miR-765 Impairs Pancreatic β -cell Function by Targeting PDX1 in type 2 Diabetes

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Research

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Abstract

Background: Type 2 diabetes (T2D) is highly connected with the defect in insulin secretion of pancreatic β -cells, which has been developing into a severe public health problem.

Methods: Here, we first detected expression of PDX1 and miR-765 in peripheral blood from 40 patients with T2D and 40 healthy volunteers. INS-1E cells (pancreatic β -cell line) were cultured as experimental model. For glucose induction, we incubated INS-1E cells with 11 mM glucose as control group and INS-1E cells with 25 mM glucose as T2D model group. For target relationship verification, we performed Luciferase reporter assay. Generally, we utilized qRT-PCR (quantitative real-time PCR), western blotting, insulin secretion detection, CCK-8, and flow cytometry in this study.

Results: The expression level of PDX1 was dramatically lower in peripheral blood from T2D patients than healthy volunteers, while miR-765 exhibited an opposite result. PDX1 expression level had an inverse correlation with blood glucose level of T2D patients whereas miR-765 exhibited a positive correlation. Furthermore, PDX1 improved insulin secretion, cell viability, and restrained cell apoptosis of INS-1E cells. PDX1 was identified as a target of miR-765 which was observed to reduce insulin secretion, cell viability, and induce cell apoptosis of INS-1E cells.

Conclusions: Taken together, we confirmed that miR-765 could cause detrimental effect on pancreatic β -cell survival and function by targeting and repressing PDX1 in T2D.

Background

Due to the advance and enrichment of diet, the public are getting rid of starvation. Unfortunately, at the same time, a variety of metabolic diseases are also coming along, of which type 2 diabetes (T2D) is extremely prevalent [1, 2]. T2D is a chronic metabolism-disorder with symptom as hyperglycemia, pancreatic β -cell dysfunction, insulin resistance [3, 4]. As one of the vital cell types of endocrine pancreas, β -cells are solely in charge of insulin production and release answering to ambient blood glucose level [5]. Recurrent hyperglycemia will lead to a rising demand for insulin secretion [6], breaking the balance of β -cell compensatory response and causing diabetes [7, 8]. Of note, not only type 1 diabetes but also type 2 diabetes is observed to have a marked reduction in β -cell mass [9]. Therefore, elucidating how relevant molecules influence β -cells in the maintenance of normal function is essential for our better understanding of the pathogenesis of T2D.

Pancreatic duodenal homeobox 1 (PDX1) gene encodes a transcription factor critically accountable for the regulation of early pancreas development β -cell survival and mature β -cell function such as insulin secretion [10–12]. The essential role of PDX1 in pancreas was highlighted by PDX1-null-mice model with pancreatic agenesis [13] and in humans with mutations of PDX1 gene [14], suggesting the vital function of this gene for human pancreas development. In addition to the part of pancreas maintenance, PDX1 is actively sustaining β -cell viability. It is reported that inadequate PDX1 impaired both insulin secretion and β -cell mass, worsening glucose tolerance and progressing diabetes [15–17]. Thus, not only does PDX1

play a vital role in pancreas development, but also a much more subtle role in β -cell survival and function conservation. More notably, declined PDX1 was believed to act as a contributor for β -cell dysfunction in T2D [18, 19]. In the present study, we continued to provide more detailed explanation for how PDX1 regulated T2D progression by utilizing pancreatic β -cell model.

MicroRNA (miRNA) is well accepted as small non-coding RNA that predominantly bind to complementary site at 3'untranslated region (3'UTR) of their target genes, leading to suppression of target gene expression post-transcriptionally [20]. In the past decade, miRNA has been attracting an increasing number of scientists with its special regulatory function in various life activities [21–24]. Among these studies, some have highlighted the essential part of miRNA in pancreatic β -cell growth and function associated with the pathogenesis of T2D. For instance, a study indicated that silence of mir-7a in β -cells improved glucose tolerance by enhancing insulin secretion [25]. In 2015, Guido *et al.* came to a conclusion that miR-124a enrichment impaired glucose-stimulated insulin secretion [26]. It is also showed that miR-335 upregulation promoted defective initiation of insulin vesicles, resulting in impaired insulin secretion [27]. As for the part of miR-765 regulating diabetes, a study observed an enrichment of miR-765 in T2D [28]. To grasp more molecular evidence for T2D development, we here examined the role of miR-765 in this area as well as its interaction with PDX1.

