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MiR-486-5p regulates pancreatic β cell function in type 2 diabetes mellitus by targeting PTEN and FOXO1

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Recently, microRNAs (miRNAs) have been suggested to play important roles in the pathophysiology of type 2 diabetes mellitus (T2DM). In this study, we explored the role of miR-486-5p in T2DM. MiR-486-5p was significantly downregulated in the serum of T2DM patients when compared to that of healthy volunteers, and miR-486-5p expression level was negative correlated with blood glucose levels of T2DM patients. Overexpression of miR-486-5p promoted cell proliferation, enhanced insulin secretion and inhibited cell apoptosis of pancreatic β-cell line (INS-1 cells). On the other hand, knockdown of miR-486-5p had the opposite effects in INS-1 cells. The bio-informatics analysis by using TargetScan revealed phosphatase and tensin homolog (PTEN) and Forkhead Box O1 (FOXO1) were downstream targets of miR-486-5p, and the interaction between miR-486-5p and PTEN (or FOXO1) was validated by luciferase reporter assay. In addition, miR-486-5p negatively regulated the mRNA and protein expression of PTEN and FOXO1. Overexpression of PTEN (or FOXO1) suppressed insulin secretion in glucose stimulation, inhibited cell proliferation, and induced cell apoptosis, and partially abolished the effects of miR-486-5p overexpression on insulin secretion, cell proliferation and cell apoptosis of INS-1 cells. In conclusion, our results revealed that miR-486-5p promoted pancreatic cell proliferation, increased insulin sensitivity and inhibited apoptosis by targeting PTEN and FOXO1.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a disease affecting about 500 million people worldwide, and incidence of T2DM is expected to increase (Petrie et al. 2018; Saliani et al. 2017). T2DM is a common endocrine and metabolic disorder, which is characterized by pancreatic β-cell dysfunction in response to elevated blood glucose levels, insulin resistance, and the failure of peripheral tissues in response to physiological levels of insulin (Kaviani et al. 2016; Zheng et al. 2017). The expenses of preventing and managing diabetes were more than billions every year. However, up to date, the molecular mechanisms underlying the pathogenesis of T2DM were not fully elucidated. Thus, to completely understand the exact mechanisms of T2DM pathogenesis and to develop effective therapy strategies is of great scientific interest.

MicroRNAs (miRNAs) are a class of small, non-coding RNAs with ~ 22 nucleotides in length, and miRNAs act to inhibit translation or directly induce degradation *via* binding to specific complementary sites within 3' untranslated region (3'UTR) of targeted genes (Feng et al. 2016). A large number of studies has illustrated the functional role of miRNAs in the development of various diseases, including cancer, neurodegenerative diseases, cardiovascular diseases and diabetes (Martinez and Peplow 2017; Steri et al. 2018; Vannini et al. 2018). Many studies have revealed the critical roles of miRNAs in pancreatic development and function related to the pathogenesis of T2DM. For example, miR-144 impairs insulin signaling by inhibiting the expression of insulin receptor substrate 1 in T2DM (Karolina et al. 2011). Forst and Olson (2011) showed that miRNA Let-7 regulated multiple aspects of glucose metabolism and insulin sensitivity and suggested knockdown of Let-7 may represent as a potential treatment for T2DM. The MiR-200 family is strongly induced in islets of diabetic mice and miR-200 overexpression is sufficient to induced β-cell apoptosis and lethal T2DM, suggesting the crucial role for the miR-200 family in β-cell survival and the pathophysiology of diabetes (Belgardt et al. 2015). MiR-19a-3p enhances the proliferation and insulin secretion, and inhibits apoptosis of pancreatic β cells via targeting SOCS3 (Li et al. 2016). Recently, the miRNA profiling in the peripheral blood from T2DM patients revealed the downregulation of miR-486-5p (Zampetaki et al. 2010). The role of miR-486-5p has been extensively examined regarding the pathophysiology of different types of cancer (Jiang et al. 2018). However, the role of miR-486-5p in the pathogenesis of T2DM has not been examined yet.

In the present study, we identified the expression of miR-486-5p in the peripheral blood from T2DM patients and healthy volunteers. The *in vitro* studies were further performed to elucidate the functional role of miR-486-5p in cell proliferation, insulin sensitivity, and cell apoptosis of pancreatic β cells. Our findings may reveal a novel role of miR-486-5p in the pathophysiology of T2DM.

2. Investigations and results

2.1. MiR-486-5p is downregulated in the peripheral blood of T2D patients

To determine the expression of miR-486-5p in the serum from healthy volunteers and T2D patients, we performed qRT-PCR to determine the expression of miR-486-5p. The qRT-PCR assay showed that miR-486-5p was significantly downregulated in the serum from T2D patients when compared with that of healthy volunteers (Fig. 1A). Furthermore, the correlation between miR-486-5p expression level and blood glucose level in T2D patients were also determined, and the expression level of miR-496-5p was negatively correlated with the blood glucose level (Fig. 1B).

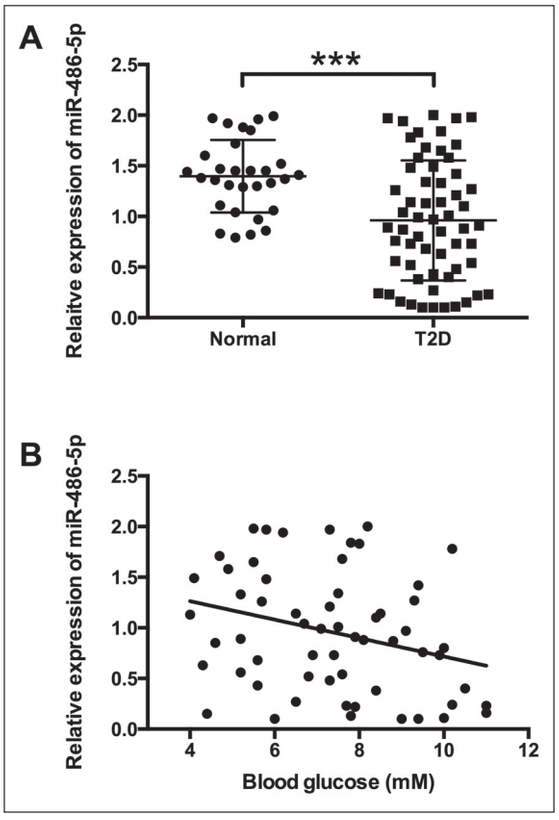

Fig. 1: MiR-486-5p is down-regulated in the peripheral blood of T2D patients. (A) The expression of miR-486-5p was measured by qRT-PCR in peripheral blood from 30 healthy volunteers and 60 patients with T2D. $P<0.001$. (B) Correlation between miR-486-5p level and blood glucose determined by Spearman correlation analysis (r = -0.1, $P<0.05$).

