

microRNA-199a-5p mediates high glucose-induced reactive oxygen species production and apoptosis in INS-1 pancreatic β -cells by targeting SIRT1

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Abstract. – **OBJECTIVE:** Hyperglycemia-induced pancreatic β -cell loss is a pathologic hallmark of type 2 diabetes mellitus (T2DM). This study was conducted to clarify the function of microRNA (miR)-199a-5p in high glucose-elicited β -cell toxicity and associated molecular mechanisms.

MATERIALS AND METHODS: INS-1 rat pancreatic β -cells were cultured under normal (11 mM) or high (30 mM) glucose for 16-72 h and examined for miR-199a-5p expression. Gain- and loss-of-function studies were performed to determine the role of miR-199a-5p in high glucose-induced apoptosis and reactive oxygen species (ROS) production. Additionally, the mechanism of SIRT1 in the action of miR-199a-5p was checked.

RESULTS: High glucose caused a significant upregulation of miR-199a-5p in INS-1 cells compared to cells under normal glucose conditions. Pre-transfection with miR-199a-5p inhibitors prevented the reduction in cell viability and inhibited ROS generation in INS-1 cells after high glucose treatment. In contrast, overexpression of miR-199a-5p significantly reduced cell viability and promoted apoptosis and ROS formation in INS-1 cells, which was coupled with a downregulation of SIRT1. Knockdown of SIRT1 led to apoptosis and death of INS-1 cells. Moreover, enforced expression of SIRT1 blocked miR-199a-5p-induced ROS generation and attenuated high glucose-mediated apoptosis in INS-1 cells.

CONCLUSIONS: miR-199a-5p is upregulated in pancreatic β -cells in response to high glucose and promotes apoptosis and ROS generation by targeting SIRT1. The miR-199a-5p/SIRT1 axis may represent a promising target for the treatment of T2DM.

Key Words:

Apoptosis, Hyperglycemia, miR-199a-5p, Oxidative stress, SIRT1.

Introduction

Pancreatic β -cell dysfunction and loss is a pathologic hallmark of type 2 diabetes mellitus (T2DM)¹. Chronic exposure to hyperglycemia is an important cause of the deterioration of pancreatic β -cells². High-glucose-induced β -cell toxicity has been closely linked to the production of excessive reactive oxygen species (ROS)³. Several antioxidant agents were reported to confer protection against glucotoxicity in pancreatic β -cells⁴. In this respect, inhibition of ROS generation may represent a promising strategy to treat T2DM.

microRNAs (miRNAs) are a class of endogenous, small non-coding RNAs that play important roles in the pathogenesis of T2DM⁵. In murine models, β -cell-specific overexpression of miR-200 causes β -cell death and lethal T2DM, whereas depletion of miR-200 prevents β -cell apoptosis and alleviates T2DM⁶. It has been shown that both miR-101a and miR-30b are implicated in inflammatory cytokine-induced dysfunction of β -cells⁷. Another study⁸ demonstrated that miR-19a-3p can augment cell proliferation and insulin secretion and reduce apoptosis in pancreatic β -cells. miR-199a-5p has a wide range of biological activities, including anticancer⁹, osteogenic¹⁰, and anti-inflammation¹¹. Arsenic-transformed bronchial epithelial cells showed downregulation of miR-199a-5p via alteration of ROS formation¹², suggesting a link between miR-199a-5p and oxidative stress. Several miRNA expression-profiling studies¹³ have reported that miR-199a-5p is deregulated in animal models of T2DM. However, the role of miR-199a-5p in the pathogenesis of T2DM is still unclear. In this work, we used an INS-1 rat pancreatic β -cell

model and investigated the expression and function of miR-199a-5p in response to high glucose. The target genes mediating the activity of miR-199a-5p were identified and functionally characterized.

Materials and Methods

Cell Culture and Treatment

INS-1 rat pancreatic β -cells were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). They were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), streptomycin (100 μ g/mL), and penicillin (100 units/mL). For high glucose treatment, INS-1 cells were exposed to 30 mM of glucose for 16–72 h. Cells cultured in fresh RPMI-1640 medium containing 11 mM glucose were used as a control.