In our current research, we collected peripheral blood from 40 T2D patients and conducted a series of in vitro experiments, aiming to elucidate the regulatory function of miR-765/PDX1 axis in pancreatic β -cells. By this effort, our observation might reveal a novel role of miR-765/PDX1 axis in T2D, thus providing new insights into the therapeutic way for T2D on molecular level.

Methods

Bioinformatics analysis

GSE38642, GSE20966 and GSE25724 were mRNA expression profiles from GEO DataSets including human pancreatic islet samples, human β -cells samples and human islet samples with or without T2D. The differentially expressed genes (DEGs) were screened by limma 3.26.8 with P-value < 0.05. GSE26168 was the miRNA expression profile from GEO DateSets including blood samples with or without T2D. Then, the two online tools (WebGestalt and Metascape) were applied to analyze the biological processes of DEGs. TargetScan and miRwalk were performed to predicted the miRNAs targeting our interested gene. Finally, the key genes and miRNAs were overlapped using Venny 2.1.0.

Blood sample collection

Peripheral blood was sampled from 40 patients with T2D and 40 non-T2D patients in Wuhan Wuchang Hospital Affiliated to Wuhan University of Science and Technology. Patients who had been diagnosed with T2D accompanied by high blood pressure, obesity and dyslipidemia were included. This study gained the approval from the Ethics Committee of Wuhan Wuchang Hospital Affiliated to Wuhan University of Science and Technology and performed in keeping with the Declaration of Helsinki. Written

informed consent from all subjects was obtained before sampling blood. Clinical characteristics of 40 patients with T2D were listed in Table 1.

Cell culture

Pancreatic β -cell line, the rat INS-1E cell line (AddexBio, US), was cultured in RPMI 1640 medium supplemented with 11 mM glucose, 5% FBS, 10 mM HEPES, 50 μ M β -mercaptoethanol and 2 mM glutamine at 37°C with 5% CO₂. For glucose induction, INS-1E cells cultured with 11 mM glucose served as control group and INS-1E cells cultured with 25 mM glucose was built up to be the model group.

Cell transfection

miR-765 mimic, miR-765 inhibitor, and negative controls (NC) were provided by RiboBio (China). PDX1 overexpression vector (PDX1-OE), knockdown of PDX1 (si-PDX1), and their NC were purchased from GenePharma (China). Before cell transfection, INS-1E cells would be seeded in different plates depending on the subsequent corresponding assay. Cell density was $2.5 \times 10^6 \cdot \text{mL}^{-1}$ for a 6-well plate, $1 \times 10^6 \cdot \text{mL}^{-1}$ for a 12-well plate, $5 \times 10^4 \cdot \text{mL}^{-1}$ for a 96-well plate, respectively. Cell transfection was performed utilizing Lipofectamine 3000 reagent (Invitrogen, US) and cell transfection concentration was 100 ng for a 96-well plate, 1000 ng for a 12-well plate, 2500 ng for a 6-well plate as stated in the protocol. After 48 h transfection, INS-1E cells were collected for further detections on condition that the transfection efficiency detected via qRT-PCR was satisfied for the present experiment.

Quantitative real-time PCR (qRT-PCR)

Total RNA from tissues or cells was extracted using RNeasy Micro Kit (QIAGEN, Germany) following manufacturer's protocol. Titan One Tube RT-PCR Kit (Roche, Switzerland) was applied to get the cDNA for mRNA. One Step miRNA cDNA Synthesis Kit (HaiGene, China) was used to obtain the cDNA for miRNA. Relative expression of PDX1 or miR-765 was measured by SYBR-Green real-time PCR kit (Takara, Japan) in Bio-Rad CFX96 instrument (Bio-Rad, US). Internal reference for PDX1 was GAPDH, and for miR-765 was U6. The relative mRNA expression level was quantified through $2^{-\Delta\Delta C_t}$ method. The sequences of primers were list in Table 2.

