16 weeks and miR-141 expression was significantly increased compared with mice fed a standard diet (P<0.01; Fig. 1C). In addition, a positive correlation was also observed in 50 patients with diabetes between the miR-141 expression and blood glucose concentration. The mean level of miR-141 in the normal subjects was used as the control for relative level of miR-141 in the diabetic patients. (P<0.05; r=0.5226; Fig. 1D).

FOXA2 is a direct target gene of miR-141 in INS-1 β cells. Bioinformatics analysis using TargetScan revealed that FOXA2 was a potential target of miR-141. As presented in Fig. 2A, at the 3'-UTR region of FOXA2, there are 6 consecutive complementary nucleotides of miR-141. In order to test whether FOXA2 was a direct target for miR-141, a series of experiments was performed as follows: The 3'-UTR of FOXA2 was cloned by PCR and inserted into the pGL3 reporter plasmid to quantify luciferase activity. In the INS-1 cells transfected with miR-141 mimic, the PGL3-FOXA2 reporter activity was significantly inhibited, whereas in the cells transfected with an miR-141 inhibitor, the relative reporter activity was increased (P<0.01; Fig. 2B). To further investigate the specific binding of miR-141 at the predicted FOXA2 seed sequences, a mutant reporter plasmid was produced using a 3-nucleotide mutation at the center of the 6 seed sequences, as presented in Fig. 2C, the mutant reporter exhibited no response to the miR-141 mimic or miR-141 inhibitor transfection. The aforementioned data demonstrated that miR-141 was able to bind to the 3'-UTR of FOXA2 and the 3 nucleotides were crucial for the binding of miR-141. In addition, the transfection of with the miR-141 mimic reduced the FOXA2 protein expression, whereas following transfection with the miR-141 inhibitor, the FOXA2 protein level was increased (Fig. 2D).

Upregulation of miR-141 results in impaired glucose-stimulated insulin secretion and INS-1 β cells proliferation. FOXA2 has been previously identified as a master regulator in pancreatic development and is involved in regulating both glucose-sensing apparatus and insulin release (25,26). It was hypothesized that miR-141 may have

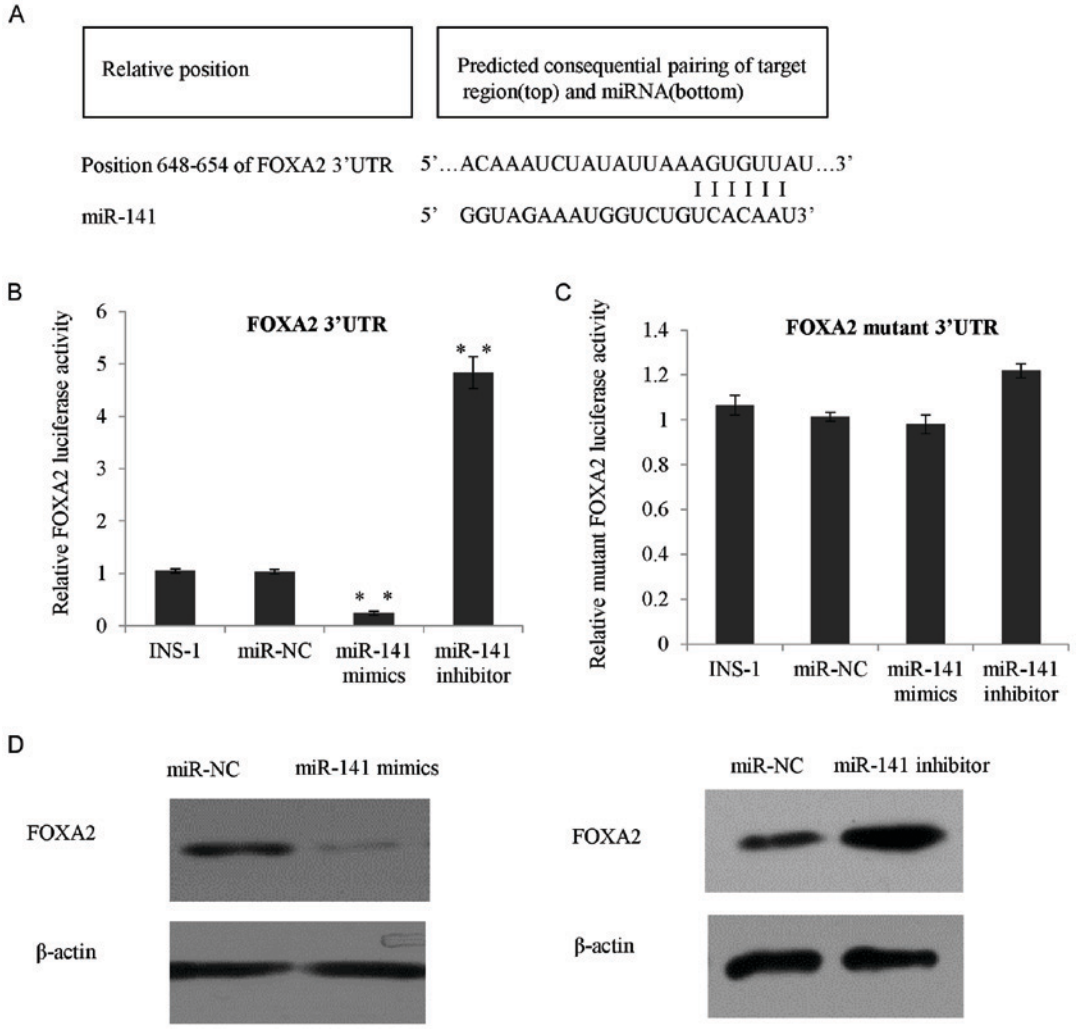

Figure 2. FOXA2 was a direct target gene of miR-141 in INS-1 β cells. (A) Bioinformatics analysis indicated the potential miR-141 binding sites in the 3'-UTR region of FOXA2. (B) Luciferase reporter assay was performed in INS-1 β cells by co-transfection of the FOXA2 luciferase reporter vector and the miR-141 mimic, miR-141 inhibitor, or with the NC. (C) Then, the luciferase reporter assay was performed in INS-1 β cells with the FOXA2 luciferase mutant reporter vector, co-transfected with the NC, miR-141 mimic or miR-141 inhibitor. Luciferase activity was tested 24 h following transfection. (D) FOXA2 protein expression was detected using western blotting in INS-1 β cells transfected NC, miR-141 mimic and miR-141 inhibitor, respectively. **P<0.01. NC, normal control; 3'-UTR, 3'-untranslated region; miR, microRNA; FOXA2, forkhead box A2.

a role in insulin secretion and β-cell proliferation. In order to verify the effects of miR-141 on glucose-stimulated insulin secretion, the cultured MIN6 pseudoislets were transfected with an miR-141 mimic, miR-141 inhibitor or scrambled negative control RNA. Insulin secretion was measured at 3.3 and 16.7 mM glucose concentration 48 h after the transfection. As presented in Fig. 3A, the expression of miR-141 was increased in the miR-141 mimic transfection group and the increase in miR-141 led to a significantly impaired insulin secretion at 16.7 mM glucose (P<0.05; Fig. 3B). Transfection with the miR-141 inhibitor, resulted in the opposite effect. Additionally, the effect of miR-141 was also tested on INS-1 β cell viability. Results from MTT assays (Fig. 3C) confirmed that overexpression of miR-141 reduced cell viability while knockdown of miR-141 significantly increased INS-1 β cell proliferation (P<0.05; Fig. 3D).

Expression of miR-141 is corrected by treatment with pioglitazone. INS-1 β cells were treated with 0, 0.01, 0.1, 1, or 10 μM pioglitazone for 24 h, the miR-141 expression level was quantified as presented in Fig. 4A, 10 μM pioglitazone resulted in the lowest miR-141 level (Fig. 4A). Furthermore, the INS-1 β cells were treated with 1 μM pioglitazone for 0, 3, 6, 12 and 24 h, the expression of miR-141 was also reduced (Fig. 4B). It is of note that the function of pioglitazone in animal models was also observed. Using db/db mice at the age of 8 weeks, or C57BL/6 mice on a HFD for 16 weeks, the mice were treated with the insulin-sensitizing pioglitazone for 4 weeks and the blood glucose level was measured once a week. As exhibited in Fig. 4C, the glucose level was decreased in the groups treated with pioglitazone. Furthermore, reduced pancreatic islet miR-141 expression was observed compared with the control groups (Fig. 4D).

miR-141 regulates glucose-stimulated insulin secretion and proliferation through FOXA2. It has been demonstrated that miR-141 may regulate glucose-stimulated insulin secretion and INS-1 β cell proliferation and FOXA2 has been identified as a direct target gene of miR-141. The axis of miR-141 targeting FOXA2 was investigated in T2D progression. As