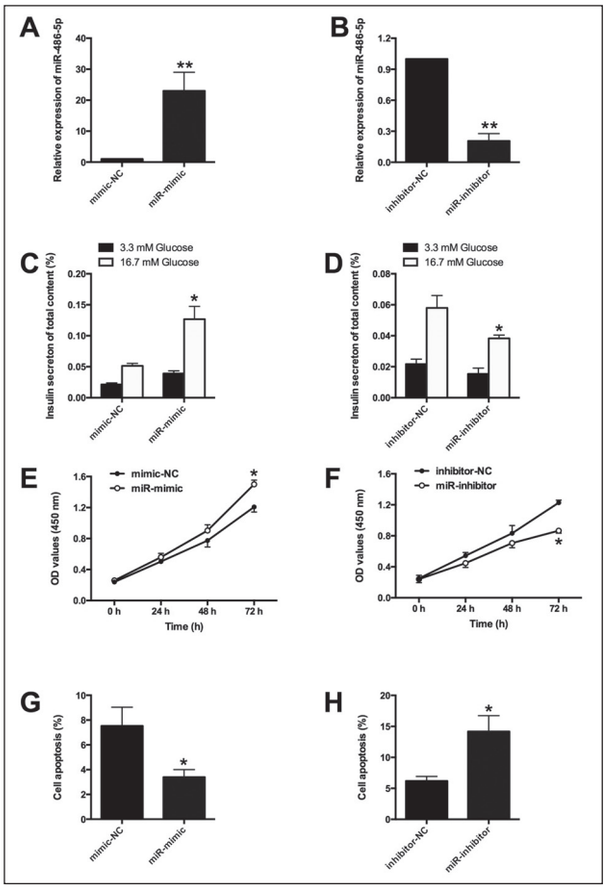

Fig. 2: Effects of miR-486-5p on the insulin secretion, cell proliferation and apoptosis of pancreatic β-cells. (A) INS-1 cells were transfected with mimic-NC or miR-486-5p mimic (miR-mimic); (B) INS-1 cells were transfected with inhibitor-NC or miR-486-5p inhibitor (miR-inhibitor). (C and D) INS-1 cells were transfected with different miRNAs (mimic-NC, miR-mimic, inhibitor-NC, or miR-inhibitor). Glucose-stimulated (3.3 or 16.7 mM glucose) insulin secretion was determined by ELISA assays. (E and F) INS-1 cells were transfected with different miRNAs (mimic-NC, miR-mimic, inhibitor-NC, or miR-inhibitor). Cell proliferation was determined by CCK-8 assay. (G-H) INS-1 cells were transfected with different miRNAs (mimic-NC, miR-mimic, inhibitor-NC, or miR-inhibitor). Cell apoptosis was determined by flow cytometry. N = 3, $*P<0.05$, $**P<0.01$.

2.2. *Effects of miR-486-5p on the insulin secretion, cell proliferation and apoptosis of pancreatic β-cells*

The functional roles of miR-486-5p in pancreatic β-cells was examined *in vitro* by using INS-1 with miR-486-5p up- or down-regulation. As shown in Figs. 2A and 2B, INS-1 cells transfected with miR-486-5p mimic had a significantly higher expression level of miR-486-5p than that transfected with mimic-NC, and transfection with miR-486-5p inhibitor suppressed the expression of miR-486-5p in the INS-1 cells. The ELISA assay showed that insulin secretion in response to glucose stimulus was increased in cells transfected with miR-486-5p mimic (Fig. 2C), and decreased in cells transfected with miR-486-5p inhibitor (Fig. 2D). The CCK-8 assay showed that overexpression of miR-486-5p increased cell proliferative ability of INS-1 cells (Fig. 2E), and knockdown of miR-486-5p suppressed cell proliferation (Fig. 2F). In addition, flow cytometry results showed that overexpression of miR-485-5p inhibited cell apoptosis of INS-1cell (Fig. 2G), and knockdown of miR-486-5p induced cell apoptosis of INS-1 cells (Fig. 2H).

2.3. *PTEN is a direct target of miR-486-5p in pancreatic β-cells*

MiRNA regulated gene expression *via* targeting the 3'UTR, and the predicted targets of miR-486-5p were identified by using Targetscan software, and PTEN and FOXO1 were found to two of the potential targets of miR-486-5p. The interaction sites between miR-486-5p and 3'UTR of PTEN are shown in Fig. 3A. To further validate the interaction between miR-486-5p and PTEN, we cloned the 3'UTR of PTEN segment (wild type or mutant) into the luciferase reporter vector. Transfection with miR-486-5p mimic dramatically suppressed the luciferase activities of the wild type 3'UTR of PTEN, but the construct containing a mutant binding site abolished the inhibitor effect of miR-486-5p overexpression (Fig.