Western blotting

INS-1E cells with a 48-h transfection were treated by RIPA buffer with 1 mM PMSF (Sigma-Aldrich, US) at 4°C for 15 to 20 min. After being gathered, the proteins (10 μ L for each hole) were separated by a 10% SDS-polyacrylamide gel at 100 V for 1 h. By the time the separation finished, proteins were transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, US) at 4°C. After the transfer and PBS washing, 5% skim milk was added to the PVDF membrane for 1-h blocking at 25°C. PBS was used to wash the PVDF membrane again when the blocking ended. Then, PVDF membrane was incubated in anti-PDX1 (1:1000, arigobio, China) and the reference β -actin (1:500, SANTA CRUZ BIOTECHNOLOGY, US) at 4°C overnight. The next morning, the secondary antibodies Goat-Anti-Rabbit IgG (1:5000, arigobio, China)

for anti-PDX1 and Goat-Anti-Mouse IgG (1:5000, arigobio, China) for β -actin were added to the proteins for 1-h incubation at 25°C. Finally, the protein bands were added ECL luminescence reagent (Sangon Biotech, China) and then exposed by Image-Pro Plus 6.0 software (Media Cybernetics, US).

Glucose-stimulated insulin secretion determination (GSIS)

GSIS was performed to confirm the function of INS-1E cells. Briefly, the transfected INS-1E cells were seeded in a 96-well plate for another 24-h culture and then incubated under the condition of 3.3 mM glucose (basal glucose) or 16.7 mM glucose (stimulatory glucose) for 1 h. After this step, cell supernatant was collected for the insulin level determination using Insulin-1 ELISA Kit (Sigma-Aldrich, US) following the manufacturer's instructions. In brief, 100 μ L of sample was added into appropriate well for a 2.5-hour incubation. Then, 100 μ L of 1 $\times$ prepared Biotinylated Detection Antibody was added to each well to incubate for 1 h. 100 μ L of prepared HRP-Streptavidin solution was added to each well to incubate for 45 minutes. 100 μ L of ELISA Colorimetric TMB Reagent was added to each well to incubate for 30 minutes avoiding light. 50 μ L of Stop Solution was added to each well. After each incubation at room temperature, the solution was discarded and the sample was washed 4 times with 1 $\times$ Wash Solution. Finally, the data was read at 450 nm in a microplate reader (BIOBASE, China) immediately.

Cell viability detection

Cell viability was detected by CCK-8 assay. INS-1E cells seeded in a 96-well plate were treated with 10 μ L CCK-8 solution per well using CCK-8 kit (TransGen Biotech, China) 1 h in advance of each detection at the point of 0, 24, 48, 72 h. The absorbance value was read in a microplate reader (BIOBASE, China) at 450 nm.

Cell apoptosis detection

Cell apoptosis was detected by flow cytometry assay. After 48-h transfection, INS-1E cells seeded in a 12-well plate were treated with a transient trypsin digestion and the digestion was terminated by complete medium. Then the cells were washed by PBS twice and then stained using Annexin V-FITC/DAPI Apoptosis Detection Kit (Elabscience, China) for 15 min protected from light at 25°C. Finally, the CytoFLEX (BECKMAN COULTER, US) was used to analyze the cell apoptosis of INS-1E cells.

Luciferase reporter assay

Rat gene PDX1 3'UTR wild type (PDX1-WT) and mutant type (PDX1-MUT) were obtained by subcloning into pmirGLO vector. PDX1-MUT contained the sequence "GGAAGG" in place of "CCUUC" which was the predicted binding site of miR-765. The predicted binding site of miR-765 remained unchanged in PDX1-WT. INS-1E cells were seeded in a 96-well plate and co-transfected with miR-765 mimic or mimic-NC and PDX1-WT or PDX1-MUT using Lipofectamine 3000 reagent (Invitrogen, US). After 48 h, luciferase activities were quantified by Luciferase reporter assay (Promega, US).

