MicroRNA-19a-3p enhances the proliferation and insulin secretion, while it inhibits the apoptosis of pancreatic β cells via the inhibition of SOCS3

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Abstract. MicroRNAs (miRNAs or miRs), a group of small non-coding RNAs, have been demonstrated to play key roles in various physiological processes and diseases, including diabetes, the most common metabolic disorder. However, the underlying mechanisms remains largely unknown. In this study, we aimed to investigate the role of miR-19a-3p in diabetes. The results of RT-qPCR demonstrated that the level of miR-19a-3p was significantly decreased in the diabetic patients, and that the decreased miR-50a-5p level was significantly associated with a high concentration of blood glucose. miR-19a-3p mimic was further used to transfect pancreatic β cells, and we found that the overexpression of miR-19a-3p promoted cell proliferation and insulin secretion, while it suppressed the apoptosis of pancreatic β cells. Suppressor of cytokine signaling 3 (SOCS3) was further identified as a direct target gene of miR-19a-3p, and its protein level was significantly decreased following the overexpression of miR-19a-3p. Moreover, the siRNA-induced downregulation of SOCS3 also enhanced cell proliferation and insulin secretion, while it inhibited the apoptosis of pancreatic β cells. In addition, the overexpression of SOCS3 reversed the effects of miR-19a-3p overexpression on cell proliferation, insulin secretion and on the apoptosis of pancreatic β cells, which further indicates that SOCS3 acts as a downstream effector in the miR-19a-3p-mediated function of pancreatic β cells. Finally, the level of SOCS3 was increased in diabetic patients, and inversely correlated with the miR-19a-3p level, suggesting that the downregulation of miR-19a-3p leads to the upregulation of SOCS3, which contributes to the dysfunction of pancreatic β cells. On the whole, the findings of this study suggest that miR-19a-3p plays an important role in β cell function, and that the miR-19a-3p/SOCS3 axis may become a potential therapeutic target for diabetes.

Introduction

Diabetes mellitus, which is a complex metabolic disease, is characterized by increased blood glucose levels, which are caused by the lack of insulin production or resistance to insulin. The most common forms are type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is mainly caused by autoimmune β cell destruction-induced insulin deficiency, while T2D often results from defects in insulin sensitivity and β cell dysfunction. However, the molecular mechanisms through which β cell dysfunction occurs have not yet been fully elucidated. Thus, the understanding of the underlying mechanisms may aid in the development of novel therapeutic strategies for diabetes.

MicroRNAs (miRNAs or miRs), a class of non-coding RNAs, 18-25 nucleotides in length, are able to suppress gene expression by targeting the complementary regions of mRNAs and inhibiting protein translation. By negatively mediating their target genes, miRs act as key regulators in a variety of physiological, pathological and biological processes, including development, metabolic disorders and tumorigenesis, as well as diseases, such as diabetes. Deregulations of miRs have been observed in patients with diabetes, and specific miRs have been demonstrated to be involved in the regulation of pancreatic development and function. For instance, Jacovetti et al found that β cell maturation was associated with alterations in the expression of miRs induced by the nutritional transition that occurs at weaning, and suggested that miRs play a central role in post-natal β cell maturation and in the determination of adult functional β cell mass. The serum levels of miR-15a have been shown to be reduced in patients with T2D and in individuals with impaired fasting glucose (IFG)/impaired glucose tolerance (IGT), and a lower miR-15a expression has been shown to be significantly associated with T2D and pre-diabetes. However, the miR-mediated effectors or signaling pathways that play key roles in diabetes have not yet been fully investigated.

miR-19a-3p, a member of the miR-17-92 miR cluster, has been previously found to be a regulator of the expression of 5-lipoxygenase, a key enzyme in leukotriene biosynthesis. Moreover, it has been previously suggested to be associated with several types of human cancer, such as breast cancer, astrocytoma, gastric cancer, skin cancer and colorectal adenocarcinoma. Recently, miR-19a-3p was found to be upregulated in patients with gestational diabetes mellitus.
However, its levels have been shown to be downregulated in the livers of db/db mice, and miR-19a-3p has been suggested to promote glycosisgenesis in hepatocytes through the downregulation of phosphatase and tensin homolog (PTEN) expression (21). Furthermore, miR-19a has been shown to promote cell proliferation and angiogenesis by regulating the PI3K/AKT pathway associated with diabetes-associated pancreatic cancer (22-24). However, the detailed role of miR-19a-3p in the regulation of β cell proliferation and function, as well as the underlying mechanisms, remain largely unknown.