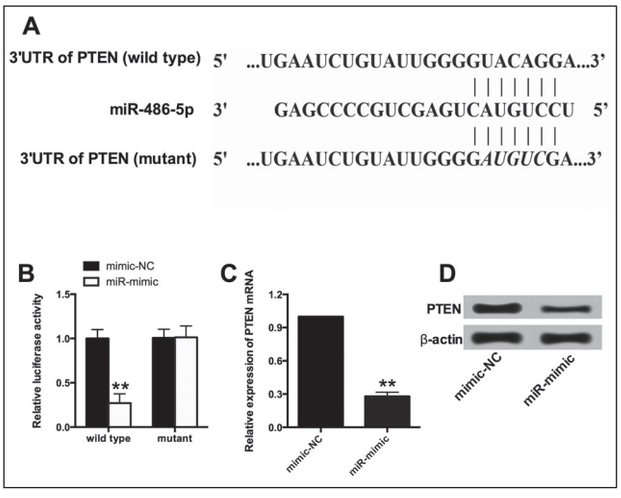

Fig. 3: PTEN is a direct target of miR-486-5p in pancreatic β-cells. (A) Schematic draw of the interaction sites between miR-486-5p and 3'UTR of PTEN. (B) INS-1 cells were co-transfected with firefly luciferase constructs containing the PTEN wild type or mutant 3'UTR and miR-486-5p mimic (miR-mimic) or mimic-NC, and at 48 h after transfection, luciferase activity was measured. (C and D) mRNA levels and protein expression of PTEN after transfected with miR-486-5p mimic (miR-mimic) or mimic-NC. N = 3, $**P<0.01$.

3B). Furthermore, qRT-PCR and western blot results showed that miR-486-5p overexpression suppressed the mRNA and protein expression of PTEN (Figs. 3C and 3D).

2.4. PTEN is involved in the miR-486-5p-mediated effects on the on the functions of pancreatic β-cells

To further examine the relevance of miR-486-5p/PTEN axis in the regulation of pancreatic β-cells, the overexpression of PTEN was performed by transfecting INS-1 cells with PTEN overexpression vector (pcDNA3.1-PTEN; Fig. 4A). As shown Figs. 4B-4D, overexpression of PTEN suppressed insulin secretion in glucose stimulation, inhibited cell proliferation, and induced cell apoptosis, and enforced expression of PTEN also partially abolished the effects of miR-486-5p overexpression on insulin secretion, cell proliferation and cell apoptosis of INS-1 cells.

2.5. FOXO1 is a direct target of miR-486-5p in pancreatic β-cells

The interaction between miR-486-5p and FOXO1 was also validated by luciferase reporter assay. Figure 5A shows the interaction sites between miR-486-5p and 3'UTR of FOXO1. The 3'UTR of FOXO1 segment (wild type or mutant) were inserted into the luciferase reporter vector. Overexpression of miR-486-5p significantly suppressed the luciferase activity of the wild type 3'UTR of FOXO1, but the construct containing the mutant binding site abolished the inhibitory effect of miR-486-5p overexpression (Fig. 5B). In addition, miR-486-5p overexpression inhibited the mRNA and protein expression of FOXO1 (Figs. 5C and 5D).

2.6. FOXO1 is involved in the miR-486-5p-mediated effects on the on the functions of pancreatic β-cells

Consistent with the findings for PTEN, overexpression of FOXO1 suppressed insulin secretion in glucose stimulation, inhibited cell proliferation, and induced cell apoptosis, and partially abolished the effects of miR-486-5p overexpression on insulin secretion, cell proliferation and cell apoptosis of INS-1 cells (Fig. 6).

