

Functional study of the upregulation of miRNA-27a and miRNA-27b in 3T3-L1 cells in response to berberine

YUE-YUE WU*, XIN-MEI HUANG*, JUN LIU, YING CHA, ZAO-PING CHEN,
FANG WANG, JIONG XU, LI SHENG and HE-YUANG DING

Department of Endocrinology, The Fifth People's Hospital of Shanghai Affiliated to Fudan University,
Shanghai 200240, P.R. China

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Abstract. Berberine is the major active component of *Rhizoma Coptidis* derived from a traditional Chinese herbal medicine and is known to regulate micro (mi)RNA levels, although the mechanism for this action remains unknown. The present study confirmed that treatment of 3T3-L1 cells with berberine inhibited cell viability and differentiation in a dose- and time-dependent manner, and significantly increased the mRNA expression levels of miRNA-27a and miRNA-27b. In addition, in 3T3-L1 cells treated with berberine, overexpression of miRNA-27a and miRNA-27b improved the berberine-mediated inhibition of cell differentiation and reduction of triglyceride contents. By contrast, miRNA-27a and miRNA-27b inhibitors attenuated the berberine-mediated inhibition of cell differentiation and reduction of triglyceride contents. Additionally, peroxisome proliferator-activated receptors (PPAR)- γ was confirmed to be a target of miRNA-27a in the 3T3-L1 cells. A dual-luciferase reporter assay indicated that the expression of PPAR- γ was negatively regulated by miRNA-27a. These findings may provide novel mechanistic insight into the antiobesity effects of certain compounds in traditional Chinese herbal medicine.

Introduction

Obesity is a major health obstacle in the industrialized world, increasing the incidence of several illness, including hypertension, diabetes and heart disease, and is characterized by a complex multifactorial chronic disease. An imbalance between

energy intake and expenditure contributes to a pathological growth of adipocytes (1). It is known that the quantity of adipose tissue can be regulated by the inhibition of adipogenesis and by the control of adipocyte size. Obesity is induced by the abnormal proliferation of adipocytes and recruits the new adipocytes from precursor cells, two of which are involved in regulating the differentiation of adipocytes (2).

Berberine is an alkaloid isolated from Chinese herbs and is currently used as a traditional medicine for the treatment of bacterial diarrhea, diabetes, hyperlipidemia, cancer, heart and inflammatory diseases (3-6). Previous studies demonstrate that berberine presents anticancer activities via the inhibition of cell proliferation and reproduction of viruses and certain tumorigenic microorganisms, and the induction of apoptosis in a variety of cancer cell lines (7-10). Also, it has been reported that berberine exhibits antiadipogenic effects in several adipocytes, although its precise mechanism remains to be elucidated (11,12). Therefore, the present study undertook a detailed study of the effect of berberine on the differentiation of 3T3-L1 cells.

Micro (mi)RNAs are non-encoding RNA molecules that regulate gene expression by suppressing the translation of target genes and degrading target mRNAs (13). miRNAs serve a critical role in a wide variety of biology processes, including proliferation, division, survival and apoptosis (14-17). In addition, miRNA-27 is one of the most important miRNAs and is associated with the differentiation of adipocytes. A previous study also suggests that both miRNA-27a and miRNA-27b act as antiadipogenic miRNAs, at least in part, by suppressing the proliferation of human adipose tissue-derived stem cells (18).

Previous studies have investigated the effects of natural compounds on the expression of miRNAs in different cancer types. Only a few reports on the effect of berberine and miRNAs have been published, and these effects remain to be fully understood. Berberine downregulates the expression of miRNA-21 in human multiple myeloma and ovarian cells, which in turn leads to apoptosis and inhibition of cell proliferation (19,20).

In the present study, the effects of berberine on miRNA-27a and miRNA-27b in 3T3-L1 cells were assessed. It was revealed that berberine increased the levels of miRNA-27a and miRNA-27b, which led to the enhancement of differentiation suppression and a reduction in triglyceride contents via

Correspondence to: Professor Jun Liu, Department of Endocrinology, The Fifth People's Hospital of Shanghai Affiliated to Fudan University, 801 Heqing Road, Minhang, Shanghai 200240, P.R. China
E-mail: liujun89189@163.com

*Contributed equally

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the targeting of peroxisome proliferator-activated receptors (PPAR)- γ .

Materials and methods

Adipocyte differentiation and treatments. The 3T3-L1 cells were obtained from Shanghai Institute of Cell Biology (Shanghai China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare, Piscataway, NJ, USA) and supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5% CO₂. After allowing 2 days for differentiation, the 3T3-L1 cells were passaged and treated at confluence with medium in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO, USA), 0.25 μ M dexamethasone (Sigma-Aldrich) and 10 μ g/ml insulin (Sigma-Aldrich) for 24 h. The 3T3-L1 cells were subsequently treated with berberine (0, 1, 10, 20, 40 and 80 μ M) for a further 24 h. The medium was changed to DMEM, supplemented with 1 μ g/ml insulin for 2 days, followed by DMEM with 10% FBS for 10 days. The medium was replaced on the cells with DMEM with 10% FBS every 2 days.

Cell viability measurement by cell counting kit (CCK)-8 following treatment with berberine. