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ORIGINAL ARTICLE

Down-Regulation of SIRT1 Expression by mir-23b Contributes to Lipid Accumulation in HepG2 Cells

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Abstract

Non-alcoholic fatty liver disease is one of the main causes of chronic liver disease and therefore is currently considered a major public health problem. Sirtuin 1 (SIRT1) is an NAD-dependent deacetylase enzyme that contributes in the regulation of metabolic processes and protects against lipid accumulation in hepatocytes. Its expression is potentially regulated by microRNAs which attach to the 3′ untranslated region (3′-UTR) of their target mRNA. HepG2 cells were incubated by glucose to induce lipid accumulation and were subsequently transfected with mir-23b mimic and inhibitor. Real-time PCR was used for measuring the expression of mir-23b and SIRT1 mRNA. Cell survival assay and intracellular triglyceride measurement were performed using colorimetric methods. Determination of SIRT1 protein level and activity were done by western blot and fluorometric analysis, respectively. The interaction of miR-23b with 3′-UTR of SIRT1 mRNA was confirmed by dual luciferase. miR-23b mimic inhibited gene and protein expression of SIRT1, while the inhibitor of miR-23b significantly elevated the expression levels of SIRT1 mRNA and protein. The results showed that the 3′-UTR of SIRT1 mRNA is a direct target for miR-23b. The intracellular triglyceride level was increased following the inhibition of SIRT1 in transfected HepG2 cell by miR-23b mimic. Cell viability was decreased in response to miR-23b upregulation compared to control cells. miR-23b reduces the expression and activity of SIRT1 and therefore may be a causative factor in the enhancement of lipid accumulation in HepG2 cells.

Keywords miR-23b-3p · Sirtuins 1 · Non-alcoholic fatty liver disease · Luciferase assay

Abbreviations

NAFLD	non-alcoholic fatty liver disease
NAD	nicotinamide adenine dinucleotide
SIRT1	silent information regulation homology 1

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UTR untranslated region
MRE microRNA response elements

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the major public health concerns worldwide (Lazo et al. 2013; Satapathy and Sanyal 2015). Metabolic syndrome which is usually associated with insulin resistance, dyslipidemia, obesity, and endothelial dysfunction is mostly accompanied by NAFLD (Albhaisi and Sanyal 2018). A combination of genetic disposition, protein factors, and environmental parameters determine the pathogenesis of NAFLD (Vickers et al. 2014; Chalasani et al. 2018).

Recently, there has been an increasing concern in finding the roles of microRNAs (miRNAs) in lipid and carbohydrate metabolism. As Fernández-Hernando (2013) have explained, various microRNAs play key roles in lipid regulation through modulation of the expression of various genes. miRNAs are small non-coding endogenous RNA molecules with ~22 nucleotides length that bind to 3′-untranslated region (3′-UTR) of target mRNA and regulate their expression by inducing their degradation or translational repression (Arner and Kulyté 2015).

miRNAs are involved in various biological processes including glucose and lipid metabolism (He et al. 2016). Several factors that modulate metabolic procedures in liver are under the control of microRNAs. miR-122 was the first miRNA identified in liver that affects lipid metabolism and its knockdown leads to the reduction in triglyceride (TG) and cholesterol levels (Esau et al. 2006). miR-33a/33b has also been shown to regulate lipid metabolism by binding to the mRNA of sterol regulatory element binding protein (SREBP), an important transcription factor in the regulation of genes involved in cholesterol, fatty acid, and TG metabolism (Najafi-Shoushtari et al. 2010).

SIRT1 is an NAD^+-dependent deacetylase enzyme that functions as a fundamental regulator of various cellular processes, including energy metabolism, stress response, cell viability, and longevity (Li et al. 2013; Kane and Sinclair 2018). SIRT1 is an important regulator of hepatic glucose and lipid metabolism. It modulates gluconeogenesis in different fasting states by deacetylating major transcription activators such as TORC2 and FOXO1 (Frescas et al. 2005; Liu et al. 2008). SIRT1 also plays an important role in hepatic lipid metabolism. Based on the evidence reviewed by Ding et al. (2017), SIRT 1 has been concluded to be beneficial in regulating hepatic lipid metabolism, controlling hepatic oxidative stress and preventing the progression of fatty liver disease through its deacetylating activity and amelioration of hepatic inflammation. It activates fatty acid oxidation through PPARα/PGC1-α pathway (Purushotham et al. 2009) and directly modulates farnesoid X receptor (FXR) and liver X receptor (LXR) and thus affects hepatic cholesterol and bile acid homeostasis (Kemper et al. 2009; Feng et al. 2010). Thereby, SIRT1 elimination increases the susceptibility to high-fat diet-induced dyslipidemia and lipid accumulation in liver (Purushotham et al. 2009), while its overexpression attenuates hepatic steatosis in animal models (Li et al. 2011).

Some microRNAs such as miR-34a and miR-21 have been shown to directly target SIRT1 expression and thereby affect metabolic homeostasis (Yang et al. 2015). As reported by Vickers et al. (2013) miR-23b, which is processed from a primary poly-cistronic transcript together with miR-27b, and miR-24–1, is among the miRNAs that is upregulated in dyslipidemic situations. Our bioinformatics analysis revealed that miR-23b aligns with the mRNA of SIRT1. However, interrelationship of SIRT1 and miR-23b has not been explored before. Thus, in this study, the role of miR-23b in the regulation of expression and activity of SIRT1 and its contribution to hepatocyte steatosis were investigated.

Materials and Methods

Cell Culture and Treatment

HepG2 and HEK-293T cell lines were obtained from the Cell Bank of the Iranian Biological Resource Center (Tehran, Iran) and cultured in Dulbecco's Modified Eagle's Medium/F12 (DMEM) (Gibco, UK) containing 5.5 mM D-glucose (normal glucose level), 10% Fetal Bovine Serum (FBS) (Gibco, UK), penicillin (100 units/ml), and streptomycin (100 µg/ml), and incubated at 37 °C in 5% CO_2 in a humidified atmosphere.

For cell treatment experiments, cells were seeded in multi-well plates that were coated by poly-D-lysine (0.1 mg/ml) to enhance cell attachment.

To develop a model of hepatic steatosis, HepG2 cells were incubated in serum-free medium with high glucose concentration (50 mM D-glucose) for 24 h. Oil Red O staining followed by microscopic evaluation as well as spectrophotometric measurement was used to confirm significant lipid accumulation. Oil Red O was also eluted from stained cells by addition of 100% isopropanol and the absorption of the mixture was measured at 500 nm (Hou et al. 2008).

Cell Transfection

Hsa-miR-23b-3p mimic (Gene Pharma, China) was used to increase the intracellular miR-23b levels and miR-23b inhibitor was used to down-regulate the endogenous intracellular miR-23b. Polyethylenimine (PEI) (Sigma-Aldrich, Germany) was used for transfection experiments. Cells were seeded into 12- and 6-well plates at the density of 1×10^5 and 2×10^5 cells per well, respectively. Briefly, a complex was formed between miRNAs and PEI after their incubation for 45 min at room temperature. The complex was mixed with Opti-MEM culture medium and the mixture was added to the cells and incubated at 37 °C with 5% CO_2. Sequences of the miR-23b mimic, inhibitor, and the relevant negative controls are shown in Table 1.

Transfection with FAM-labeled microRNAs followed by examination by a fluorescent microscope 24–48 h after transfection, was used to evaluate the efficiency of transfection and the resulting microscopic images were analyzed with ImageJ software 1.17 (NIH, USA).

Table 1 Sequences of microRNAs

microRNA	Sequences
miR-23b-3p mimic	5′-AUCACAUUGCCAGGGAUUACC-3′
miR-23b-3p inhibitor	5′-GGUAAUCCCUGGCAAUGUGAU-3′
Negative control mimic	5′-UUGUACUACACAAAAGUACUG-3′
Negative control inhibitor	5′-CAGUACUUUUGUGUAGUACAA-3′

Intracellular Triglyceride Measurement

In order to measure the intracellular TG by enzymatic method, total TG was extracted from the cells by NP-40 (Calbiochem, USA) and then measured by a triglyceride assay kit (Pars Azmoon, Iran) using glycerol kinase and glycerol 3-phosphate oxidase, according to the manufacturer's instructions. Intracellular triglyceride content was normalized according to the protein concentration and reported as μg of TG/mg of protein.

RNA Extraction and Real-Time PCR

Total cellular RNA was extracted using the miRCURY RNA Isolation Kit (Exiqon, USA) and quantified with a NanoDrop spectrophotometer (NanoDrop, Thermo Fisher Scientific). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used for the synthesis of cDNA.

In order to assess miRNA expression, a poly (A) tail was added to the 3′-end of the miRNA transcripts to elongate them using *E. coli* poly (A) polymerase (Biolabs, New England) prior to cDNA synthesis. Real-time PCR was performed by specific primers (Table 2) using a SYBR Green Master Mix Kit (Ampliqon, Denmark). The expression levels of miR-23b and SIRT1 were normalized by U6 small nuclear RNA and β-Actin, respectively.

Relative quantification was performed by ΔΔCt method.

Table 2 Sequences of primers for real-time PCR

Primers	Sequences
miR-23b-3p	5′-CAGGCAAGATGCTGTTGCA-3′
Universal (Reverse)	5′-GCGAGCACAGAATTAATACGACTC-3′
SIRT1 (Forward)	5′-AGTCCTGCTCCTTCCAAAAC-3′
SIRT1 (Reverse)	5′-CTTCGGTGTAGCCCATTTGT-3′
U6 (Forward)	5′- CTCGCTTCGGCAGCACA-3′
U6 (Reverse)	5′- AACGCTTCACGAATTTGCGT-3′
β-ACTIN (Forward)	5′-TGGACTTCGAGCAAGAGATG-3′
β-ACTIN (Reverse)	5′-GAAGGAAGGCTGGAAGAGTG-3′

Cell Survival Assay

Cell survival was examined using a Water Soluble Tetrazolium (WST-1) assay kit (Roche Applied Science, Germany). Cells were seeded at the density of 5×10^3 in each well of a 96-well plate and the plate was incubated overnight and the cells were transfected with miRNA mimic, inhibitor, and the relevant negative controls. Subsequently, 10 µl of the WST-1 reagent was added and incubated at 37 °C for 4h. The absorbance of the resulting soluble formazan was measured using a plate reader at 450 nm against 650 nm.

Western Blotting

Cells were lysed using RIPA buffer with added protease inhibitors (Sigma-Aldrich, Germany). After centrifugation at 12,000 rpm for 15 min, total protein of the cell lysate was assessed using a BCA protein assay kit (Thermo Scientific, UK). The protein samples were analyzed by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by blotting onto polyvinylidene difluoride membranes (PVDF) (Roche Applied Science, Germany). Blocking was performed at room temperature by incubation with 5% skimmed milk for 5 h. Membranes were then incubated with 1:1000 dilution of primary antibodies against SIRT1 or GAPDH (cell signaling technology, USA) and subsequently 1:5000 dilution of anti-Rabbit HRP-conjugated secondary antibody (cell signaling technology, USA). Visualization was carried out with Clarity Western ECL Substrate (Bio-Rad, USA) and ImageJ software was used for the densitometric analysis of the resulting bands. GAPDH was used as the normalizer.

Luciferase Reporter Gene Assay

Interaction of miR-23b with its microRNA response element in the 3′-UTR of the SIRT1 mRNA was evaluated by co-transfection of HEK-293T cells with miR-23b mimic or inhibitor together with the psiCHECK2 vector including the sequence of the 3′-UTR SIRT1 mRNA. psiCHECK2 vector alone or the vector containing a mutant form of miR-23b-3p response element (MRE) on the 3′-UTR of SIRT1 (MRE-tandem-mut) were used as the negative controls. Dual luciferase assay kit (Promega, Germany) was used to determine the relative luciferase activity based on the manufacturer's instructions. The firefly luciferase activity was used for normalization (Zhao et al. 2016).

SIRT1 Activity Assay

SIRT1 activity was assessed with a fluorometric assay kit (Abcam, USA) using a peptide substrate containing a fluorophore and a quencher that emitted fluorescence only after deacetylation reaction and subsequent action of a protease that separates fluorophore from the quencher. Recombinant SIRT1 was used as the

positive control. The reaction was terminated by adding developer and the fluorescence intensity was measured by a plate fluorometer with excitation and emission wavelength of 460 nm and 360 nm, respectively, and the rate of reaction was calculated.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 7 Software. Data are represented as mean ±SD of at least three separate triplicate experiments. Statistical significance was analyzed using one-way analysis of variance (ANOVA).

Results

miR-23b-3p Mimic Down-regulated SIRT1 mRNA and Protein Expression

In order to investigate the influence of miR-23b on SIRT1 expression, we first generated a model of hepatocyte steatosis by incubating HepG2 cells with high glucose concentration. After treating the cells with glucose, TG accumulation in the HepG2 cells were evaluated by Oil Red O staining and the results indicated that the intracellular TG content was increased significantly (Supplementary Fig. 1).

In order to alter the level of miR-23b, miR-23b mimic or miR-23b inhibitor was introduced into HepG2 cells. As shown in Fig. 1a, level of miR-23b was significantly

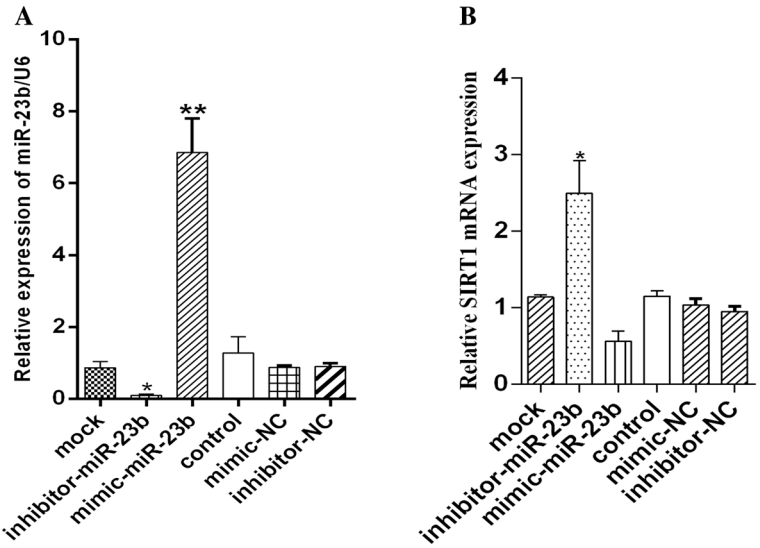


Fig. 1 Expression of miR-23b (**a**) and SIRT1 mRNA (**b**) in HepG2 cells treated by glucose followed by transfection with miR23b mimic and inhibitor. Data are shown as mean ± SD from at least three separate assays (*$p < 0.05$)

augmented in cells transfected by miR-23b mimic compared with control cells. On the other hand, miR-23b inhibitor generated a significant decline in the level of miR-23b in HepG2 cells.

SIRT1 mRNA expression was evaluated by real-time PCR in transfected cells. miR-23b inhibitor dramatically raised SIRT1 mRNA expression. Although SIRT1 mRNA expression was reduced in response to miR-23b mimic, this change was not significant compared to control.

Protein level of SIRT1 was assessed by western blotting in cells transfected by miR-23b mimic, inhibitor, or their negative controls. As shown in Fig. 2, SIRT1 protein level was reduced in response to miR-23b upregulation by its mimic oligonucleotide. Nevertheless, transfection by miR-23b inhibitor caused a significant increase in SIRT1 protein levels.

miR-23b-3p Changed SIRT1 Activity

SIRT1 activity was assessed in transfected cells by a specific fluorometric substrate and the SIRT1 activity was compared with that of control cells. Results showed a significant increase in SIRT1 activity in response to transfection with miR-23b-3p inhibitor. Conversely, SIRT1 activity was significantly declined after transfection with miR-23b mimic (Fig. 3).