3. Discussion

T2DM is one of the most severe threats to human health worldwide, and various miRNAs such as let-7, miR-7, miR-9, miR-21 may play a role in its pathogenesis (Hashimoto and Tanaka 2017). In our current study, we showed the downregulation of miR-486-5p in the peripheral blood of T2DM patients, and miR-486-5p expression level was negatively correlated with blood glucose levels of T2DM patients. The *in vitro* functional studies revealed that miR-486-5p increased insulin sensitivity, promoted cell proliferation, inhibited cell apoptosis of pancreatic β cells. The bioinformatics analysis and luciferase reporter assay further identified PTEN and FOXO1 were downstream targets of miR-486-5p, and PTEN and FOXO1 are involved in the effects of miR-486-5p overexpression on insulin sensitivity, cell proliferation and cell apoptosis of pancreatic β cells.

The functional role of miR-486-5p has been mainly studied in human cancers. MiR-486-5p was found to be downregulated in lung cancer tissues and downregulation of miR-486-5p contributed to tumor progression and metastasis by targeting protumorigenic ARHGPA5 in lung cancer (Wang et al. 2014). Zhang et al. (2014) showed that miR-486-5p suppressed cell proliferation in breast cancer cells *via* targeting PIM-1. MiR-486-5p was identified as tumor suppressor in colorectal cancer and attenuated tumor growth and lymphangiogenesis by targeting neuropilin-2 (Liu et al. 2016). MiR-486-5p can also act as a tumor suppressor in esophageal squamous cell carcinoma (Yi et al. 2016). Zampetaki et al. (2010) showed that lower plasma levels of miR-486 was found in prevalent diabetes mellitus. In another cohort of study, lower serum levels of miR-486 were determined in T2DM patients (Yang et al. 2014). Consistently, our results revealed a downregulation of miR-486 in the serum from T2DM patients and was negatively correlated with blood glucose levels, suggesting that downregulation of miR-486-5p plays a role in the pathophysiology of T2DM. Our functional studies further revealed that overexpression of miR-486-5p increased insulin sensitivity, promoted cell proliferation and inhibited cell apoptosis of pancreatic β cells, suggesting that miR-486-5p was important in attenuate pancreatic β cell dysfunction in T2DM.

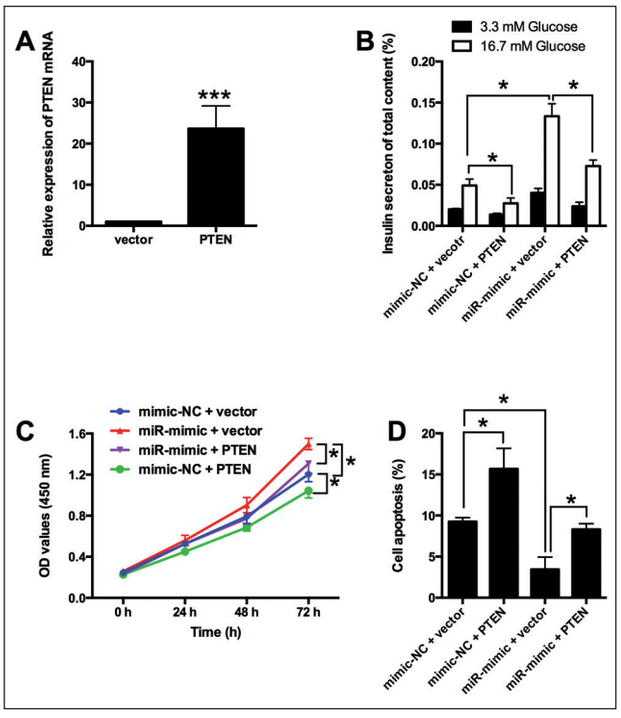

Fig. 4: PTEN is involved in the miR-486-5p-mediated effects on the on the functions of pancreatic β-cells. (A) INS-1 cells were transfected with pcDNA3.1 (vector) or pcDNA3.1-PTEN (PTEN), and the mRNA expression of PTEN was determined by qRT-PCR. INS-1 cells were co-transfected with pcDNA3.1-PTEN (or pcDNA3.1 vector) and miR-486-5p mimic (or mimic-NC), (B) glucose-stimulated insulin secretion was determined by ELISA assay, (C) cell proliferation was determined by CCK-8 assay, and (D) cell apoptosis was determined by flow cytometry. N = 3, **P*<0.05, ****P*<0.001.