In this study, we aimed to examine the role of miR-19a-3p in diabetes. We found that miR-19a-3p was significantly downregulated in diabetic patients, and that it enhanced cell proliferation and insulin secretion, while it inhibited the apoptosis of pancreatic β cells by directly targeting suppressor of cytokine signaling 3 (SOCS3). Accordingly, we suggest that miR-19a-3p may serve as a potential candidate for the clinical management of diabetes.

Materials and methods

Blood sample collection. This study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China), and was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study subjects prior to enrollment. Blood samples from patients with T2D (n=45) and normal subjects (n=20) were collected from the First Affiliated Hospital of Chongqing Medical University from November, 2012 to February, 2014. Patients with serious liver or kidney diseases, malignancy and acute heart failure were excluded.

Determination of glucose and SOCS3 levels in blood samples. The glucose levels in the blood samples were determined by the routine laboratory method. The blood glucose levels were examined at the Department of Clinical Laboratory of our hospital using the Glucose Assay kit (BioVision, San Francisco, CA, USA), according to the manufacturer's instructions. Plasma SOCS3 levels were analyzed by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions using the SOCS-3 ELISA kit (Ybiotech, Shanghai, China), siRNA targeting SOCS3 (SOCS3 siRNA), non-specific siRNA (NC siRNA), the pc-DNA3.1-SOCS3 plasmid and pc-DNA3.1 vector (NC), and Lipofectamine 2000 were diluted with OPTI-MEM (both from Life Technologies). The diluted Lipofectamine 2000 was added to the respective diluted plasmid, miR, or siRNA. Following incubation at room temperature for 20 min, the above mixture was added to the cell suspension, which was then incubated at 37°C, 5% CO₂ for 6 h. Subsequently, the transfection mixture was replaced by DMEM with 10% FBS. Untransfected cells were used as controls. Following transfection for 48 h, the following assays were conducted:

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using TRIzol reagent (Life Technologies). RT-qPCR was used to examine the relative miR-19a-3p expression using the mirVana™ real-time RT-PCR microRNA detection kit (Life Technologies), in accordance with the manufacturer's instructions. U6 was used as an internal reference. The specific primers for miR-19a-3p and U6 were purchased from Genecopoeia, Guangzhou, China. The relative mRNA expression of SOCS3 was detected by quantitative PCR (qPCR) using the standard SYBR-Green RT-PCR kit (Takara, Otsu, Japan) in accordance with the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. The specific primers for SOCS3 were as follows: forward, 5′-ATGGTCACCCACAGCAAGTTT-3′ and reverse, 5′-TCC AGTAGAATCCGCTCTCCT-3′. The specific primers for GAPDH were as follows: forward, 5′-TTGCCTCCTCGGT TTCCCTAC-3′ and reverse, 5′-GAGTTGCTTTGAAGT CGCA-3′. The relative expression level was quantified using the the 2^ΔΔCt method.

Western blot analysis. The cells were lysed in the protein lysis buffer [50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 1% NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% sodium dodecylsulfate, 1% PMSF and 1X phosphatase inhibitor cocktail]. The protein concentration was determined using the BCA Protein assay kit (Pierce Chemical, Rockford, IL, USA). Protein (60 µg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a PVDF membrane (Life Technologies), and then blocked in 5% non-fat dried milk (Yili, Beijing, China) in TBST for 2 h. The PVDF membrane was then incubated with primary antibodies against SOCS3 (ab16030) and GAPDH (ab37168; both from Abcam,
**Bioinformatics analysis.** Bioinformatics analysis was conducted to predict the putative targets of miR-19a-3p using TargetScan 4.2 online software (www.targetscan.org).