SIRT1 was a Direct Target of miR-23b

Bioinformatics analysis showed that miR-23b-3p aligns with 8 nucleotides in 899–906 position of SIRT1 3′-UTR. In order to experimentally evaluate this interaction, cells were co-transfected by miR-23b mimic or inhibitor together with psiCHECK-2 vector containing the whole sequence of SIRT1 3′-UTR. As shown in Fig. 4, luciferase activity was significantly decreased in response to miR-23b mimic up-regulation. On the other hand, mir-23b inhibitor caused a dramatic increase in luciferase activity. No alteration in luciferase activity was observed following transfection with the vector containing the mutated form of SIRT1 3′-UTR or the vector alone, further confirming the specific binding of miR-23b to the 3′-UTR of SIRT1 mRNA.

miR-23b-3p Reduced Cell Viability

To assess the effect of up-regulation of miR-23b on cell survival, cells were transfected and their viability was checked by WST-1 assay. Cell viability was decreased in response to miR-23b mimic compared with control cells while miR-23b inhibitor enhanced the cell viability compared with control cells (Fig. 5).

miR-23b-3p Up-regulation Enhanced Steatosis in HepG2 Cells

After incubating HepG2 cells with high concentration of glucose and transfection with miR-23b mimic or inhibitor, intracellular TG content was evaluated by an

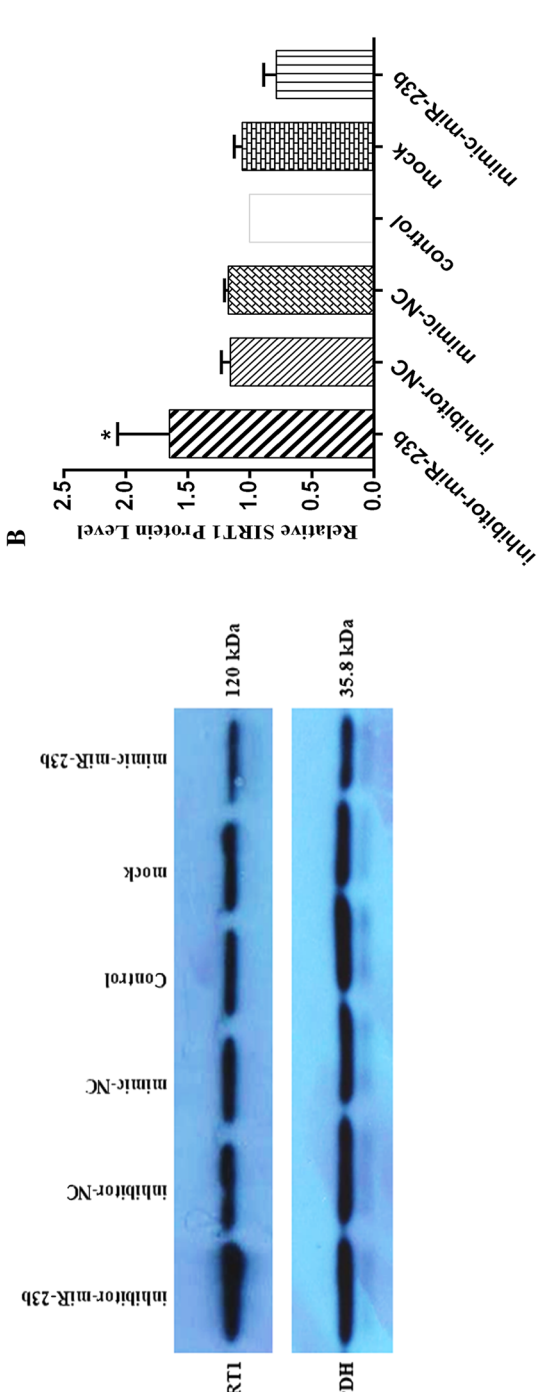


Fig. 2 SIRT1 protein level in HepG2 cells following transfection by miR-23b mimic, inhibitor, and the negative controls (NC). **a** A representative western blot is shown. **b** Densitometric analysis of western blotting results. Data are shown as mean ± SD. $^{*}p < 0.01$ versus control

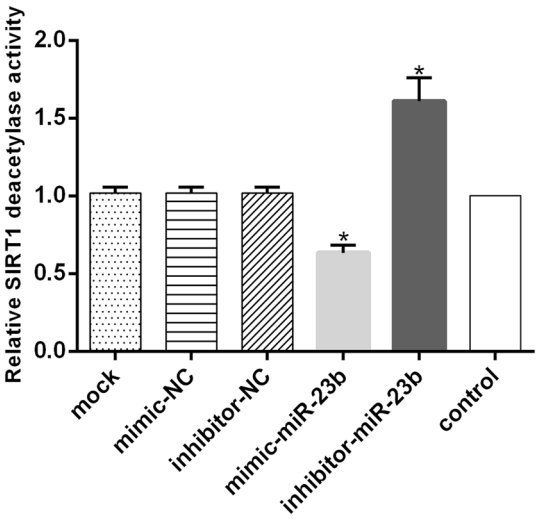


Fig. 3 Deacetylase activity of SIRT1 in HepG2 cells treated with glucose after transfection with miR-23b mimic, inhibitor, or their negative controls (NC). Data are shown as mean ±SD (triplicate assays) of at least three separate experiments. *$p < 0.05$ versus control

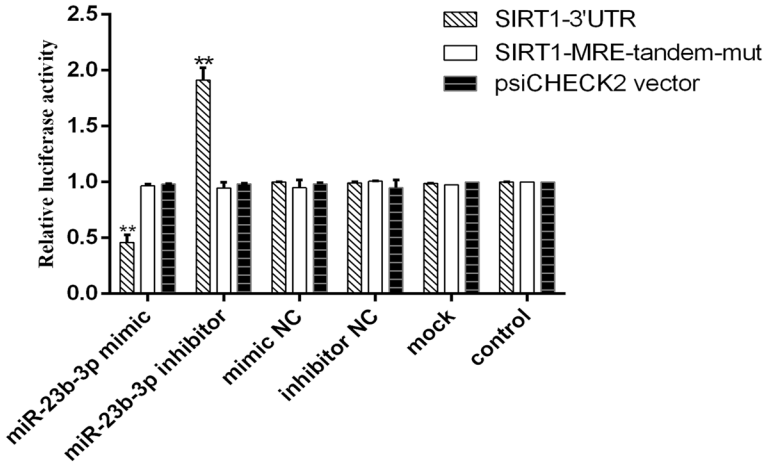


Fig. 4 Luciferase reporter assay to investigate direct binding of miR-23b-3p to the 3′-UTR of SIRT1. psiCHECK2 vectors containing SIRT1 3′-UTR or mutated sequence of miRNA response element (MRE-tandem-mut) or psiCHECK2 alone were co-transfected with miR-23b-3p mimic, inhibitor, or their negative controls (NC) into HEK-293T cells. Dual luciferase assay was performed to determine luciferase activity. The data are shown as mean ± SD of at least three separate experiments. (**$p < 0.001$)

enzymatic method. As depicted in Fig. 6, miR-23b mimic increased TG accumulation in cells compared with control cells. miR-23b inhibitor did not exert any significant effect on the TG content of the treated cells.

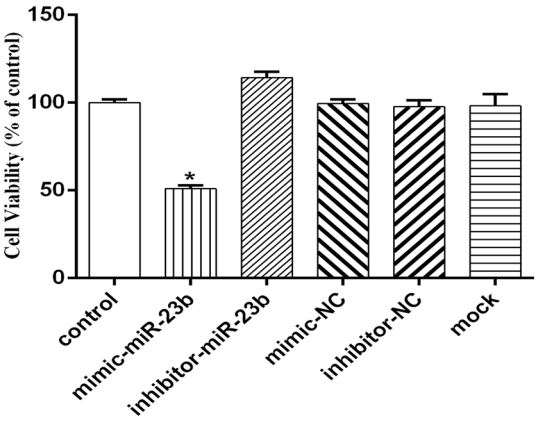


Fig. 5 Evaluation of cell viability by Water Soluble Tetrazolium Salt (WST) assay performed 1h after transfection. The viability of untreated cells (control) in each time point was considered as 100% and the viability of other groups was reported as percentage of untreated cells at the same time point. Data are mean ± SD of three independent experiments. $*p < 0.05$ versus control