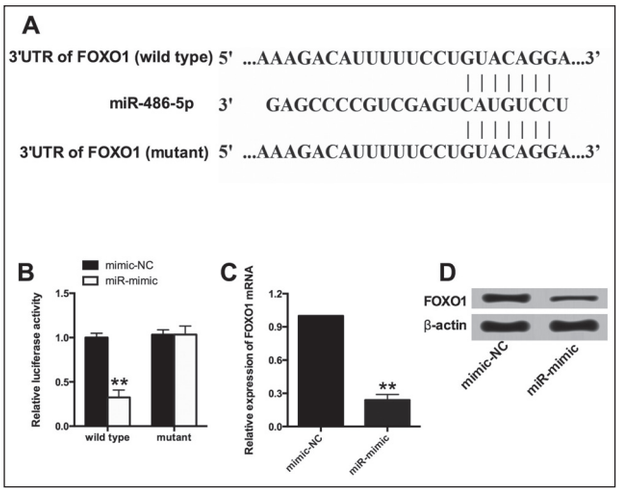

Fig. 5: FOXO1 is a direct target of miR-486-5p in pancreatic β-cells. (A) Schematic draw of the interaction sites between miR-486-5p and 3'UTR of FOXO1. (B) INS-1 cells were co-transfected with firefly luciferase constructs containing the FOXO1 wild type or mutant 3'UTR and miR-486-5p mimic (miR-mimic) or mimic-NC, and at 48 h after transfection, luciferase activity was measured. (C and D) mRNA levels and protein expression of FOXO1 after transfected with miR-486-5p mimic (miR-mimic) or mimic-NC. N = 3, ***P*<0.01.

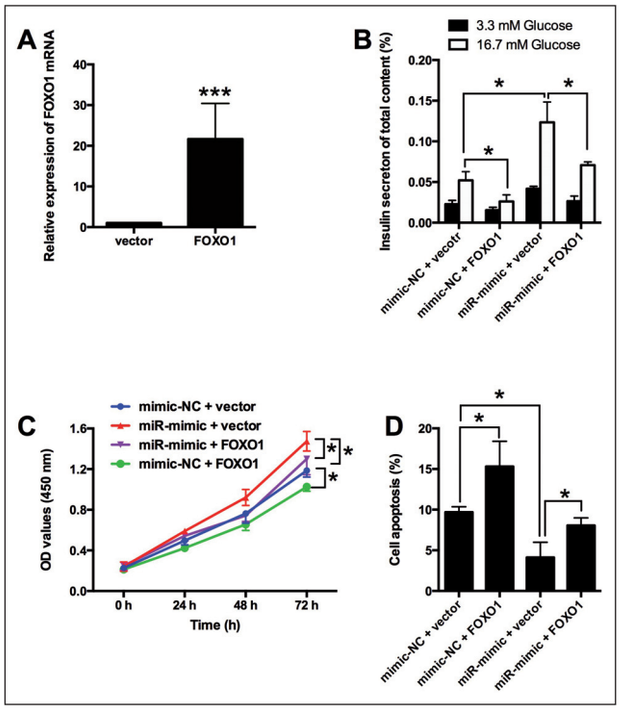

Fig. 6: FOXO1 is involved in the miR-486-5p-mediated effects on the on the functions of pancreatic β-cells. (A) INS-1 cells were transfected with pcDNA3.1 (vector) or pcDNA3.1-FOXO1 (FOXO1), and the mRNA expression of PTEN was determined by qRT-PCR. INS-1 cells were co-transfected with pcDNA3.1-FOXO1 (or pcDNA3.1 vector) and miR-486-5p mimic (or mimic-NC), (B) glucose-stimulated insulin secretion was determined by ELISA assay, (C) cell proliferation was determined by CCK-8 assay, and (D) cell apoptosis was determined by flow cytometry. N = 3, **P*<0.05, ****P*<0.001.

