

Inhibition of microRNA-9-3p reduces lipid accumulation in HepG2 cells by targeting the expression of sirtuin type 1

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Abstract. Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder caused by the interaction of environmental factors and multiple genes. The genetic background of T2DM is complex and remains to be fully elucidated. MicroRNAs (miRNAs) are negative regulators of gene expression and several miRNAs are associated with the development of T2DM. However, the expression and biological function of miRNA-9-3p in lipid metabolism of patients with T2DM remain to be fully elucidated. The predominant aim of the present study was to examine the effect of miRNA-9-3p on lipid accumulation in HepG2 cells. To investigate this, an MTT assay was used to determine cell proliferation, and the effects of miRNA-9-3p on triglycerides (TG) and total cholesterol (TC) in the HepG2 cells were also examined. Reverse transcription-quantitative polymerase chain reaction and western blot analyses were used to measure the expression levels of SIRT1 at the gene and protein levels, respectively. The data revealed that downregulation of miRNA-9-3p inhibited the proliferation of HepG2 cells, and significantly reduced the accumulation of lipids, and decreased TG and TC content. In addition, the present study demonstrated that inhibition of miRNA-9-3p increased the protein expression of sirtuin type 1 (SIRT1), but had no effects on the gene expression of SIRT1. Therefore, these findings demonstrated that the inhibition of miRNA-9-3p reduced the proliferation of HepG2 cells and

lipid accumulation by upregulating the expression of SIRT1, indicating its potential as a therapeutic target.

Introduction

Diabetes mellitus (DM) is a common endocrine and metabolic disease, and presents a major medical problem worldwide. The World Health Organization has predicted that DM will be the seventh leading cause of mortality by 2030 (1). In addition, it is the underlying cause of microvascular disorders, including macrovascular diseases, diabetic nephropathy and retinopathy (2). With the rapid development of social economy and continuous improvement of living standards, the prevalence of DM continues to increase. Currently, >347,000,000 individuals worldwide have been diagnosed with DM (3). Type 2 DM (T2DM) accounts for 90-95% of all these cases. It leads to a higher risk of chronic kidney disease (4), which may develop into end-stage renal disease and has serious consequences on morbidity and the mortality rates in this population. It is known that effective glycemic control may delay the deterioration in kidney function (5). T2DM is also one of the leading causes of cardiovascular disease, as atherosclerosis is the important pathological mechanism underlying macrovascular disease in T2DM, resulting in major life-threatening events, including myocardial infarction and stroke (6). Furthermore, clinical investigations have suggested that certain intensive therapies are ineffective in the prevention of cardiovascular-associated mortality in T2DM (7). Atherosclerosis in diabetic patients has a complex pathogenesis, which is initiated and modified by environmental risk factors and a number of genes, several of which remain to be elucidated (8). The overall genetic contribution is reported to be ~50-70%, and a substantial number of genes have been reported to be associated with T2DM (9-11). To date, a number of the most important mechanisms have been reported, including lipid metabolism disorders (12), endothelial dysfunction (13), chronic inflammation (14) and imbalance in redox homeostasis (15). Notably, lipid metabolism disorders are critical in atherosclerosis in T2DM patients.

MicroRNAs (miRNAs) represent the largest family of short non-coding RNA molecules, with a length of 19-23 nucleotides (16). It is well known that miRNAs regulate

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Abbreviations: DM, diabetes mellitus; T2DM, type 2 diabetes mellitus; miRNAs, microRNAs; SIRT1, sirtuin type 1; TG, triglycerides; TC, total cholesterol; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

Key words: type 2 diabetes mellitus, microRNA-9-3p, HepG2, sirtuin type 1, triglycerides, total cholesterol

the expression of target genes in a post-transcriptional manner, through incomplete base pairing with the 3'-untranslated region of target mRNAs, inhibiting translation and/or causing mRNA degradation (17). These may contribute to the regulation of a wide variety of cellular functions. It is now well established that miRNAs account for ~1% of predicted genes in higher eukaryotic genomes, and that up to 30% of protein-coding genes may be regulated by miRNAs (18). In addition, previous studies have demonstrated that miRNAs are critical regulators of cell cycle, proliferation, differentiation, development and apoptosis during embryogenesis (16,19,20). As well as in the cell, miRNAs also exist in the circulatory system, therefore, circulating miRNAs in the plasma are expected to become novel biomarkers for molecular diagnostic and therapeutic agents for various disease.

In a previous study (21), high throughput gene chips were used to screen differential gene expression profiles in healthy controls, patients with T2DM and its complication with patients with unstable angina. The findings indicated that the occurrence of T2DM is a complicated and dynamic process affected by various miRNAs. Among the differentially expressed miRNAs, miR-9-3p was one of the genes with the most significant difference in expression. To the best of our knowledge, there are no reports evaluating the association between miR-9-3p and lipid metabolism in T2DM combined with atherosclerosis. It is well-known that the liver, as the main organ of energy metabolism, is the most frequently involved organ in glucose and lipid metabolic disorders (22). HepG2 cell lines have been used to examine lipid metabolism disorder in several diseases. Therefore, the aim of the present study was to evaluate the effects of miRNA-9-3p on lipid metabolism in HepG2 cells, and investigate its molecular mechanisms by cell transfection, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses.

The present study revealed that miRNA-9-3p has an important role in development of T2DM, indicating its potential as a therapeutic target. The future applications of miRNA-based diagnostics and therapeutics in metabolic diseases requires further research.

Materials and methods

Cell culture. The HepG2 cells were maintained in high glucose-Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin/streptomycin (XiangBo Biological Technology Co., Ltd., Guangzhou, China) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The media was replaced every 2-3 days.

Cell transfection. The miRNA-9-3p mimic, miRNA-9-3p inhibitor (anti-miRNA-9-3p) and their corresponding negative control (NC) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The HepG2 cells were transfected with the miRNA-9-3p mimic (50 nM), miRNA-9-3p inhibitor (100 nM) or their negative controls for 48 h using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions. The HepG2 cells were divided into a normal group, NC group, miRNA-9-3p mimic group and anti-miRNA-9-3p group.

MTT assay. Cell proliferation was determined using an MTT assay 24, 48 and 72 h following transfection. The HepG2 cells were plated in 96-well plates at a density of 3x10⁴ per well. Subsequently, 10 µl 5 mg/ml MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well of the 96-well plate, and the plates were incubated in the dark at 37°C for 2 h. The absorbance was measured at a wavelength of 490 nm with a microplate reader (BIORAD680; Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the results were expressed as a percentage of the control.

Intracellular triglyceride (TG) and total cholesterol (TC) measurements. The concentrations of TG and TC in the HepG2 cells were measured using a Total cholesterol Test kit and a Triglyceride Test kit (BioVision, Inc., Milpitas, CA, USA), according to the manufacturer's instructions, based on phosphoglycerol oxidase/peroxidase enzymatic reactions. Briefly, HepG2 cells at 80% confluence were washed three times with 1 ml cold phosphate-buffered saline (PBS; Zhongshan Jinqiao Biological Technology Co., Ltd., Beijing, China). The concentrations of TG and TC were measured in the HepG2 cell lysates. The cell lysates, working liquid and distilled water blended at 37°C for 6 min. After cooling, the absorbent density was tested.

RNA extraction. Total RNA was isolated from the HepG2 cells using TRIzol reagent (Invitrogen Life Technologies) and an miRNeasy mini kit (Invitrogen Life Technologies), according to the manufacturer's instructions. The concentration and purity of total RNA samples was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), with an average 260/280 ratio of 2.02, and an average 260/230 ratio of 1.79. RNA integrity was determined by agarose gel electrophoresis. Agarose (0.5 g; Zhongshan Jinqiao Biological Technology Co., Ltd.) was dissolved in electrophoresis buffer (50 ml; Zhongshan Jinqiao Biological Technology Co., Ltd.), and the samples were added to the gel following blending. The voltage was adjusted to 100 V, to allow RNA migration from the anode to the cathode. The gel was treated with EB dyeing liquid (Applygen Technologies Inc. Beijing, China) for 5 min.

RT-qPCR. The concentration, purity and integrity of the RNA samples in the present study met the criteria (A260/A280 ratio >2.0 and an A260/A230 ratio >1.7) for evaluation using RT-qPCR analysis. For RNA analysis, single-strand cDNA synthesis was performed using a PrimeScript II 1st strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's instructions. The reaction volume was 50 µl: RNase-DNase free water 32.5 µl, buffer 10 µl, dNTP mix (2.5 mM) 4 µl, forward primer (10 µM) 1 µl, reverse primer (10 µM) 1 µl, cDNA 1 µl and PrimeStar HS DNA polymerase 0.5 µl. RT-qPCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA), with the following conditions: One cycle of initial denaturation at 95°C for 10 min; followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The primers designed in the present study (Sangon Biotech Co., Ltd., Shanghai, China) were as follows: sirtuin type 1 (SIRT1), sense 5'-CTACTGGTCTTACTTTGAGGG-3', and antisense 5'-CAAGGGATGGTATTTATGCT-3'; and

GAPDH, sense 5'-TCCACCACCCTGTTGCTGTA-3' and anti-sense 5'-ACCACAGTCCATGCCATCAC-3'. GAPDH was used as an internal control. The threshold cycle (Ct), defined as the cycle at which PCR amplification reached a significant value, was determined as the mean value. The relative expression levels for each mRNA were determined using the $2^{-\Delta\Delta Ct}$ method (23).

Western blot analysis. The cells were washed in fresh PBS, homogenized in radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich) containing protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and centrifuged at $10,000 \times g$ for 15 min at 4°C . Protein concentration was determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc.). Equal quantities of total protein ($40 \mu\text{g}$) were subjected to electrophoreses on 10% SDS-PAGE (OriGene Technologies, Inc., Beijing, China), and then transferred onto polyvinylidene difluoride membranes (Roche Diagnostics GmbH, Mannheim, Germany). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline-Tween 20 (0.1%) (TBST; Zhongshan Jinqiao Biological Technology Co., Ltd.), and incubated with mouse anti-SIRT1 monoclonal antibody (A-4111-100; 1:200; Abcam, Cambridge, MA, USA) or mouse anti- β -actin monoclonal antibody (sc-47778; 1:500; Abcam) at 37°C for 2 h. Subsequently, the proteins were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (111-035-003; 1:5,000; Abcam) at 37°C for 1 h and then washed three times with TBST for 15 min. The proteins were detected using an enhanced chemiluminescence (ECL) system (GE Healthcare Life Sciences). Densitometric quantification of the bands was performed using ImageJ software (version 1.41; National Institutes of Health, Bethesda, MD, USA). The loading volume of each sample was normalized by the β -actin protein band density.

Statistical analysis. Statistical analysis in the present study was performed using SPSS (version 18.0; SPSS, Inc., Chicago, IL, USA). All data from the experiments are presented as the mean \pm standard error of the mean. Significant differences in the mean values were evaluated using Student's t-test, and one-way analysis of variance was performed to compare the means among groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miRNA-9-3p in HepG2 cells. To determine the potential cellular effects of miRNA-9-3p on HepG2 cells, the HepG2 cells were transfected with the miRNA-9-3p mimic or miRNA-9-3p inhibitor, and their NCs for 48 h. To confirm whether miR-9-3p was upregulated or downregulated in the HepG2 cells, the expression levels of miRNA-9-3p in the HepG2 cells were determined using RT-qPCR analysis (Fig. 1). The results revealed that miRNA-9-3p was expressed at significantly higher levels in the HepG2 cells transfected with the miRNA-9-3p mimic, compared with the levels in the normal control group and NC group ($P < 0.05$; Fig. 1A). By contrast, miRNA-9-3p was downregulated in the HepG2 cells transfected with the miRNA-9-3p inhibitor, compared with the normal control group and NC group ($P < 0.05$; Fig. 1B).