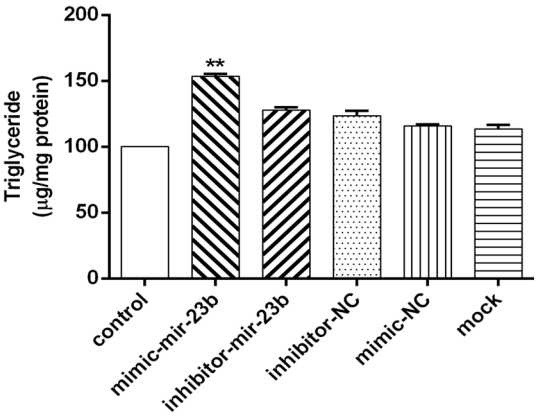
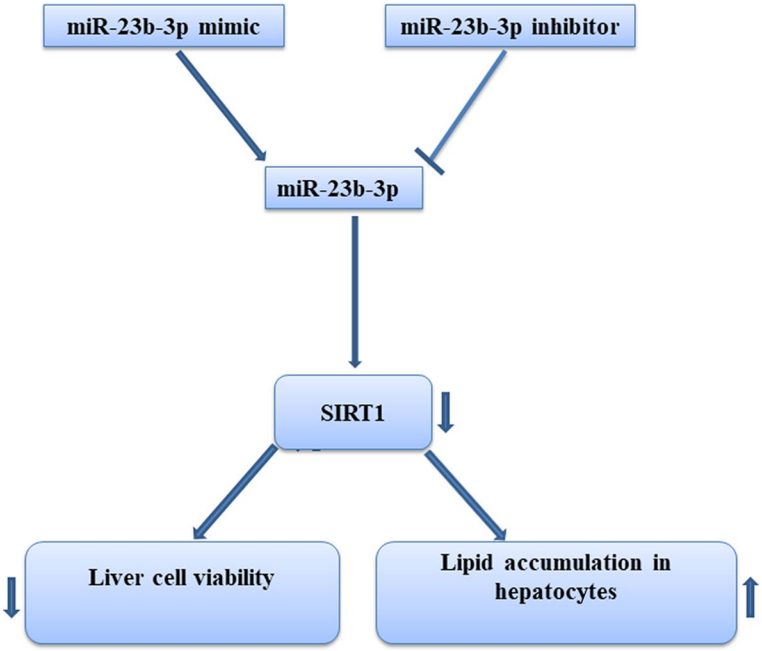


Fig. 6 Triglyceride (TG) concentration in cells treated with high concentrations of glucose followed by transfection with miR23b mimic, inhibitor, or their negative controls (NC). Data are the mean ± SD of at least three independent experiments. $*p < 0.01$ versus control



Discussion

Non-alcoholic fatty liver disease has the potential to progress from intracellular triglyceride accumulation, to steatohepatitis, liver fibrosis, cirrhosis, and ultimately hepatocellular carcinoma, which increases the risk of morbidity and mortality (Chalasani et al. 2018; Matteoni et al. 1999). Several studies have shown a potential relationship between insulin resistance, hepatic lipid metabolism, and miRNAs (Alisi et al. 2011; Zhang et al. 2017). Moreover, there are studies that link the function of miRNAs with the pathogenesis of NAFLD (Bala et al. 2009; Schueller et al. 2018). For example, NAFLD gene expression profile analysis has revealed that the expression of microRNAs such as miR-146, miR-200, and miR-152 are aberrant in liver (Feng et al. 2014).

In the present study, we aimed to assess the role of miR-23b in intracellular lipid accumulation in non-alcoholic fatty liver model. A few studies recently demonstrated that miR-23b is involved in glucose and lipid metabolism (Zhao et al. 2016). It has also been shown that miR-23b is elevated in obesity and is associated with increased body mass index and adiposity (Xiong et al. 2017; Ferrante et al. 2015). The relationship between miR-23b and liver pathology has also been the concept of some recent studies. miR-23b has been suggested as a molecular diagnostic and prognostic factor in hepatocellular carcinoma (HCC) and also a responsive target

for limiting HCC progression (Grossi et al. 2017). miR-23b is also involved in liver fibrosis such that its knockdown can block or revert TGF-β-induced liver fibrosis (Rogler et al. 2017). However, there is no study about the role of mir-23b in hepatic steatosis with focus on SIRT1 expression.