Quantification of Mature miR-199a-5p Expression

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized using a specific stem-loop primer. Quantitative real-time PCR (qRT-PCR) was carried out on the Applied Biosystems StepOne Plus Detection System (Applied Biosystems, Foster City, CA, USA). The Taqman microRNA assay (Applied Biosystems) was used to detect mature miR-199a-5p expression. The results were normalized to the level of U6.

Plasmids and Oligonucleotides

A fragment containing miR-199a-5p precursor was yielded by PCR amplification of genomic DNA and cloned into the pSUPER expression vector. Full-length human cDNA of SIRT1 lacking the 3' untranslated region (3'-UTR) was purchased from OriGene (Rockville, MD, USA) and cloned into pcDNA3.1(+) expression vector. Locked nucleic acid (LNA)-modified anti-miR-199a-5p inhibitor and negative control oligonucleotides were purchased from Exiqon (Vedbaek, Denmark). Small interfering RNA (siRNA) targeting *SIRT1* gene and negative control siRNA were provided by GenPharm (Shanghai, China).

Cell Transfection

INS-1 cells were seeded at a density of 5×10^3 cells/well in 96-well plates or 2×10^5 cells/well in

24-well plates. Cells were individually transfected with miR-199a-5p-expressing plasmid (1 μ g), *SIRT1* siRNA (50 nM), and corresponding controls using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). In some experiments, cells were pre-transfected with anti-miR-199a-5p inhibitors (40 nM) or pcDNA3.1/SIRT1 plasmid (1 μ g) 24 h before high glucose treatment. To reverse the effect of miR-199a-5p, cells were co-transfected with miR-199a-5p-expressing plasmid (0.2 μ g) and pcDNA3.1/SIRT1 plasmid (1 μ g) 24 h before ROS measurement.

MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done to determine cell viability. In brief, 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells. After incubation for 4 h at 37°C, dimethyl sulfoxide (DMSO) was used to dissolve the formazan. Absorbance was recorded at a wavelength of 570 nm.

Apoptosis Detection by Flow Cytometry

Cell apoptosis was detected using the Annexin-V/propidium iodide (PI) Apoptosis Detection kit (Becton Dickinson Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. After staining with FITC-conjugated annexin-V and PI, apoptotic cells were analyzed by a flow cytometer using the CellQuest software (Becton Dickinson Biosciences, San Jose, CA, USA).

Western Blot Analysis

Cells were lysed in ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% NP-40, and 0.5% sodium deoxycholate) supplemented with proteinase inhibitors (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using the Bradford assay. Protein samples (40 μ g) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated at 4°C overnight with primary antibodies (1: 400 dilution) against cleaved caspase-3 and -9, Bcl-2, SIRT1, and β -actin (Cell Signaling Technology, Beverly, MA, USA). After washing three times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA). Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

Measurement of ROS Production

Measurement of ROS formation was performed as described previously¹⁵. A permeable probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used in this assay, which can be oxidized to generate the fluorescent product dichlorofluorescein (DCF). Cells were incubated with 20 μ M of DCFH-DA for 15 min at 37°C in the dark. After washing, cells were re-suspended in phosphate buffered saline and analyzed by flow cytometry.

Statistical Analysis

Data are expressed as means $\pm$ standard deviation (SD). Statistical differences were determined using the Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey's test. *p* < 0.05 were considered statistically significant.