**Detection of glucose-stimulated insulin secretion.** The cells were seeded in a 96-well plate and cultured overnight. The cells were then treated with basal glucose (3.3 mmol/l) or stimulatory glucose (16.7 mmol/l) for 1 h. Subsequently, the insulin level was measured by ELISA. In brief, the cells in each well were sonicated in acid ethanol, followed by 3 freeze/thaw cycles, and then centrifuged for 5 min at 10,000 x g. The supernatant was used to measure the insulin level by ELISA as described above.

**Determination of cell proliferation.** The cells (2x10^5) in each group (miR-NC, miR-19a-3p mimic, SOCS3 siRNA, NC siRNA, miR-19a-3p mimic + SOCS3, ormiR-19a-3p mimic + NC vector) were plated into a 96-well plate and cultured for 1 to 5 days at 37˚C with 5% CO_2_. At each time point, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Sigma, St. Louis, MO, USA) were added. Following incubation at 37˚C for 4 h, 150 µl of dimethyl sulfoxide (DMSO) were added. Following incubation at room temperature for 10 min, the formazan production was detected by determining the optical density (OD) at 570 nm using the Elx800 enzyme immunoassay analyzer (BioTek Instruments, Inc., Winooski, VT, USA).

**Apoptosis assay.** The cell apoptotic levels were examined using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. The cells in each group were re-suspended in 1X binding buffer solution with Annexin V-FITC and PI and incubated for 15 min at room temperature in the dark. The apoptotic rate was determined using the EPICS Altra flow cytometer (Beckman Coulter, Brea, CA, USA).

**Luciferase reporter assay.** The wild-type (WT) of SOCS3 3'-UTR was constructed by PCR and inserted into the pMIR-REPORT miRNA Expression Reporter vector (Ambion, Carlsbad, CA, USA). The mutant type (MT) of SOCS3 3'-UTR was constructed using the Easy Mutagenesis system kit (Promega, Madison, WI, USA), in accordance with the manufacturer's instructions, and inserted into the pMIR-REPORT miRNA Expression Reporter vector. 293 cells (Cell Bank of the Chinese Academy of Sciences) were plated in 96-well plates, and co-transfected with the WT SOCS3-3’UTR or MT SOCS3-3’UTR plasmid (300 ng), and miR-NC or miR-19a-3p mimic (100 nM), using Lipofectamine 2000, in accordance with the manufacture's instructions. The dual-luciferase reporter assay system (Promega) was used to determine the activity of Renilla luciferase and Firefly luciferase following co-transfection for 48 h. The Renilla luciferase activity was normalized to the Firefly luciferase activity.

**Statistical analysis.** The data of at least 3 independent experiments are expressed as the means ± SD. GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analysis. The differences were analyzed using the Student's t-test or one-way ANOVA. The association between the miR-19a-3p level and the blood glucose or SOCS3 levels was analyzed using Spearman's correlation coefficient. A value of P<0.05 was considered to indicate a statistically significant difference.

**Results**

**miR-19a-3p expression is significantly downregulated in diabetic patients.** To reveal the function of miR-19a-3p in diabetes, we first conducted RT-qPCR to examine the plasma miR-19a-3p level in diabetic patients and normal subjects. Our data demonstrated that the miR-19a-3p level was significantly decreased in the blood of diabetic patients, when compared with that of normal subjects (Fig. 1A). In addition, we observed a significant inverse correlation between the plasma miR-19a-3p level and the blood glucose concentration among these diabetic patients (Fig. 1B). These data suggest that the downregulation of miR-19a-3p expression plays a role in the progression of diabetes.

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glucose-responsive pancreatic β cell lines, INS-1 and MIN6, were used in our in vitro experiments to investigate the exact role of miR-19a-3p in pancreatic β cells. As miR-19a-3p was found to be significantly downregulated in diabetic patients, we transfected the INS-1 and MIN6 cells with miR-19a-3p mimic to restore its expression level. Following transfection, qPCR was conducted to examine the expression level of miR-19a-3p. Our data indicated that the level of miR-19a-3p was significantly higher in the cells transfected with miR-19a-3p mimic compared to the control or negative control (NC)-transfected cells.