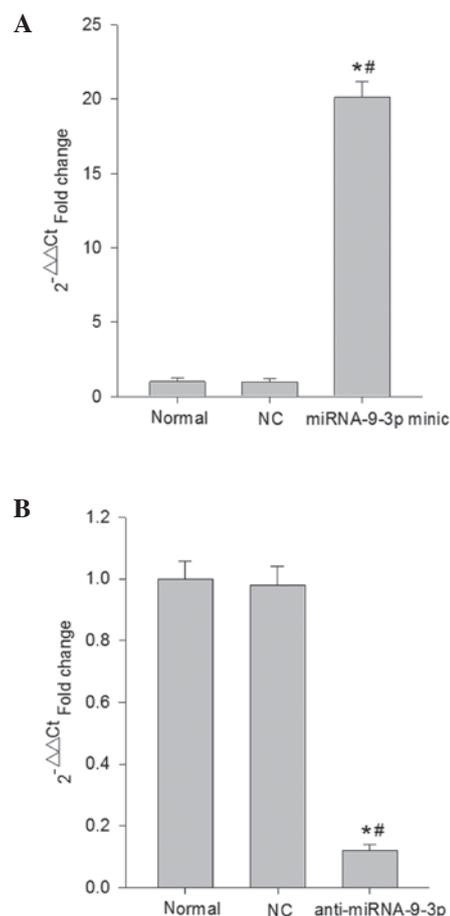


Figure 1. Levels of miRNA-9-3p in HepG2 cells following transfection. (A) Expression of miRNA-9-3p was significantly higher in the HepG2 cells transfected with the miRNA-9-3p mimic. (B) Expression of miRNA-9-3p was effectively inhibited by transfection with the miRNA-9-3p inhibitor. Data are presented as the mean \pm standard error of the mean of triplicate determinations. * $P < 0.05$, vs. normal group; # $P < 0.05$, vs. NC group. miRNA, microRNA; NC, negative control.

Effects of miRNA-9-3p on HepG2 cell proliferation. The present study investigated the effect of miRNA-9-3p on cell proliferation using MTT growth assays at 24, 48 and 72 h post-transfection. The data revealed that miRNA-9-3p overexpression significantly enhanced cell proliferation, compared with the normal group and NC group ($P < 0.05$; Fig. 2A). However, in the HepG2 cell lines, transfection with the miRNA-9-3p inhibitor markedly inhibited cell proliferation ($P < 0.05$; Fig. 2B). Collectively, these results demonstrated that the inhibition of miRNA-9-3p reduced the growth of the HepG2 cells.

Effects of miRNA-9-3p on the levels of TG and TC in HepG2 cells. Subsequently, the present study analyzed the effect of miRNA-9-3p on lipid accumulation in the HepG2 cells. The TG and TC contents in the cells were measured using phosphoglycerol oxidase/peroxidase enzymatic reactions. As shown in Fig. 3A and B, the levels of TG and TC in the miRNA-9-3p group were significantly increased, compared with the normal group and NC group ($P < 0.05$). By contrast, transfection with the miRNA-9-3p inhibitor caused downregulation in the levels of TG and TC ($P < 0.05$). These results indicated that inhibition

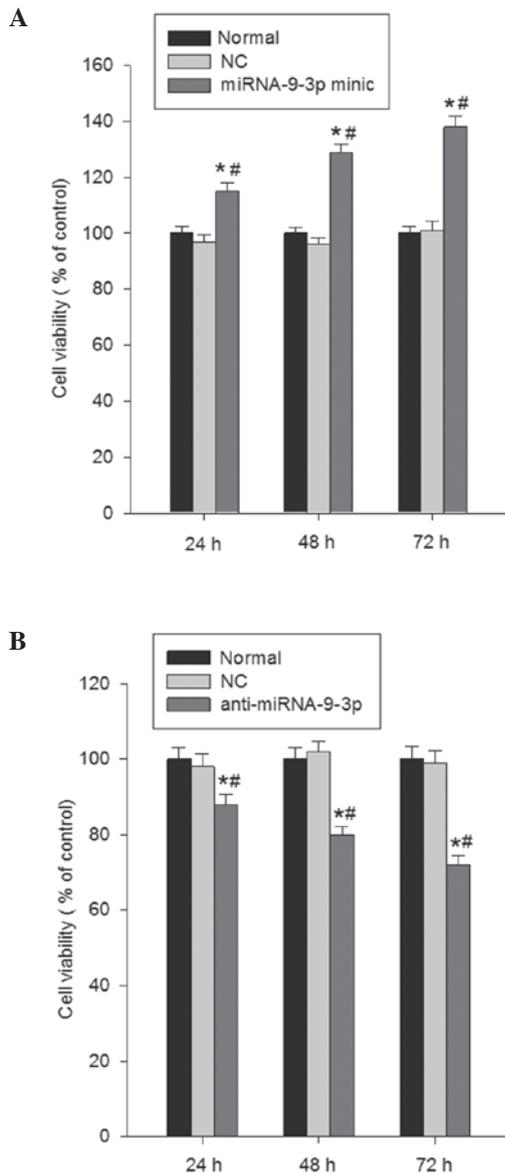


Figure 2. miRNA-9-3p affects HepG2 cell proliferation. Cell viability was measured using an MTT assay. (A) miRNA-9-3p transfection enhanced cell growth, compared with the normal group and NC group. (B) Inhibition of miRNA-9-3p reduced the growth of the HepG2 cells. Data are presented as the mean \pm standard error of the mean of triplicate determinations. * $P < 0.05$, vs. normal group; # $P < 0.05$, vs. NC group. miRNA, microRNA; NC, negative control.

of the expression of miRNA-9-3p significantly decreased the TG and TC contents, and relieved lipid deposition in the HepG2 cells.