Statistical analysis

All data from at least three repeated experiments were depicted as mean $\pm$ SD in this study. We applied GraphPad Prism 6 software (US) to deal with statistical analysis as well as process correspondent graphs. The correlation between PDX1 or miR-765 expression and blood glucose was obtained by Spearman correlation analysis. Student's t-test or one-way ANOVA was used to analyze differences. $P < 0.05$ was accounted as statistically significant difference between value.

Results

PDX1 rather than SLC2A2 is a potential regulator involved in T2D development

To explore the key gene affecting T2D, GSE38642, GSE20966 and GSE25724 were mRNA expression profiles to be used. By Venny 2.1.0, a total of 286 DEGs were overlapped from GSE38642, GSE20966 and GSE25724 (Fig. 1a). Then, the 286 DEGs were uploaded to WebGestalt and Metascape, respectively. The Type II diabetes mellitus involving four genes was identified as the key biological processes based on the results of WebGestalt (Fig. 1b), while the regulation of beta-cell development involving six genes was identified as the key biological processes based on the results of Metascape (Fig. 1c). Finally, PDX1 and SLC2A2 were overlapped involving Type II diabetes mellitus and the regulation of beta-cell development (Fig. 1d). To identified our interested gene to be investigated, qRT-PCR was performed to detect the expression of PDX1 and SLC2A2 in our clinical samples. The result showed that PDX1 mRNA significantly expressed higher in peripheral blood sample from healthy tissue than T2D (type 2 diabetes) tissue, but the differential expression of SLC2A2 was not that significant as compared to PDX1 (Fig. 1e). Thus, we chose PDX1 as our interested gene to explore the development of T2D.

Pdx1 Plays A Protective Role In Pancreatic β -cells

According to the results above which indicated PDX1 might play a role in T2D development, we further designed the following verifications. First, we observed PDX1 expression exhibited a strong negative correlation with the level of blood glucose (Fig. 2a). Subsequently, we cultured INS-1E cells in the presence of 25 mM glucose to establish T2D model group and INS-1E cells cultured with 11 mM glucose served as control group. Both PDX1 mRNA and protein expression levels were dramatically lower in relatively high-glucose-treated INS-1E cells, as low as only around 50% of the control group (Fig. 2a, b). Following this, the effect of PDX1 on glucose-induced INS-1E cells was investigated with transfection of PDX1-OE (for PDX1 overexpression) or si-PDX1 (for PDX1 knockdown). As expected, PDX1-OE successfully upregulated both mRNA and protein expression of PDX1 and si-PDX1 successfully downregulated both mRNA and protein expression of PDX1 in INS-1E cells (Fig. 2d, e). Specifically, PDX1-OE dramatically increased PDX1 mRNA expression to a more than three-time level of the control group, and si-PDX1 remarkably suppressed PDX1 mRNA expression to an approximate 30% level of the control group (Fig. 2d). From Fig. 2e, we could observe PDX1-OE dramatically increased PDX1 protein expression to a more than 1.5-time level of the control group, and si-PDX1 significantly suppressed PDX1 protein

expression to an approximate 60% level of the control group. As depicted in Fig. 2f, insulin level in INS-1E cells was increased by PDX1-OE by almost 70% while decreased by si-PDX1 by around 45% compared to the control group. In addition, insulin secretion answering to glucose stimulus in INS-1E cells transfected with PDX1-OE doubled compared to the control group whereas insulin secretion level in si-PDX1 group was only 60% of the control group (Fig. 2g). CCK-8 assay was conducted to determine cell viability of INS-1E cells. We found that PDX1-OE remarkably enhanced cell viability while si-PDX1 exerted an opposite effect (Fig. 2h). Finally, we carried out flow cytometry to examine cell apoptosis of INS-1E cells and found PDX1 overexpression restrained cell apoptosis by about 70% compared to the control group while silenced PDX1 dramatically accelerated this process to an about 2.25-time level of the control group (Fig. 2i). All these results demonstrated that PDX1 could protect and enhance the survival and function of pancreatic β -cells.