The bioinformatics analysis in our study showed that PTEN and FOXO1 were potential targets of miR-486-5p. PTEN was originally identified as a tumor suppressor and was one of most frequently mutated genes in various types of cancers (Kim et al. 2017). Qu et al. (2011) showed that variation in the PTEN-induced putative kinase 1 gene associated with the increase risk of type 2 diabetes in Northern Chinese. PTEN deltein in RIP-Cre neurons protects against T2DM by activating the anti-inflammatory flex (Wang et al. 2014), and upregulation of PTEN was associated with the development of insulin resistance in T2DM (Birnbaum et al. 2014). Downregulation of miR-449-5p contributed to hepatic insulin resistance by targeting PTEN (Wang et al. 2015). In our study, we showed that overexpression of miR-486-5p suppressed the expression of PTEN, and PTEN overexpression attenuated the effects of miR-486-5p overexpression on pancreatic β cell functions. Collectively, these results may suggest that miR-486-5p-mediated effects on pancreatic β cell function is partially via modulating PTEN.

FOXO1 is an important transcriptional factor that belongs to the forkhead family, and FOXO1 played an essential role in regulating oxidative stress response, cell death and cellular metabolism (Guo et al. 2014). Studies have demonstrated that FOXO1 was associated with pancreatic β-cell dysfunction in T2DM (Lee et al. 2013). Luo et al. (2017) also showed that miR-21 regulated hepatic glucose metabolism by targeting FOXO1. In addition, long non-coding RNA Gomafu upregulated FOXO1 expression to promote hepatic insulin resistance by sponging miR-139-5p (Yan et al. 2018). Our results showed that miR-486-5p negatively regulated the expression of FOXO1, and FOXO1 overexpression abolished the effects of miR-486-5p overexpression on pancreatic β cell proliferation, insulin sensitivity and apoptosis, suggesting that miR-486-5p regulated the function of pancreatic β cells partially via targeting FOXO1.

In conclusion, our results showed that patients with T2DM presented lower expression levels of miR-486-5p in serum and miR-486-5p expression level was negatively associated with blood glucose levels. *In vitro* studies revealed that miR-486-5p promoted pancreatic cell proliferation, increased insulin sensitivity and inhibited apoptosis by targeting PTEN and FOXO1. Our results may implicate the potential therapeutic role of miR-486-5p as a candidate for T2DM treatment, and the therapeutic effects of miR-486-5p may require further exploration.

4. Experimental

4.1. Blood sample collection

Blood samples were collected from 60 patients with T2D and 30 healthy volunteers in the Shenzhen Third People's Hospital during June 2016 to June 2017. Patients with malignancy, acute heart failure, severe kidney or liver diseases were excluded from this study. This study was approved the Ethics Committee of Shenzhen Third People's Hospital, and was carried out in accordance with Declaration of Helsinki. Written informed consent was obtained from all subjected prior to blood collection.

4.2. Cell culture

The INS-1 cell line (ATCC, Manassas, USA) was cultured in Dulbecco's modified Eagle's Medium containing high glucose (Thermo Fisher Scientific, Waltham, USA), supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific) and 70 μM β-mercaptoethanol (Sigma, St. Loius, USA) at 37 °C with 5% CO_2 in a humidified incubator.