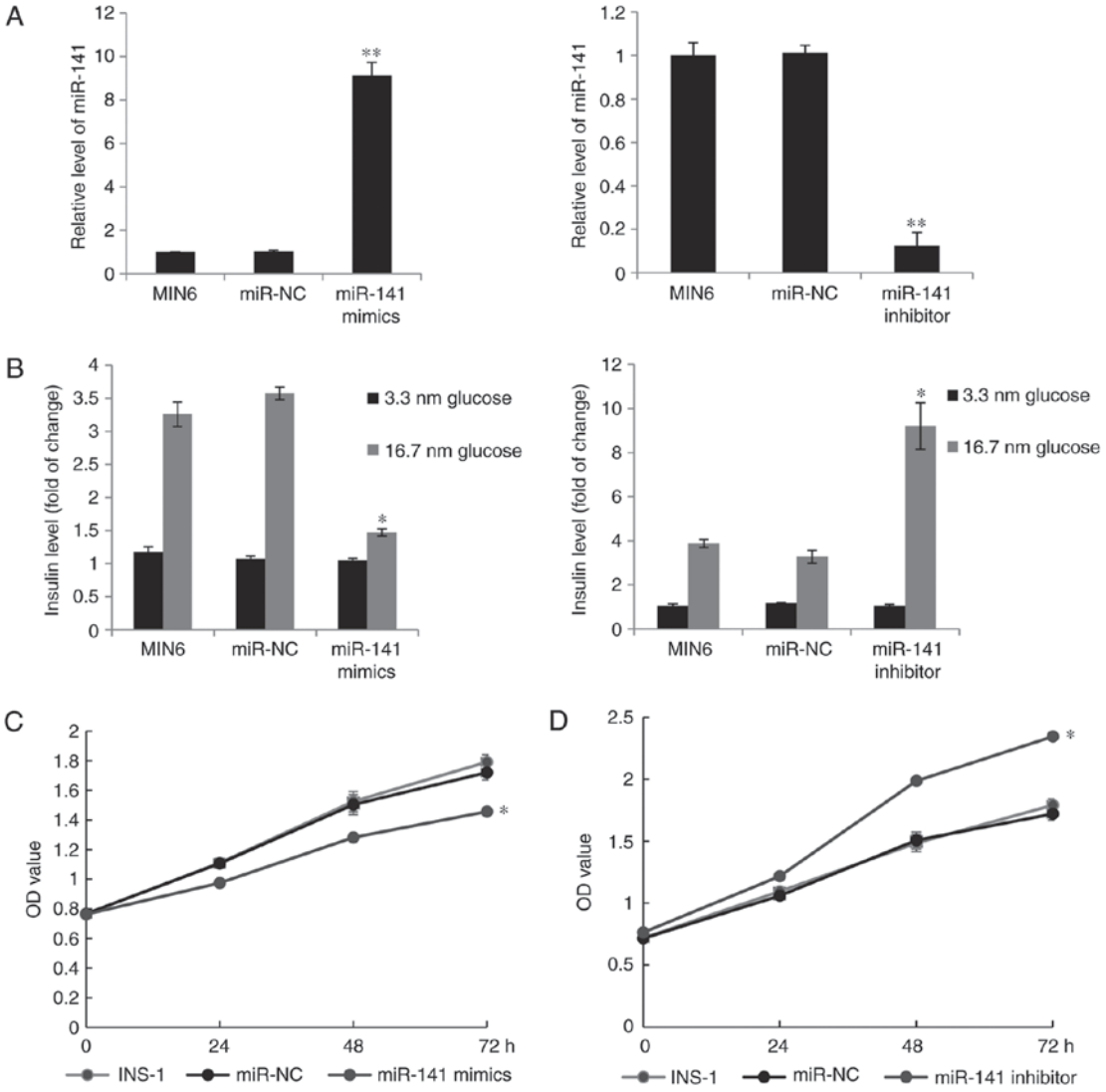

Figure 3. miR-141 overexpression resulted in impaired glucose-stimulated insulin secretion and INS-1 β cell proliferation. (A) MIN-6 pseudoislets were transfected with miR-141 mimic or miR-141 inhibitor or with a scrambled sequence, the expression level of miR-141 was quantified using reverse transcriptase-quantitative polymerase chain reaction. (B) Transfected pseudoislets were incubated with 3.3 or 16.7 mM glucose and insulin secretion was determined at 60 h. Data are presented as the mean ± standard deviation, of three different experiments. MTT assay was performed in INS-1 β cells transfected with (C) miR-141 mimic or mimic controls and (D) miR-141 inhibitor or mimic and NC to examine cell proliferation. *P<0.05, **P<0.01. miR, microRNA; NC, negative control; OD, optical density.

presented in Fig. 5A, overexpression of FOXA2 in MIN6 pseudoislets increased the effect of the miR-141 inhibitor on glucose-stimulated insulin secretion. Conversely, cells co-transfected with the miR-141 mimic and the knockdown of FOXA2 synergistically inhibited glucose-stimulated insulin secretion (Fig. 5B). Additionally, the INS-1 cells co-transfected with the miR-141 mimic and FOXA2 plasmid exhibited an increase in cell proliferation compared with the cells transfected with miR-141 (Fig. 5C). The INS-1 cells co-transfected with miR-141 inhibitor and shFOXA2 had reduced cell proliferation potential compared with the miR-141 inhibitor transfection groups (Fig. 5D). The results of the present study demonstrated that miR-141 inhibited the proliferation and insulin secretion of pancreatic β cells by directly targeting FOXA2.

Discussion

Several miRs have been reported to be associated with insulin resistance and/or diabetes. For example, increased expression of miR-429 may downregulate the expression of occludin and induce impaired intestinal barrier function in diabetes mellitus mice (27). MiR-593-3p negatively regulated insulin-regulated glucose metabolism in hepatocellular carcinoma cell lines such as HepG2 (28). As Chen *et al* (29) reported, under metabolic stress, miR-17-92 regulated glucose-stimulated insulin secretion and pancreatic β-cell adaptation. By inhibiting glycerol kinase, miR-451 negatively regulated hepatic gluconeogenesis and blood glucose levels in diabetes (30). Fu *et al* (31) reported that miR-26a was downregulated in two obese mouse models and regulated insulin signaling and metabolism of glucose. A previous study revealed that in the diabetic kidney, the renal expression of miR-141 was reduced in mouse models representing early and advanced kidney disease, which indicated miR-141 may have a role in diabetes (32).