Pdx1 Gene Is A Target Of Mir-765

Plenty of genes can be regulated by miRNAs which in return are able to target various genes in cell life process. Therefore, we first used GSE26168 to screen the differentially expressed miRNAs with P-value < 0.05. At the same time, TargetScan and miRwalk were used to predict the miRNAs targeting PDX1. Then, miR-1258, miR-583 and miR-765 were screened out (Fig. 3a). After literature review, miR-765 with high expression in T2D attracted our attention [28]. Next, we examined the expression level of miR-765 in peripheral blood of healthy tissue or T2D tissue. As a result, miR-765 exhibited a higher mRNA expression in T2D tissue, completely reversal of PDX1 (Fig. 3b). Then, we analyzed the correlation expression between PDX1 and miR-765 and the data depicted miR-765 expression was negatively correlated with PDX1 expression (Fig. 3c). After that, we generated miR-765 mimic (for miR-765 overexpression), wild type PDX1 3'UTR (PDX1-WT) containing the predicated miR-765 binding site "CCUUC" and mutant type PDX1 3'UTR (PDX1-MUT) containing the predicated miR-765 binding site replaced by "GGAAGG" (Fig. 3d). Luciferase reporter assay was then performed, suggesting that co-transfection of miR-765 mimic with PDX1-WT repressed luciferase activity by about 50% compared to the co-transfection of mimic NC with PDX1-WT (Fig. 3e). On the contrast, co-transfection of miR-765 mimic with PDX1-MUT failed to affect luciferase activity (Fig. 3e). Furthermore, we observed an obvious inverse correlation between miR-765 expression and blood glucose level (Fig. 3f). miR-765 expression in INS-1E cells treated with 25 nM glucose was increased by over 50% compared to cells cultured in 11 nM glucose (Fig. 3g). Together, we reached a conclusion that miR-765 directly targeted PDX1 gene in pancreatic β -cells.

miR-765 exerts detrimental impact on the survival and function of pancreatic β -cells through mediating PDX1

To further determine whether miR-765 made an impact on the survival and function of pancreatic β -cells by repressing PDX1, we designed the following experiments with or without si-PDX1 transfection. Before the determination, we first synthesized miR-765 inhibitor to inhibit the expression of miR-765. From Fig. 4a, we observed that miR-765 mRNA expression was only about 30% of the control group when INS-

1E cells were transfected with miR-765 inhibitor or si-PDX1 plus miR-765 inhibitor, indicating a successful inhibitory effect of miR-765 inhibitor on miR-765 expression in INS-1E cells. When miR-765 was inhibited by its inhibitor, PDX1 mRNA expression was upregulated, doubling the level compared to the control group (Fig. 4a). However, this double enhancement could be compromised by si-PDX1 (Fig. 4a). In addition to mRNA expression, miR-765 inhibitor also affected the expression of PDX1 on protein level. As we could see from Fig. 4b, PDX1 protein expression was 1.7 times as much as that of the control group when cells were treated with miR-765 inhibitor and this increase was also restored by si-PDX1. Insulin concentration was increased in INS-1E cells transfected with miR-765 inhibitor by almost 70% compared to the control group, while this promotion was reversed when si-PDX1 existed (Fig. 4c). Insulin secretion level in miR-765 inhibitor group was twice as much as that of the control group, but such increment was also blocked by silenced PDX1 (Fig. 4d). What's more, from the results of CCK-8 and flow cytometry, we observed that miR-765 inhibitor enhanced cell viability (Fig. 4e) and restrained cell apoptosis by 60% compared to the control group (Fig. 4f), whereas the impact was controlled when co-transfected with si-PDX1. Taken together, our data confirmed that miR-765 impaired the survival as well as function of pancreatic β -cells by interacting with PDX1.