Effects of miRNA-9-3p on the expression of SIRT1 mRNA in HepG2 cells. RT-qPCR analysis of the mRNA levels of SIRT1 was performed 48 h post-transfection. As shown in Fig. 4, the RT-qPCR results demonstrated no significant difference in the mRNA expression levels of SIRT1 relative to the mRNA expression of GAPDH in any group.

Effects of miRNA-9-3p on the protein expression of SIRT1 in HepG2 cells. The protein expression of SIRT1 was analyzed using western blot analysis 48 h post-transfection. The data

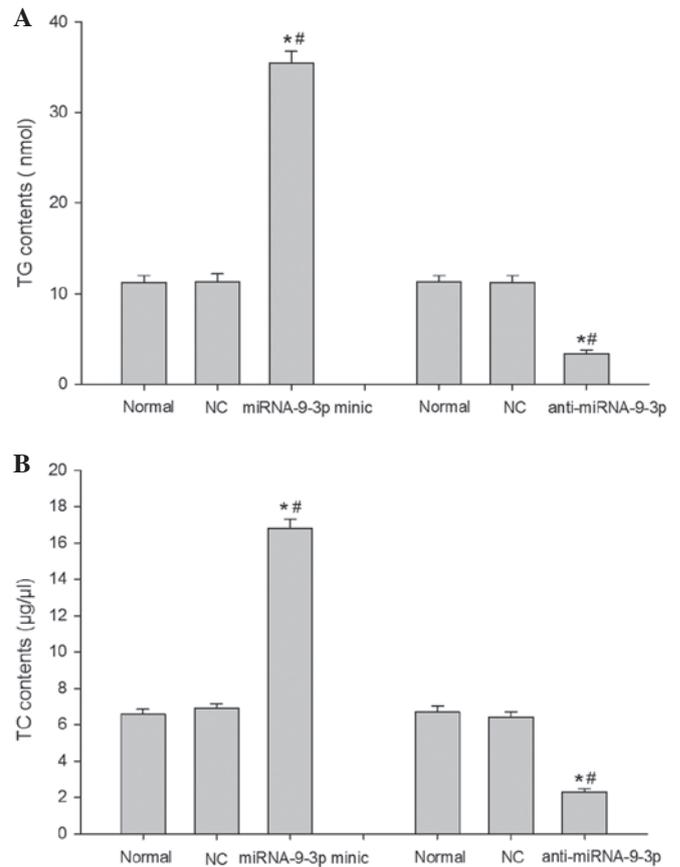


Figure 3. Effects of miRNA-9-3p on lipid accumulation in HepG2 cells. (A) TG and (B) TC levels were measured using commercial kits 48 h post-transfection. TG and TC were significantly reduced following the transfection of miRNA-9-3p inhibitor into the HepG2 cells. Data are presented as the mean \pm standard error of the mean of triplicate determinations. * $P < 0.05$, vs. normal group; # $P < 0.05$, vs. NC group. miRNA, microRNA; NC, negative control; TG, triglyceride; TC, total cholesterol.

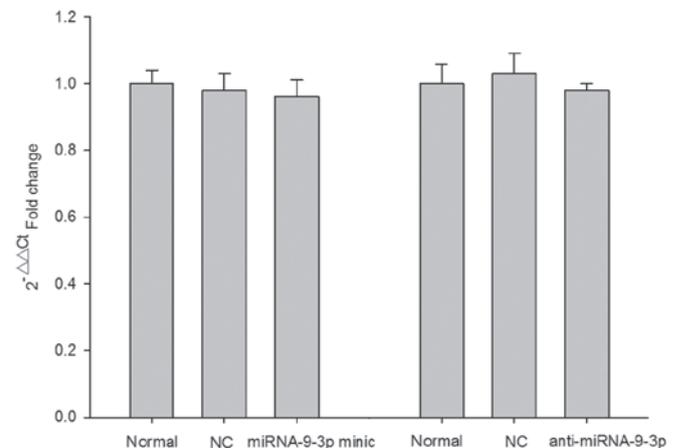


Figure 4. mRNA expression of sirtuin type 1 in each group. Gene expression levels were normalized to the reference gene (GAPDH). No statistically significant differences were observed between any group. miRNA, microRNA; NC, negative control.

were normalized to the expression of β -actin. As shown in Fig. 5, the protein levels of SIRT1 were significantly decreased in the miRNA-9-3p-transfected HepG2 cells, compared with the cells in the normal group and NC group ($P < 0.05$).