In this study, for the first time, we demonstrated that miR-23b significantly increased TG content and lipid accumulation in HepG2 cells. In consistent with our findings, several studies have reported that miRNAs control lipid metabolism in liver. Adlakha et al. (2013) revealed that miR-128 regulated lipid metabolism by targeting the expression of SIRT1 in HepG2 cells. Sun et al. (2015) found that the inhibition of miR-9-3p reduced lipid accumulation in HepG2 cells by targeting the expression of SIRT1. Zhang et al. (2017) have also demonstrated that a high level of miR-200a results in inhibition of hepatic SIRT1 by directly binding to its 3′-UTR, which may indicate a potential mechanism for the regulation of SIRT1 in fructose-related metabolic disorders. Therefore, we investigated whether SIRT1 inhibition is responsible for the effects of miR-23b in liver.

We first examined the alignment of miR-23b with the 3′-UTR of SIRT1 mRNA and found high complementarity between these two. Then we analyzed the expression of SIRT1 gene and protein and revealed that miR-23b controls SIRT1 levels at post-transcriptional and translational levels. We also showed that miR-23b mimic reduced the enzyme activity of SIRT1 in HepG2 cells. Nevertheless, transfection with miR-23b inhibitor rescued the inhibitory effect of miR-23b and enhanced the enzyme activity of SIRT1, further confirming the effect of miR-23b on SIRT1. The results of the luciferase assay with the vector containing the 3′-UTR of SIRT1 mRNA showed that inhibition of SIRT1 by miR-23b is a direct effect of miR-23b and not an off-target influence.

Consistent with our findings, Zhao et al. (2016) have reported the targeting of SIRT1 by miR-23b and its down-regulation after transfection with miR-23b mimic. However, they have examined this interaction in retinal cells to investigate the importance of SIRT1 regulation in diabetic retinopathy, while our findings have implications in non-alcoholic fatty liver disease.

Studies have identified that SIRT1 plays a major role in regulation of lipid metabolism, oxidative stress, and adaptation. SIRT1 has a prominent role as a metabolic sensor and participates in pathophysiology of fatty liver disease in animal and human models (Nassir and Ibdah 2016; Stefanowicz et al. 2018). SIRT1 activates AMP-activated protein kinase (AMPK) through liver kinase B1 (LKB1) and hence functionally inhibits fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) that are key regulators of lipid metabolism pathway (Sun et al. 2015; Guarente and Picard 2005). Thus, SIRT1 reduces lipid accumulation, possibly through increased fatty acid oxidation and/or decreased fatty acid synthesis (Zang 2006; Reznick 2007). The relationship between SIRT1 and lipid metabolism supports the role of SIRT1 in mediating the effect of miR-23b on lipid accumulation in hepatocytes.

In the current study, we showed that miR-23b-3p suppressed the survival of HepG2 cells. SIRT1 is considered a longevity-associated enzyme and increases cell survival by prevention of apoptosis (Cohen et al. 2004; Zullo et al. 2018). The effect of calorie restriction on cell longevity is mediated by SIRT1 while down-regulation of SIRT1 causes accelerated aging and apoptosis (Lu et al. 2014; Sun et al. 2007).

Consistently, our results show that inhibition of SIRT1 expression by miR-23b reduced the survival of hepatocytes emphasizing the importance of SIRT1 in cell viability.

The results presented here indicate that miR-23b-3p causes lipid accumulation in hepatocytes by regulating the protein level, mRNA expression, and activity of SIRT1 and therefore a causative role for miR-23b in NAFLD might be plausible. This new finding may help to understand the potential roles of miR-23b-3p in non-alcoholic fatty liver and to design new therapeutic or diagnostic strategies for NAFLD and related metabolic disorders.

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Compliance with Ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent This article does not contain any studies with human participants and there is no need for informed consent.

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