In the present study, miR-141 expression was analyzed in plasma from T2D and non-diabetic donors using RT-qPCR and an upregulation of miR-141 was detected in elderly diabetic

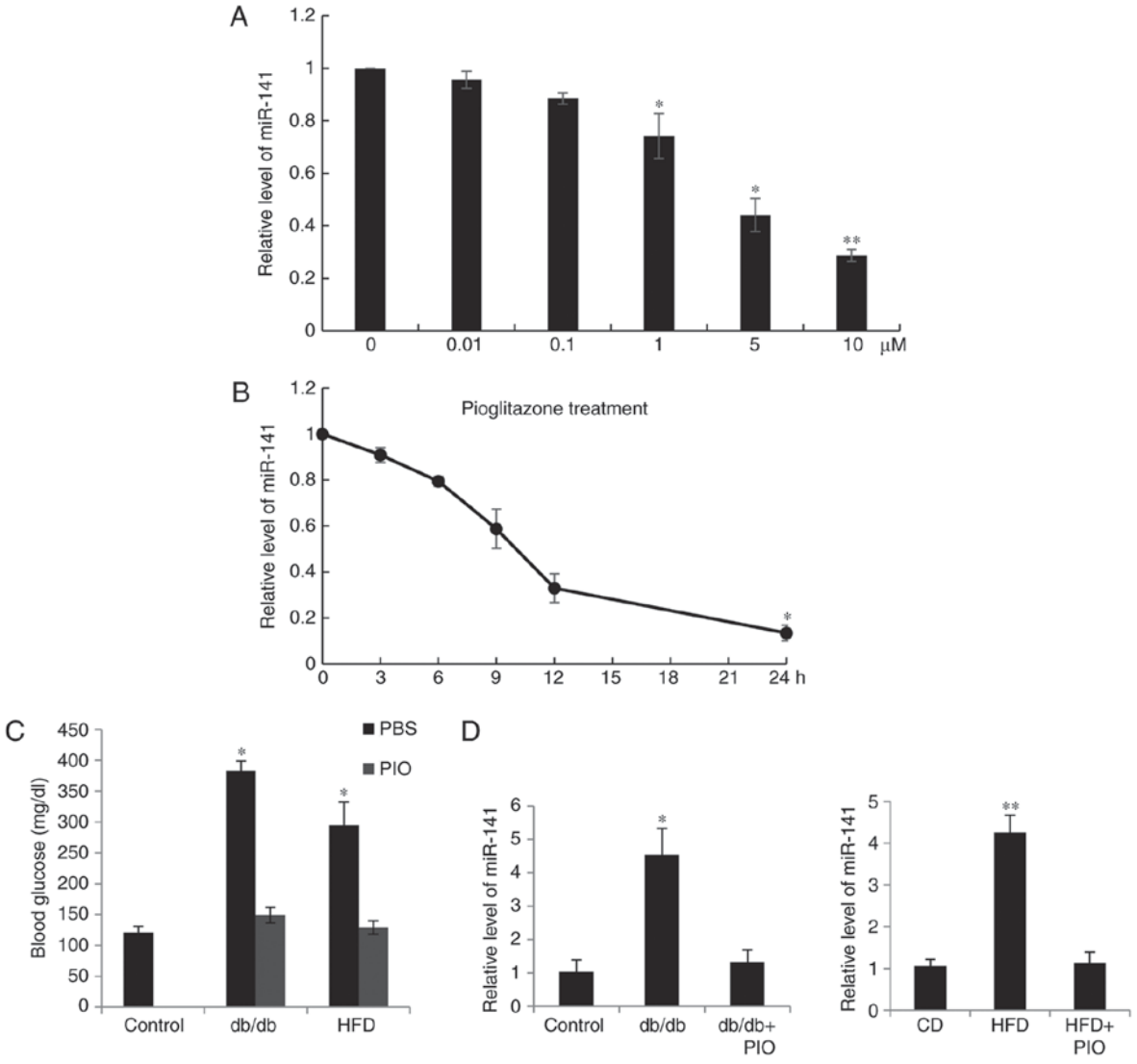

Figure 4. Expression of miR-141 was altered by treatment with PIO. (A) INS-1 β cells were treated with (A) 0, 0.01, 0.1, 1, 5 and 10 μM PIO for 24 h and (B) 1 μM PIO for 0, 3, 6, 12 and 24 h. The expression of miR-141 was quantified and all the data was presented as the mean ± standard deviation. (C) Fasting blood glucose levels were measured in diabetic model db/db mice, or C57BL/6 mice fed with a HFD, followed by treatment with PIO. (D) Relative hepatic levels of miR-141 were measured by reverse transcriptase-quantitative-quantitative polymerase chain reaction in the mice. *P<0.05, **P<0.01. HFD, high fat diet; PIO, pioglitazone; CD, control diet; miR, microRNA.

patients. However, as sufficient plasma was not collected from the mice, the plasma miR-141 level was not measured in the animal model, this was a limitation for the present study. The direct binding of miR-141 to the FOXA2 3'-UTR was confirmed by luciferase assay. The therapeutic effects of pioglitazone were considered to be the result of the regulation of multiple pathways (33-35). The expression of miR-141 was corrected by treatment with pioglitazone, suggesting that the dysregulation of miR-141 was associated with the progression of diabetes. INS-1 and MIN6 pseudoislets are glucose-responsive pancreatic β cells, in order to