Figure 2. RT-qPCR was used to detect the miR-19a-3p level in (A) INS-1 and (B) MIN6 cells transfected with scramble miR mimic (miR-NC) or miR-19a-3p mimic. Untransfected INS-1 or MIN6 cells were used as a control. (C and D) Glucose-stimulated insulin secretion was then determined in each group. (E and F) MTT assay and (G and H) flow cytometry were then performed to examine cell proliferation and apoptosis. **P<0.01 vs. INS-1 or MIN6 untransfected cells (bars labeled as INS-1 or MIN6) or negative control (NC)-transfected cells.
further examined the effect of miR-19a-3p on the proliferation and apoptosis of pancreatic β cells. Our data indicated that the overexpression of miR-19a-3p markedly enhanced cell proliferation (Fig. 2E and F), while it suppressed the apoptosis of the INS-1 and MIN6 cells (Fig. 2G and H), when compared with the control and NC groups. Based on these data, it is thus evident that miR-19a-3p enhances the proliferation and insulin secretion, while it inhibits the apoptosis of pancreatic β cells.

**SOCS3 is a target gene of miR-19a-3p in pancreatic β cells.** As miRs function by regulating the expression of their target genes, we then used TargetScan software to analyze the putative target genes of miR-19a-3p. SOCS3 was predicted to be a potential target gene of miR-19a-3p with evolutionary conservation (Fig. 3A and B). To verify their targeting relationship, the WT SOCS3 3'-UTR containing the predicted miR-19a-3p binding sites or the mutant type (MT) SOCS3 3'-UTR without the predicted miR-19a-3p binding sites were subcloned into the pMiR-REPORT miRNA Expression Reporter vector. (E) Luciferase reporter data indicated that co-transfection with the WT SOCS3 3'-UTR vector and miR-19a-3p mimic caused a significant decrease in luciferase activity; however, the luciferase activity was not altered in the 293 cells co-transfected with the MT SOCS3 3'-UTR vector and miR-19a-3p mimic. **P<0.01 vs. controls (untransfected cells or miR ‑NC‑transfected cells). (F and G) The protein expression of SOCS3 was examined by western blot analysis in INS-1 and MIN6 cells transfected with negative control miR mimic (miR ‑NC) or miR-19a-3p mimic. **P<0.01 vs. INS‑1 or MIN6 untransfected cells (bars labeled as INS ‑1 or MIN6) or negative control (NC)‑transfected cells.
After that, we analyzed the correlation between the plasma level of miR-19a-3p and the blood glucose concentration among the diabetic patients, suggesting that the dysregulation of miR-19a-3p is associated with the progression of diabetes. INS-1 and MIN6 are glucose-responsive murine pancreatic β cells. Therefore, to further reveal the role of miR-19a-3p in pancreatic β cell function, the INS-1 and MIN6 cells were transfected with miR-19a-3p mimic. We found that the overexpression of miR-19a-3p promoted the proliferation and insulin secretion, while it suppressed the apoptosis of INS-1 and MIN6 cells, which further supports the notion that miR-19a-3p plays a role in diabetes, consistent with the clinical data.