Results

miR-199a-5p Mediates High Glucose-Induced Toxicity in INS-1 Cells

qRT-PCR analysis revealed that high glucose-treated INS-1 cells displayed an induction of miR-199a-5p expression, compared to control cells under normal glucose conditions (*p* < 0.05; Figure 1A). The levels of miR-199a-5p were elevated by 1.8- and 3.2-fold after exposure to high glucose for 16 and 72 h, respectively. To determine the role of miR-199a-5p in high glucose-induced toxicity in INS-1 cells, we transfected with anti-miR-199a-5p inhibitors before exposure to high glucose. MTT assay showed that high glucose treatment for 72 h led to a 54% decline in the viability of INS-1 cells (*p* < 0.05; Figure 1B). Pre-transfection with anti-miR-199a-5p inhibitors significantly prevented the toxic effect of high glucose on INS-1 cells. Next, we asked whether overexpression of miR-199a-5p could affect the viability of INS-1 cells. Notably, miR-199a-5p-overexpressing INS-1 cells had a 42% lower viability than empty vector-transfected cells (*p* < 0.05; Figure 1C). These results suggest that miR-199a-5p is involved in high glucose-induced toxicity in INS-1 cells.

Enforced Expression of miR-199a-5p Triggers Apoptosis in INS-1 Cells

Next, we investigated the effect of miR-199a-5p overexpression on the apoptosis of INS-1

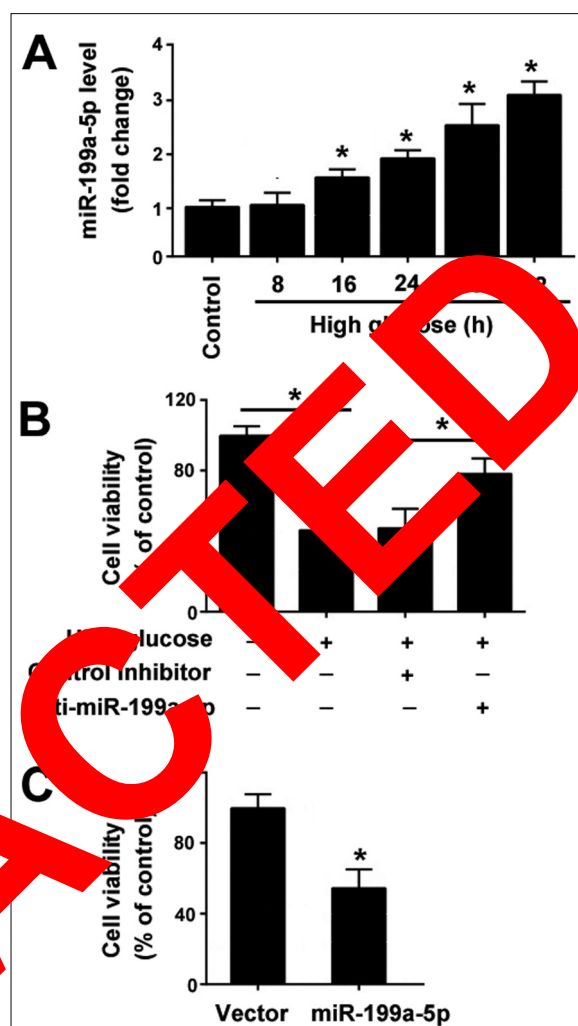


Figure 1. miR-199a-5p mediates high glucose-induced toxicity in INS-1 cells. **A**, Measurement of miR-199a-5p levels in high glucose-treated INS-1 cells by qRT-PCR analysis. **p* < 0.05 vs. control cells under normal glucose conditions. **B**, MTT assays were done to measure the viability of INS-1 cells pre-transfected with control or anti-miR-199a-5p inhibitors before high glucose treatment for 72 h. **p* < 0.05. **C**, Measurement of the viability of INS-1 cells transfected with vector or miR-199a-5p-expressing plasmid by MTT assays. **p* < 0.05 vs. vector-transfected cells.

cells. Flow cytometric analysis showed that miR-199a-5p-overexpressing INS-1 cells had a significantly greater percentage of apoptotic cells than empty vector-transfected cells ($21.2 \pm 1.9\%$ vs. $4.8 \pm 0.8\%$, *p* < 0.05; Figure 2A). Western blot analysis confirmed that miR-199a-5p overexpression led to an enhancement of caspase-3 and caspase-9 cleavage, as well as decrease in Bcl-2 expression (Figure 2B).