Discussion

T2D and its accompanying diseases have been affecting an increasing number of populations worldwide [29]. Thus, it is indeed a tricky public health issue in demand of new and effective treatments. More efforts are supposed to be put in scientific researches so as to address such complex issue, bringing good news as well as health to patients who are suffering from T2D. In our present study, PDX1 and miR-765 were identified as our interested gene and miRNA by bioinformatics analysis. Then, the down-regulation of PDX1 and up-regulation of miR-765 were observed in peripheral blood of T2D patients. Besides, PDX1 expression level had inverse correlation with blood glucose level of T2D patients whereas miR-765 exhibited a positive correlation here. Our in vitro verification revealed that PDX1 enhanced insulin secretion, cell viability, and restrained cell apoptosis of pancreatic β -cells. By luciferase reporter assay, we identified miR-765 as an upstream regulator of PDX1. As a target gene of miR-765, PDX1 was here verified to participate in the process in which miR-765 reduced insulin secretion, cell viability, and induced cell apoptosis of pancreatic β -cells.

It is reported that mutations of PDX1 could lead to insulin secretion defect [30–32]. Similarly, insufficiency of PDX1 expression in pancreatic β -cells in animal models also accounted for pancreatic β -cell impairment and T2D [13]. Here, we also identified knockdown of PDX1 was a contributor to the dramatical reduction of insulin secretion in pancreatic β -cells, while overexpression of PDX1 was beneficial for the insulin secretion. In other words, PDX1 was verified as a protective factor in the function of pancreatic β -cells. Previous research demonstrated that not only was PDX1 a key mediator of insulin gene expression, but also critical for the support of pancreas normal growth [33–35]. This statement has underscored the importance of PDX1 in the survival and viability of pancreatic β -cells. Consistently, our findings depicted that PDX1 helped to facilitate cell viability and repressed cell apoptosis of pancreatic β -

cells. Thus, PDX1 is a gene that is able to prevent T2D through protecting the survival and function of pancreatic β -cells.

miRNAs are widely accepted to be involved in the damage of insulin secretion from pancreatic β -cells and the promotion of T2D progression, such as miR-375 that suppressed glucose-induced insulin secretion [36], miR-7 that was a negative regulator of insulin secretion [25], and miR-200 that induced development of T2D [37]. Not only do these previous systematic researches concerning the mediation of aberrant miRNAs in T2D help to broaden our horizon in the molecular activity involved in T2D progression, but also advance the development of new diagnostic biomarkers and therapeutic strategies for T2D. However, because of the complicity of T2D pathogenesis, a deep, comprehensive understanding of other aberrant miRNAs is still needed. In a study [28], researchers explored the aberrantly expressed miRNAs in T2D by performing pathway enrichment analysis and establishing mRNA-miRNA interaction networks, and then they found miR-765 was one of the enriched miRNAs in T2D development. Here, we further determined how this insulin-secretion related miRNA, miR-765 acted in the development of T2D through a series of cellular and molecular experiments. As a result, we found miR-765 could block insulin secretion in INS-1E cells, damaging cell viability, inducing cell apoptosis and facilitating T2D development. Of note, these effects of miR-765 on pancreatic β -cells were exerted by targeting and repressing PDX1 gene which has been already identified in many studies as a strong associated marker in pancreatic β -cell protection and T2D prevention [12, 16, 38]. Therefore, miR-765 could be a marked damage factor that induced pancreatic β -cell dysfunction and promoted T2D development.

Considering PDX1 is an essential regulator that influences substantial genes and pathways in β -cell sustainability, we hoped to dig out more mechanisms of PDX1 underlying the protection of pancreatic β -cell survival and function interacting with these regulated genes. This attempt didn't implement in this study which simply focused on the interaction between PDX1 and its upstream regulator miR-765 through epigenetic mechanism.