4.3. Oligo nucleotides and cell transfection

The miRNAs including miR-586-5p mimic (miR-mimic), miR-586-5p inhibitor (miR-inhibitor), and their respective negative controls (mimic-NC and inhibitor-NC) were purchased from RiboBio (Guangzhou, China). The control vector (pcDNA3.1) and the phosphatase and tensin homolog (PTEN)-overexpressing vector (pcDNA3.1-PTEN) and and Forkhead Box O1 (FOXO1)-overexpressing vector (pcDNA3.1-FOXO1) were obtained from GenePharma company (Shanghai, China). Cell transfection or co-transfection was performed by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) in accordance with the manufacturer's protocol. At 48 h after transfection, INS-1 cells were collected for further analysis.

4.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from peripheral blood (serum) or cells were extracted by using TRIzol reagent (Invitrogen) in accordance with the manufacturer's protocol. The relative expression was determined using the mirVana qRT-PCR detection kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. The relative mRNA expression of PTEN and FOXO1 was detected by using the standard SYBR-Green real-time PCR kit (Takara, Dalian, China) in according with the manufacturer's instructions. U6 was used as an internal reference for miR-486-5p, and GAPDH was used as an internal reference for PTEN and FOXO1 mRNA expression. The relative expression levels were quantified using the comparative Ct method.

4.5. Measurement of glucose-stimulated insulin secretion

The INS-1 cells were seeded in a 96-well plate with further culturing for 24 h, and the cells were treated with 3.3 mM glucose (basal glucose) or 16.7 mM glucose (stimulatory glucose) for 1 h. After that, the insulin level was measured by Insulin ELISA kit (Abcam, Cambridge, UK) in accordance with the manufacturer's instructions.

4.6. Cell viability assay

Transfected INS-1 cells were seeded in a 96-well plate with further culturing for 24 h. After that, cells were incubated with 20 μl of MTT solution (5 mg/ml) for 4 h at room temperature, and then 200 μl of DMSO was added to each well to dissolve the formazan for 10 min at room temperature. The cell viability was detected by measuring the absorbance at 490 nm using a microplate reader (BioTek, Winooski, USA) in according with the manufacturer's instructions.

4.7. Flow cytometry to detect cell apoptosis

The cell apoptotic rate was measured by using the Annexin V-FITC apoptosis detection kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Transfected cells were collected, and washed in PBS, and resuspended in binding buffer containing Annexin-FITC and propidium iodide and incubated for 15 min at room temperature in the dark. The cell apoptosis of INS-1 cells was analyzed by using a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, USA).

4.8. Dual-luciferase reporter assay

The pmiRGLO vector was used to construct the wild type and mutated type 3'UTR of PTEN and FOXO1. MiR-486-5p mimic or mimic-NC and wild type or mutated type of PTEN 3'UTR or FOXO1 3'UTR were co-transfected into INS-1 cells by using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. At 48 h after transfection, luciferase activities were quantified by Dual-Luciferase reporter assay (Promega, Madison, USA).

4.9. Western blot assay

Cells were lysed with RIPA buffer containing protease inhibitors (Sigma), and the extracted protein levels were determined by BCA method (Bio-Rad, Hercules, USA). The proteins were separated on a 10% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, USA). The PVDF membrane was blocked with 5% skimmed milk for 1 h at room temperature. After that, the PVDF membrane was further incubated with primary antibodies against PTEN, FOXO1 and β-actin (Cell Signaling Technology, Danvers, USA) at 4 °C overnight. After incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, the blots were visualized by ECL reagent (Thermo Fisher Scientific).

4.10. Statistical analysis

All the data are expressed as mean±standard error mean, and GraphPad Prism 6 software (GraphPad Software, La Jolla, USA) was used to plot graphs and to perform statistical analysis. The association between miR-486-5p expression level and blood glucose level was analyzed by using Spearman correlation analysis. Significant differences were analyzed using Student's t-test or one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

Authors' contributions: JY performed the experiments. ZX and HT recorded and analyzed the data, JL is a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate: This study was carried out in accordance with Declaration of Helsinki. Written informed consent was obtained from all subjected prior to blood collection.

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Competing interests: The authors declare no conflict of interest.

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