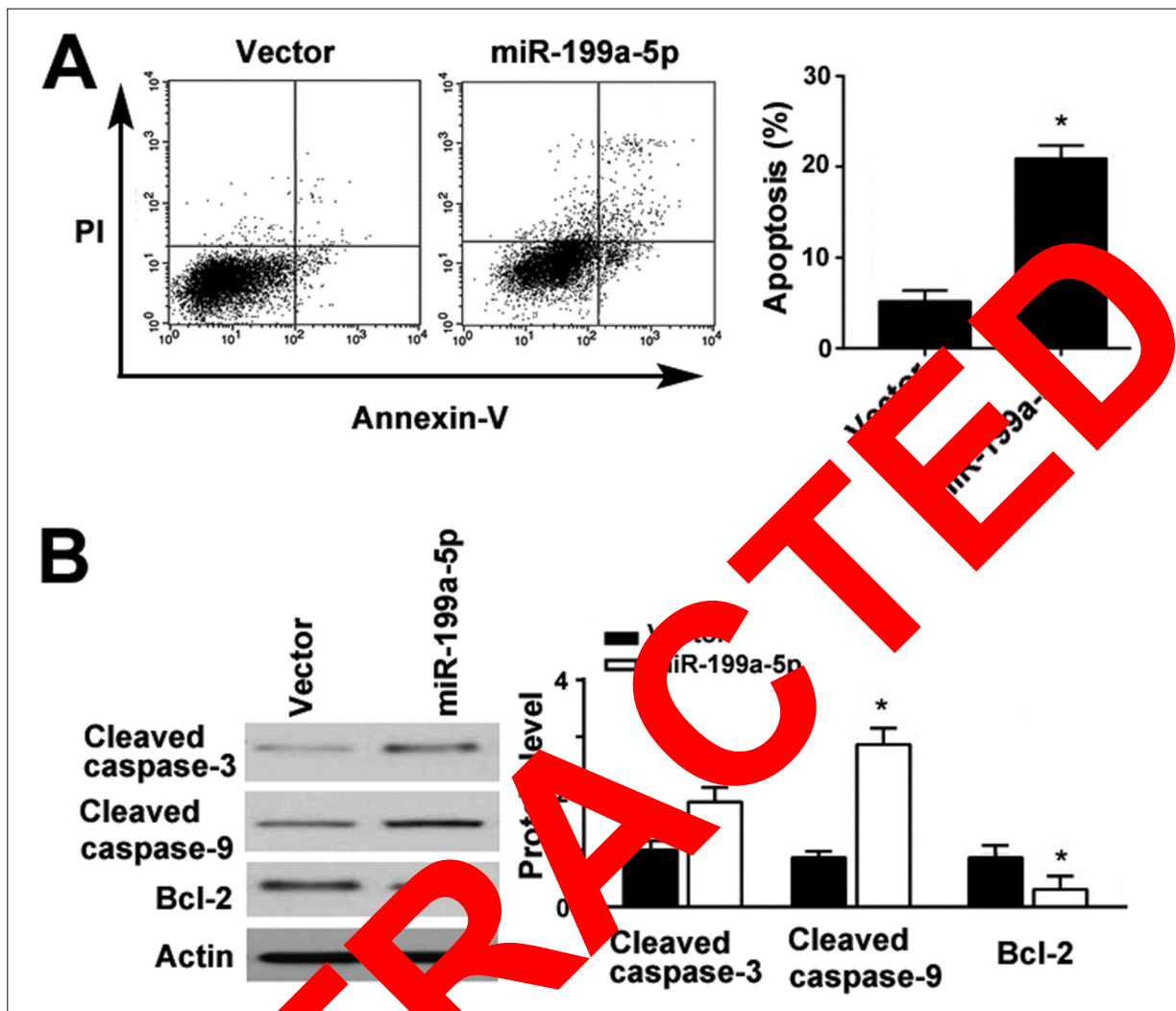


Figure 2. Enforced expression of miR-199a-5p triggers apoptosis in INS-1 cells. **A**, Flow cytometric analysis of apoptosis in INS-1 cells transfected with vector or miR-199a-5p-expressing plasmid. *Left*, representative flow cytometry dot plots after Annexin-V/PI staining. *Right*, quantification of apoptosis from three independent experiments. **B**, Western blot analysis of indicated proteins in INS-1 cells transfected with vector or miR-199a-5p-expressing plasmid. Bar graphs show densitometric analysis of protein intensities from three independent experiments. * $p < 0.05$ vs. vector-transfected cells.