Conclusions

Overall, we observed that 40 T2D patients displayed higher expression of miR-765 in peripheral blood, and miR-765 expression was reversely related to blood glucose level, which was completely opposite to PDX1. We also further verified that miR-765 gave rise to the damage of pancreatic β -cells and the progression of T2D by targeting and interacting with PDX1. Our all results provided a novel insight into the potential molecular therapeutic strategies in T2D from the point of PDX1 and miR-765.

Abbreviations

T2D

Type 2 diabetes; INS-1E cells: Pancreatic β -cell line; qRT-PCR: Quantitative real-time PCR; PDX1: Pancreatic duodenal homeobox 1; miRNA: MicroRNA; 3'UTR: 3'untranslated region; DEGs: Differentially expressed genes; NC: Negative controls; PDX1-OE: PDX1 overexpression; si-PDX1: Knockdown of PDX1; GSIS: Glucose-

stimulated insulin secretion determination; PDX1-WT:PDX1 3'UTR wild type; PDX1-MUT:PDX1 3'UTR mutant type.

Declarations

Ethics approval and consent to participate

This study gained the approval from the Ethics Committee of Wuhan Wuchang Hospital Affiliated to Wuhan University of Science and Technology and performed in keeping with the Declaration of Helsinki. Written informed consent from all subjects was obtained before sampling.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interest

The authors declare that there is no conflict of interest.

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Authors' contributions

LZ and YLW conducted the study, collected and analyzed the data. YL designed the study and methods and collected the funds. YHL analyzed and interpreted the data. LL collected materials and resources, conducted literature analysis and prepared manuscript. XHW conducted literature analysis and prepared the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Clinical characteristics of 40 patients with type 2 diabetic.

characteristics	Total = 40	Percentage (%)
Gender		
Male	24	60.0%
Female	16	40.0%
Age(years)		
>55	15	37.5%
≤55	25	62.5%
Duration of disease (years)		
>10	11	27.5%
≤10	29	72.5%
Body mass index (kg/m ²)		
>25	12	30.0%
≤25	28	70.0%
Co-morbidities		
High blood pressure	14	35.0%
Obesity	17	42.5%
Dyslipidemia	9	22.5%

Table 2. The primer sequences for RT-qPCR.

GENE	Primer sequences (5'-3')
miR-765	Forward: TGGAGGAGAAGGAAGGTGATG
	Reverse: GTGCAGGGTCCGAGGT
U6	Forward: <i>ATTGGAACGATACAGAGAAGATT</i>
	Reverse: <i>GGA ACGCTTCACGAATTTG</i>
PDX1	Forward: CCACCCCAGTTTACAAGCTC
	Reverse: TGTAGGCAGTACGGGTCCTC
SLC2A2	Forward: TACTCAATGAACCCAAAACCAAC
	Reverse: GGCCTGAAATTAGCCCTTCC
GAPDH	Forward: AGCCACATCGCTCAGACAC
	Reverse: GCCCAATACGACCAAATCC

Figures

Figure 1

PDX1 rather than SLC2A2 is a potential regulator involved in T2D development. (A) 286 DEGs were overlapped from three mRNA expression profiles (GSE38642, GSE20966 and GSE25724). (B) The enrichment results of 286 DEGs by WebGestalt analysis. (C) The enrichment results of 286 DEGs by Metascape. (D) Two genes were overlapped from Regulation of beta-cell development involving 6 genes from Metascape and Type II diabetes mellitus involving 4 genes from WebGestalt. (E) PDX1 was dramatically down-regulated in peripheral blood of patients with type 2 diabetes (T2D) but SLC2A2 was not as significant as PDX1 measured by qRT-PCR. Normal (n=40): peripheral blood from 40 healthy volunteers, T2D (n=40): peripheral blood from 40 patients with type 2 diabetes. * $P < 0.05$, ** $P < 0.001$. Data are presented as the mean $\pm$ SD.