investigate the role of miR-141 in pancreatic β cell function, INS-1 cells or MIN6 pseudoislets were transfected with the miR-141 mimic or miR-141 inhibitor. The overexpression of miR-141 inhibited the proliferation and insulin secretion, whereas knockdown of miR-141 promoted the proliferation and insulin secretion, which further supported the notion that miR-141 served a role in diabetes, which is consistent with the results of the present study, where the expression of miR-141 was increased in T2D patients. The present study revealed that upregulation of miR-141 may lead to impaired glucose-stimulated insulin secretion and INS-1 β cell proliferation though targeting FOXA2. However, it is of note that the MIN6 pseudoislets used in the present study were obtained from mice and INS-1 b were from rats, and it is preferable to use the two different cell lines from the same species for more reliable findings. This is a limitation of the present study. As Sebastiani *et al* (36) previously reported, miR-124a was also increased in T2D human pancreatic islets and has a role in the control of FOXA2 and myotrophin. Therefore, it is interesting to investigate the association between miR-124a and miR-141. To the best of the authors knowledge, this is the first study to reveal the regulatory mechanism of pioglitazone/miR-141/FOXA2 axis in pancreatic β cells proliferation and insulin secretion. The findings of the present study provide a plausible alternative for the treatment of T2D. However, the specific function of this