miR-199a-5p Promotes ROS Production via Targeting SIRT1

Excessive ROS is an inducer of apoptosis in pancreatic β -cells in response to high glucose^{16,17}. We next investigated the role of miR-199a-5p in ROS production. As shown in Figure 3A, there was 8.3-fold increase in ROS levels in miR-199a-5p-overexpressing INS-1 cells, compared to vector-transfected cells ($p < 0.05$). Delivery of anti-miR-199a-5p inhibitors was noted to impair ROS generation in INS-1 cells after high glucose exposure (Figure 3B). These results suggest that miR-199a-5p contributes to high glucose-induced ROS formation.

Next, we attempted to identify the target genes involved in the induction of ROS by miR-199a-5p. It has been reported¹⁸ that SIRT1 is a direct target of miR-199a-5p in the hippocampus. Since SIRT1 shows antioxidant activity in various biological settings^{19,20}, we hypothesized that miR-199a-5p may promote ROS generation in INS-1 cells via targeting of SIRT1. In line with this hypothesis, we showed that enforced expression of miR-199a-5p significantly suppressed the endogenous expression of SIRT1 in INS-1 cells (Figure 4A). Most importantly, overexpression of SIRT1 (Figure 4B) significantly impaired miR-199a-5p-induced ROS formation (Figure 4C).