Figure 2

PDX1 plays a protective role in pancreatic β -cells. (A) Correlation between PDX1 level and blood glucose determined by Spearman correlation analysis ($R^2=0.5708$, $P<0.0001$). (B) PDX1 mRNA expression in the glucose-induced INS-1E cells. (C) PDX1 protein expression in the glucose-induced INS-1E cells. (D) Transfection efficiency of PDX1 overexpression vector or knockdown by qRT-PCR. (E) Transfection efficiency of PDX1 overexpression vector or knockdown by western blotting. (F, G) PDX1 overexpression vector or knockdown was transfected into INS-1E cells for 24 h. Insulin content (F) and insulin secretion (G) were determined by glucose-stimulated insulin secretion assay. (H) Cell viability of INS-1E cells was detected by CCK-8 assay after transfection of PDX1 overexpression vector or knockdown. (I) Cell apoptosis of INS-1E cells was detected by flow cytometry after transfection of PDX1 overexpression vector or knockdown. Normal: INS-1E cells cultured with 11 mM glucose, Model: INS-1E cells cultured with 25 mM glucose. OE: PDX1 overexpression vector, Si: si-PDX1 for PDX1 knockdown, CON: blank control, NC: negative control. * $P < 0.05$, ** $P < 0.001$. Data are presented as the mean $\pm$ SD.

Figure 3

PDX1 gene is a target of miR-765 in pancreatic β -cells. (A) miR-1258, miR-583 and miR-765 were overlapped from GSE26168, TargetScan and miRwalk. GSE26168 was a miRNA expression profile involving T2D. TargetScan and miRwalk were the online tools for the prediction of miRNAs targeting PDX1. (B) miR-765 was dramatically up-regulated in peripheral blood of patients with type 2 diabetes (T2D). Normal (n=40): peripheral blood from 40 healthy volunteers, T2D (n=40): peripheral blood from 40 patients with type 2 diabetes. (C) Correlation between PDX1 mRNA expression and miR-765 mRNA expression ($R^2=0.6104$, $P<0.0001$). (D) PDX1 wild type containing the predicated miR-765 binding site "CCUUC" and PDX1 mutant type containing the predicated miR-765 binding site replaced by "GGAAGG". (E) Luciferase reporter assay was conducted to determine the target relationship between miR-765 and PDX1. (F) Correlation between miR-765 level and blood glucose determined by Spearman correlation analysis ($R^2=0.5329$, $P<0.0001$). (G) miR-765 mRNA expression in the glucose-induced INS-1E cells. Normal: INS-1E cells cultured with 11 mM glucose, Model: INS-1E cells cultured with 25 mM glucose.

PDX1-WT: wild type PDX1 3'UTR, PDX1-MUT: mutant type PDX1 3'UTR. *P < 0.05, **P < 0.001. Data are presented as the mean ± SD.

Figure 4

miR-765 exerts detrimental impact on the survival and function of pancreatic β-cells through mediating PDX1. (A) Transfection efficiency of miR-765 inhibitor in INS-1E cells and PDX1 mRNA expression was detected by qRT-PCR after the transfection of miR-765 inhibitor or si-PDX1 plus miR-765 inhibitor. (B) PDX1 protein expression was detected by western blotting after the transfection of miR-765 inhibitor or si-PDX1 plus miR-765 inhibitor. (C, D) miR-765 inhibitor or si-PDX1 plus miR-765 inhibitor was transfected into INS-1E cells for 24 h. Insulin content (C) and insulin secretion (D) were determined by glucose-stimulated insulin secretion assay. (E) Cell viability of INS-1E cells was detected by CCK-8 assay after transfection of miR-765 inhibitor or si-PDX1 plus miR-765 inhibitor. (F) Cell apoptosis of INS-1E cells was detected by flow cytometry after transfection of miR-765 inhibitor or si-PDX1 plus miR-765 inhibitor. Inhibitor: miR-765 inhibitor, Si+Inhibitor: si-PDX1 plus miR-765 inhibitor. CON: blank control, NC: negative

control. *P < 0.05, **P < 0.001 versus control group. #P < 0.05, ##P < 0.001 versus Si+Inhibitor group. Data are presented as the mean $\pm$ SD.