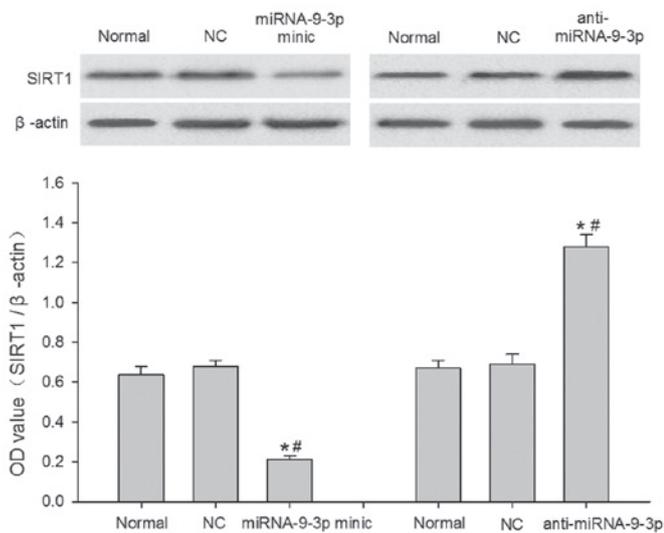


Figure 5. Protein expression of SIRT1 in each group. Western blot analysis revealed that the protein expression of SIRT1 was downregulated following transfection with the miRNA-9-3p mimic, and was upregulated following transfection with the miRNA-9-3p inhibitor. Data are presented as the mean \pm standard error of the mean of triplicate determinations. * $P < 0.05$, vs. normal group; # $P < 0.05$, vs. NC group. miRNA, microRNA; NC, negative control; SIRT1, sirtuin type 1; OD, optical density.

However, the protein levels of SIRT1 were significantly increased when miRNA-9-3p was inhibited via transfection with anti-miRNA-9-3p, compared with the normal group and NC group ($P < 0.05$). These results indicated that the protein expression of SIRT1 is endogenously regulated by miRNA-9-3p in HepG2 cells, suggesting that the expression of SIRT1 was post-transcriptionally regulated by miRNA-9-3p in the HepG2 cells.

Discussion

miRNAs are a group of small non-coding RNAs, which modulate the expression of target genes (24). Increasing evidence has revealed that miRNAs are critical in cell cycle regulation, cell differentiation, proliferation and apoptosis (25,26). miRNAs control these cellular biological functions by targeting the expression of genes. Therefore, the understanding and elucidation of functional targeted genes is a focus of investigations. Additionally, miRNAs are involved in several physiological and pathological processes as negative regulators. It has been reported that miRNAs are important role in insulin signaling and insulin secretion, however, the association between miRNAs and insulin resistance remains to be fully elucidated (27). In HepG2 cells lines, a previous study demonstrated that the overexpression of miRNA-145 inhibits glucose uptake, decreases the phosphorylation of protein kinase B (Akt) and insulin receptor substrate-1 (IRS-1), and induces resistin-induced insulin resistance (28). The IRS-1/phosphoinositide 3-kinase (PI3K)/AKT pathway is the predominant insulin signaling pathway involved in the regulation of blood glucose in T2DM. Another study revealed that miRNA-126 overexpression inhibited the PI3K/AKT pathway by regulating the expression of IRS-1, suggesting that miRNA-126 may be associated with glucose metabolism (29).

The knockout of miRNA-375 in mice reduces the secreting ability of pancreatic islet B cells, causing insulin resistance and severe clinical symptoms (30), whereas the overexpression of miRNA-29a and miRNA-29b may inhibit insulin induced glucose uptake (31). In previous years, studies have reported that miRNA not only directly regulates insulin secretion, pancreatic development and pancreatic islet cell differentiation, but also indirectly regulates lipid metabolism and is involved in diabetes and its complications (32,33,34). However, whether miRNAs are involved in lipid metabolism in diabetes remain to be elucidated. Therefore, the purpose of the present study was to investigate whether miRNA-9-3p is involved in the process of lipid metabolism.