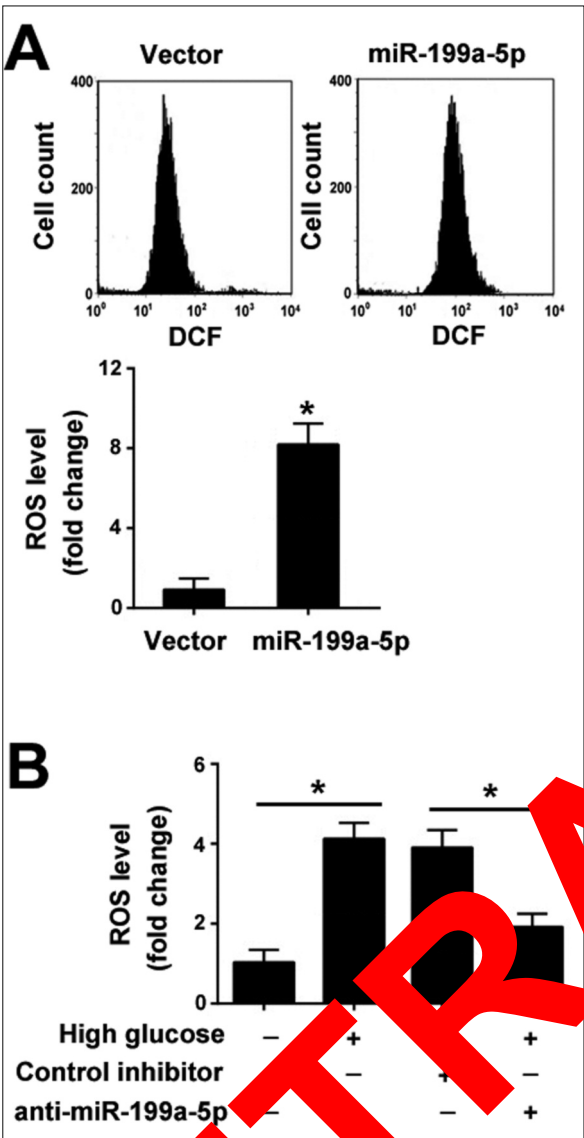


Figure 3. miR-199a-5p contributes to high glucose-induced ROS production. **A**, Measurement of ROS levels in INS-1 cells transfected with vector or miR-199a-5p-expressing plasmid. Representative flow cytometry histograms showing ROS levels determined by DCF fluorescence. Below, quantitative data from three independent experiments. $*p < 0.05$ vs. vector-transfected cells. **B**, Measurement of ROS production in INS-1 cells pre-transfected with vector or anti-miR-199a-5p inhibitors before high glucose treatment for 72 h. $*p < 0.05$.

SIRT1 Downregulation Contributes to High Glucose-Induced Apoptosis in INS-1 Cells

Finally, we clarified the roles of SIRT1 in high glucose-induced an apoptotic response. Similar to high glucose treatment, silencing of SIRT1 (Figure 5A) was found to cause significant apoptosis in INS-1 cells (Figure 5B). In contrast,

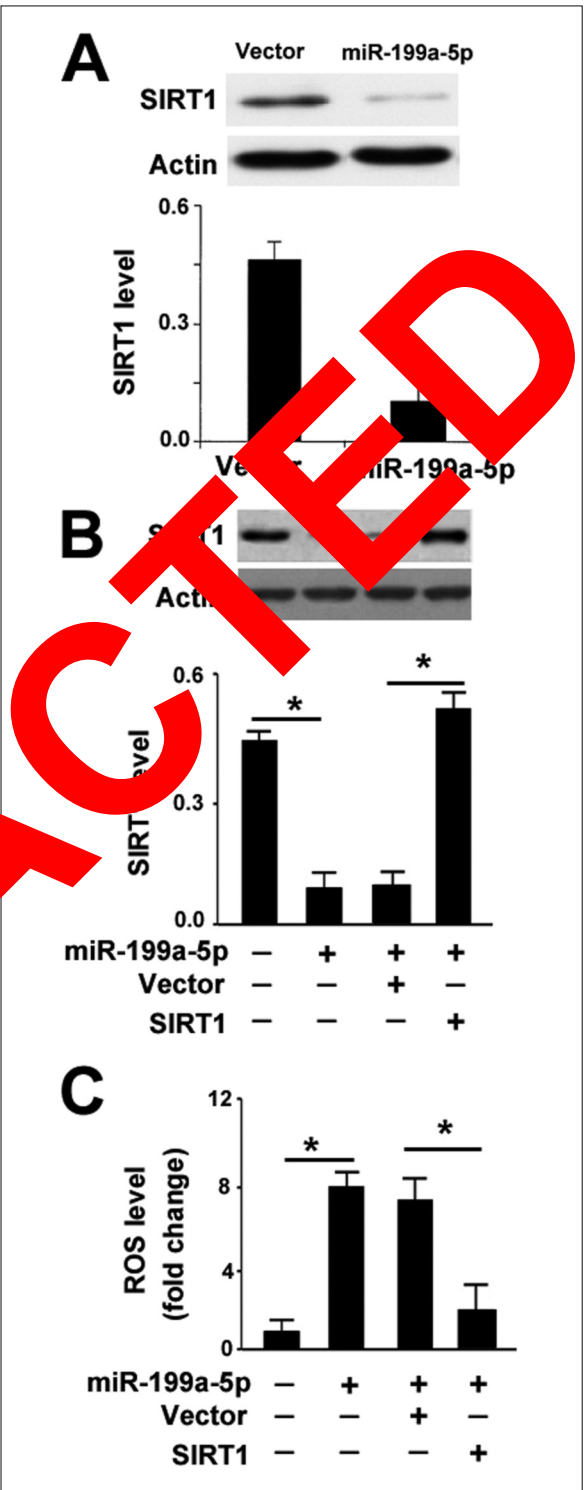


Figure 4. miR-199a-5p promotes ROS production by targeting SIRT1. **A**, Western blot analysis of SIRT1 protein levels in INS-1 cells transfected with vector or miR-199a-5p-expressing plasmid. $*p < 0.05$ vs. vector-transfected cells. **B**, Western blot analysis of SIRT1 protein levels in INS-1 cells transfected with indicated constructs. $*p < 0.05$. **C**, Measurement of ROS production in INS-1 cells transfected with indicated constructs. $*p < 0.05$.