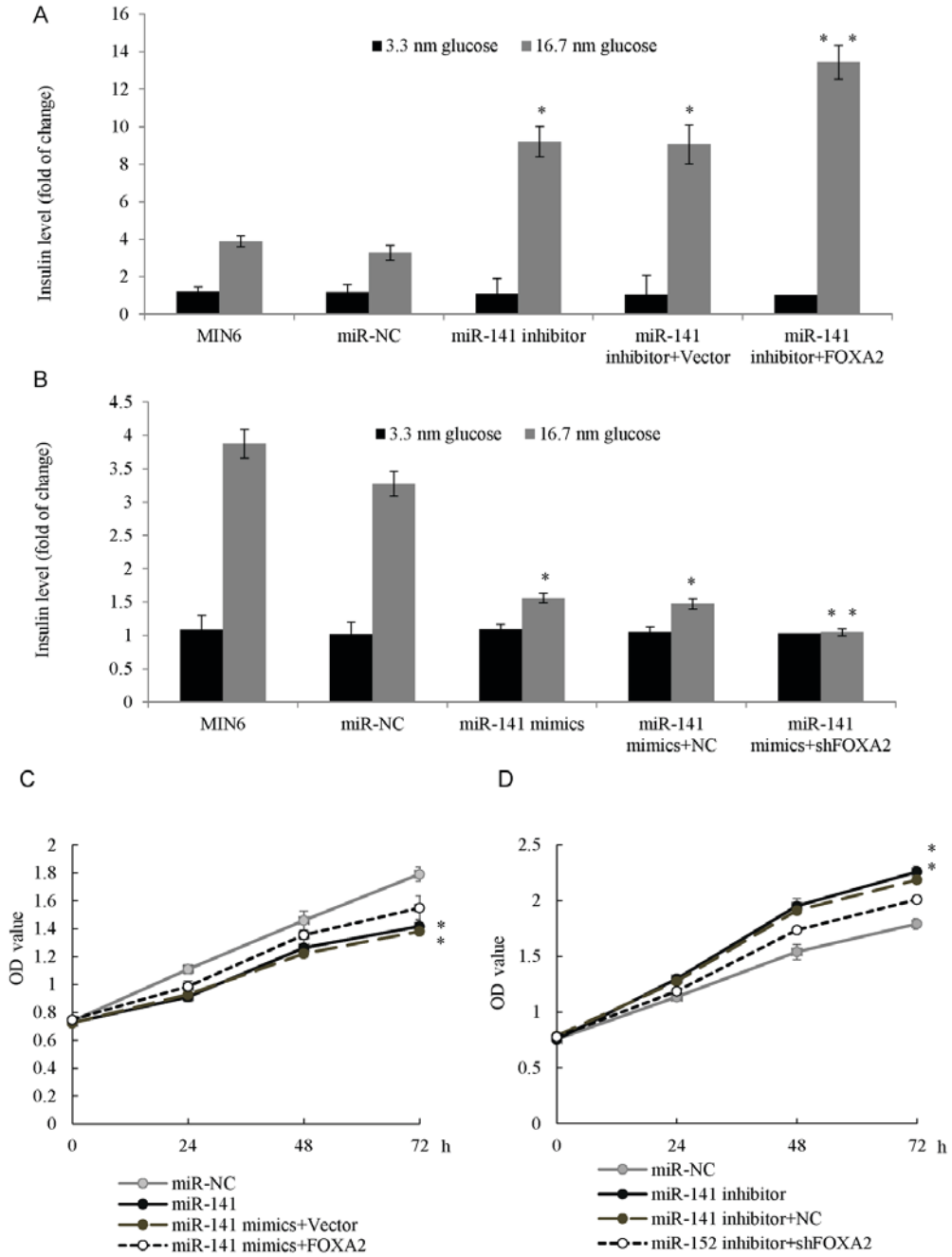

Figure 5. miR-141 regulated GSIS and cell proliferation through FOXA2. (A) MIN6 pseudoislets were transfected with an miR-141 inhibitor, or co-transfected with miR-141 inhibitor and FOXA2 overexpression plasmid. GSIS was determined in each group at 3.3 or 16.7 nM glucose. (B) MIN6 pseudoislets were transfected with the miR-141 mimic, or co-transfected with miR-141 mimic and shFOXA2. GSIS was determined in each group. (C) INS-1 cells were transfected with the miR-141 mimic, or co-transfected with the miR-141 mimic and FOXA2 plasmid and an MTT assay was performed in each group. (D) INS-1 cells were transfected with the miR-141 inhibitor or co-transfected with the miR-141 inhibitor and shFOXA2, MTT assay was performed in each group. *P<0.05, **P<0.01 vs. the miR-NC group. GSIS, glucose-stimulated insulin secretion; miR, microRNA; FOXA2, forkhead box A2; NC, negative control; sh, short hairpin; OD, optical density.

axis requires further investigation in terms of clinical characteristics in the future.

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Availability of data and materials

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

Authors' contributions

XY conceived and designed the present study. LZ provided technical assistance and analyzed the data. XY and LZ

drafted the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All human tissues are collected under Institutional Review Committee (IRB) and Health Insurance Portability and Accountability Act (HIPAA) approved protocols.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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