The human miRNA-9 gene locates on chromosome 15, and includes miRNA-9-3p and miRNA-9-5p. miRNA-9, with a brain source, is highly expressed in the nervous system and is conserved among species, exhibiting 100% similarity between *Drosophila melanogaster* and vertebrates (35). miRNA-9 has been demonstrated to be crucial in the repair of the myelin sheath following hypoxic-ischemic brain damage (36). Several studies have demonstrated that miRNA-9 regulates the differentiation of oligodendrocyte progenitor cells by regulating serum response factor (37), and maintain myelinogenesis (38). Importantly, the expression of miR-9-3p in islet β cells is involved in the physiological processes of insulin secretion and glucose metabolism (39). A study by Plaisance demonstrated that miR-9-3p overexpression increased the secretion of granuphilin-a protein, and reduced the exocrine abilities of the islet β cells (40). In addition, miRNA-9-3p in the plasma may be involved in cholesterol metabolism and angiogenesis, adjusting the biological function of the myocardium (41). However, whether there is a regulatory association between miRNA-9-3p and lipid metabolism disorder in T2DM remains to be elucidated.

In the present study, the effect of miRNA-9-3p on lipid accumulation in the HepG2 cells was examined by transfection of miRNA-9-3p mimic, miRNA-9-3p inhibitor and their corresponding NCs into HepG2 cells. For the first time, to the best of our knowledge, the present study demonstrated that the inhibition of miRNA-9-3p suppressed the proliferation of HepG2 cells and significantly decreased TG and TC contents, reducing lipid accumulation in the HepG2 cells. These results indicated that miRNA-9-3p may offer potential as a therapeutic target for diseases associated with lipid metabolism. Subsequent investigations aim to identify biological or molecular tools to alter the expression of miRNA-9-3p for further elucidation of its function and therapeutic implications.

To examine the molecular mechanisms underlying the inhibition of lipid deposition, the present study assessed the expression of SIRT1, at mRNA and protein levels, in the HepG2 cells. The results of the RT-qPCR and western blot analyses revealed that the inhibition of miRNA-9-3p upregulated the protein expression of SIRT1 protein, but had no effect on the mRNA expression of SIRT1. SIRT1 is an NAD⁺-dependent deacetylase, which has been linked to the regulation of cell aging, life span and energy metabolism (42). SIRT1 function has been reported to regulate a wide range of cellular functions affecting metabolic homeostasis. SIRT1 not only exerts anti-oxidative, anti-apoptotic, and anti-inflammatory effects against cellular injury, it also inhibits sugar dysplasia effects

and improves insulin sensitivity in liver, muscle and adipose tissue (43-45). Furthermore, the activation and upregulation of SIRT1 may be beneficial in age-associated disorders, including T2DM and neurodegenerative diseases (46). Similarly, down-regulation in the expression of SIRT1 has been observed in insulin-resistant subjects (47). In liver tissue, SIRT1 is key in the regulation of lipid metabolism balance. Lovis *et al* (48) found that SIRT1-knockout in the liver increases the level of glucose in the plasma, reduces insulin sensitivity and upregulates the levels of free fatty acids and cholesterol. These effects may be associated with the downregulation of ABCA1 and low density lipoprotein receptor (LDLR), reported by Balasubramanyam *et al* (49). In addition, Adlakha *et al* (50) revealed that miRNA-128 regulated lipid metabolism by targeting the expression of SIRT1 in HepG2 cells. These findings indicate that the expression SIRT1 regulates lipid metabolism. Therefore, maintaining SIRT1 levels or stimulating upregulation in the expression of SIRT1 may be beneficial in the treatment of a variety of diseases with lipid metabolic disorders.

In conclusion, the findings of the present study indicated that the inhibition of miRNA-9-3p exerted a regulatory effect on lipid accumulation, reducing TG and TC contents, by regulating the protein expression of SIRT1 in the HepG2 cells. This novel evidence may assist in understanding the potential roles of miRNA-9-3p in T2DM, and supports the possibility of a synthetic inhibitor of miRNA-9-3p as a novel therapeutic strategy for lipid metabolism disorder in T2DM.

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