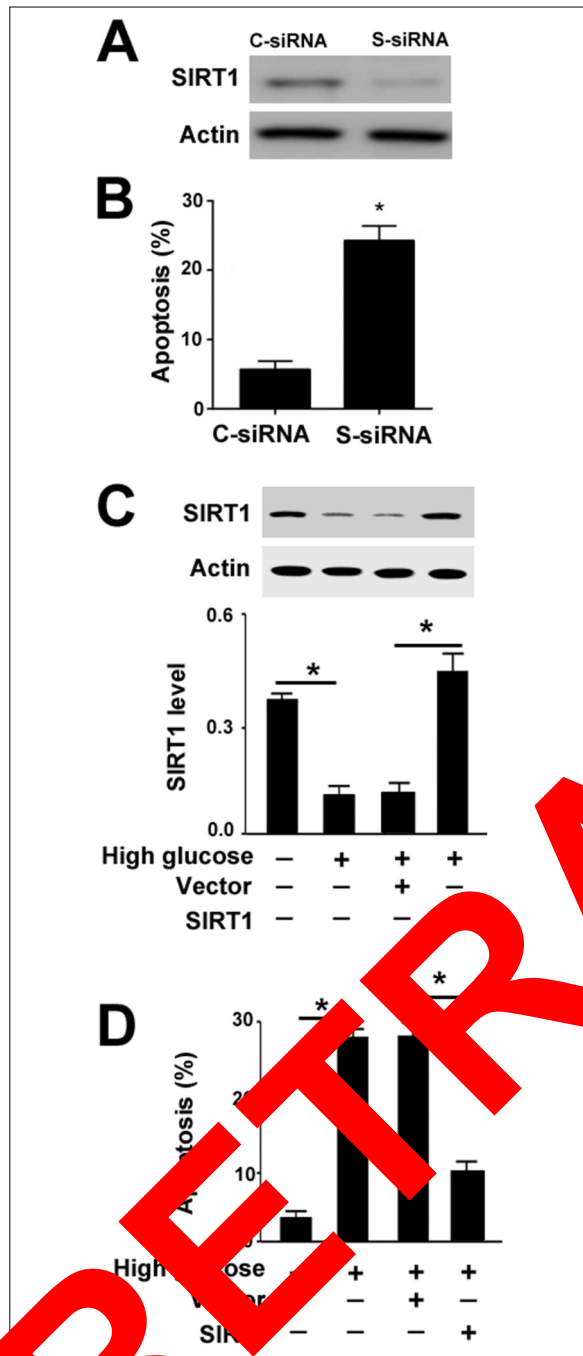


Figure 5. SIRT1 downregulation contributes to high glucose-induced apoptosis in INS-1 cells. **A**, Western blot analysis of SIRT1 protein levels in INS-1 cells transfected with control siRNA (C-siRNA) or SIRT1-targeting siRNA (S-siRNA). **B**, Apoptosis detection in INS-1 cells transfected with C-siRNA or S-siRNA by flow cytometry after Annexin-V/PI staining. * $p < 0.05$ vs. C-siRNA-transfected cells. **C**, Western blot analysis of SIRT1 protein levels in INS-1 cells pre-transfected with vector or SIRT1-expressing plasmid before high glucose treatment. **D**, Flow cytometric analysis of apoptosis in INS-1 cells treated as in **(C)**. Bar graphs represent quantitative data from three independent experiments. * $p < 0.05$.

overexpression of SIRT1 (Figure 5C) decreased the percentage of apoptosis from $28.9 \pm 2.3\%$ to $10.2 \pm 1.9\%$ in high glucose-treated INS-1 cells ($p < 0.05$, Figure 5D), suggesting an involvement of SIRT1 in the survival of β -cells.