Previous studies have mainly focused on the role of miR-19a-3p in human cancers. Yang et al demonstrated that miR-19a-3p inhibited breast cancer progression and metastasis by inducing macrophage polarization through the targeting of the oncogene, Fra-1 (15). These data indicate that miR-19a-3p participates in the regulation of cell motility. Moreover, miR-19a-3p expression has been shown to be significantly downregulated following multifractionated radiation in breast cancer cells, and that it may contribute to radiosensitivity and can be used as a biomarker for radiotherapy (26). Furthermore, miR-19a-3p has been shown to be downregulated in non-melanoma skin cancer, suggesting a tumor suppressor role (18). On the contrary, a high serum level of miR-19a-3p was found to be significantly associated with a poor survival of astrocytoma patients, suggesting that it may play an oncogenic role in astrocytoma (16). In addition, Zheng et al identified a four-miRNA panel, including miR-19a-3p, miR-223-3p, miR-92a-3p and miR-422a with a high diagnostic accuracy for early-stage
Figure 4. Western blot analysis was used to detect the protein expression of SOCS3 in (A) INS-1 and (B) MIN6 cells transfected with negative control miR mimic (miR-NC) or miR-19a-3p mimic. Untransfected INS-1 or MIN6 cells were used as controls. (C and D) Glucose-stimulated insulin secretion was then determined in each group. (E and F) MTT assay and (G and H) flow cytometry were then performed to examine cell proliferation and apoptosis. *P<0.01 vs. INS-1 or MIN6INS-1 or MIN6 untransfected cells (bars labeled as INS-1 or MIN6) or negative control (NC)-transfected cells.
Figure 5. Western blot analysis was used to detect the protein expression of SOCS3 in (A) INS-1 and (B) MIN6 cells transfected with miR-19a-3p mimic, or co-transfected with miR-19a-3p mimic and SOCS3 expression plasmid. (C and D) Glucose-stimulated insulin secretion was then determined in each group. (E and F) MTT assay and (G and H) flow cytometry were then performed to examine cell proliferation and apoptosis. **P<0.01 vs. cells transfected with miR-19a-3p and the negative control vector (bars labeled as miR-19a-3p).
cololectal adenocarcinoma (19). In the present study, we found that miR-19a-3p promoted the proliferation and insulin secretion, while it suppresses the apoptosis of pancreatic β cells. Jiang et al reported that miR-19a protected endothelial cells from lipopolysaccharide (LPS)-induced apoptosis through the apoptosis signal-regulating kinase 1 (ASK1)/p38 pathway (27). Therefore, the ASK1/p38 pathway may also be involved in the protective effects of miR-19a-3p against pancreatic β cell apoptosis; this warrants further investigation in future studies.

SOCS3 is a member of the SOCS family, which are negative regulators of cytokine signal transduction and inhibit the cytokine-induced activation of signal transducer and activator of transcription (Stat) signaling (28). SOCS3 has been demonstrated to be associated with the development of leptin resistance and the inhibition of insulin (29,30). The muscle-specific overexpression of SOCS3 in mice has been shown to lead to impaired systemic and muscle-specific glucose homeostasis and insulin function, as well as to decreased basal and leptin-stimulated activity and the phosphorylation of α2 AMP-activated protein kinase (α2AMPK) and acetyl-CoA carboxylase (31). Moreover, muscle SOCS3 overexpression also suppresses leptin-regulated genes involved in fatty acid oxidation and mitochondrial function (31). In the present study, we identified SOCS3 as a direct target gene of miR-19a-3p, and the overexpression of miR-19a-3p led to a significant decrease in the protein level of SOCS3. Moreover, we found that the effects of miR-19a-3p on the proliferation, apoptosis and insulin secretion of pancreatic β cells occurred directly through the targeting of SOCS3. it has also previously been reported that SOCS3 is involved in the regulation of pancreatic cell proliferation and apoptosis. The restoration of SOCS3 expression using a demethylating agent (5-aza-2'-deoxycytidine), was shown to markedly suppress the proliferation and to induce the apoptosis of methylated pancreatic cells (32). In addition, we observed a negative correlation between miR-19a-3p expression and the SOCS3 level in the plasma samples of patients with diabetes, which further supports the notion that the protective role miR-19a-3p in diabetes at least partly involves the inhibition of SOCS3 expression.

In conclusion, the findings of the present study demonstrate that miR-19a-3p significantly downregulated in diabetic patients, and that its expression is inversely correlated with the blood glucose concentration. Moreover, our data indicate that miR-19a-3p significantly enhances the proliferation and insulin secretion, while it inhibits the apoptosis of pancreatic β cells (INS-1 and MIN6) by directly targeting SOCS3. Accordingly, miR-19a-3p may become a potential candidate for the treatment of diabetes. However, further studies are warranted to verify our findings.

References