Discussion

In this work, we showed that miR-199a-5p was upregulated in INS-1 cells after exposure to high glucose. Functional studies revealed that inhibition of miR-199a-5p attenuated high glucose-induced toxicity. In contrast, overexpression of miR-199a-5p significantly reduced the viability of INS-1 cells. These results indicate that miR-199a-5p functions in pancreatic β -cells to mediate the cytotoxicity induced by high glucose. Consistent with our findings, high glucose-induced upregulation of miR-199a-5p has also been noted in rat mesangial cells, where miR-199a-5p modulates high glucose-elicited fibrotic and inflammatory responses²¹.

Our data further demonstrated that miR-199a-5p overexpression significantly induced apoptosis in INS-1 cells. At the molecular level, there was an increase in the cleavage of caspase-3 and caspase-9 in miR-199a-5p-overexpressing cells. The caspase-9/caspase-3 cascade can be activated by cytochrome c released from the mitochondria²². Bcl-2 acts as an anti-apoptotic protein through blocking mitochondrial cytochrome c efflux²³. Notably, we found that miR-199a-5p overexpression led to a downregulation of Bcl-2 in INS-1 cells. Therefore, miR-199a-5p may activate the mitochondria-dependent apoptotic pathway in INS-1 cells. Accompanying induction of apoptosis, ectopic expression of miR-199a-5p augmented the production of ROS in INS-1 cells. It has been suggested that high glucose exposure triggers apoptotic response in pancreatic β -cells by facilitating ROS formation^{16,17}. Notably, we found that depletion of miR-199a-5p impaired the production of ROS in high glucose-exposed INS-1 cells. These results suggest that induction of ROS formation may account for miR-199a-5p-mediated apoptosis in pancreatic β -cells. SIRT1 has been identified to be a target gene of miR-199a-5p in the hippocampus¹⁸. Consistently, we showed that miR-199a-5p also had the ability to downregulate SIRT1 expression in INS-1 cells. Most importantly, overexpression of SIRT1 reversed the effect of miR-199a-5p on ROS formation. Taken together, we provide evidence that miR-199a-5p promotes ROS generation

in INS-1 cells likely through downregulating SIRT1.

Given the important role of SIRT1 in the regulation of ROS production^{19,20}, we tested the hypothesis that SIRT1 is involved in glucotoxicity in pancreatic β -cells. Interestingly, knockdown of SIRT1 significantly promoted apoptosis in INS-1 cells, which phenocopied the effect of miR-199a-5p overexpression on INS-1 cells. Moreover, overexpression of SIRT1 reduced apoptotic response in INS-1 cells after treatment with high glucose. Altogether, these results suggest that the miR-199a-5p/SIRT1 axis regulates the survival of β -cells in response to high glucose. In agreement with our observations, previous reports showed that overexpression of SIRT1 confers protection against cytokine toxicity²⁴ and hexosamine-induced apoptosis in pancreatic β -cells²⁵.

However, it should be noted that miR-199a-5p can regulate multiple target genes other than SIRT1^{26,27}. It has been reported that FZD6 is negatively regulated by miR-199a-5p in human colorectal cancer cells²⁶. Another study²⁷ demonstrated that miR-199a-5p could target GLUT4 to repress glucose uptake in rat L6 myoblast cells. Therefore, it is possible that miR-199a-5p simultaneously coordinate some target genes including SIRT1, consequently contributing to high glucose-induced β -cell toxicity.

Conclusions

We provide evidence that miR-199a-5p is induced in pancreatic β -cells upon high glucose exposure and facilitates ROS generation and apoptotic death, largely through the negative regulation of SIRT1 expression. Knockdown of miR-199a-5p or overexpression of SIRT1 confers protection against high glucose-induced apoptosis in pancreatic β -cells. The miR-199a-5p/SIRT1 axis may constitute a promising target for reduction of β -cell loss in the treatment of T2DM.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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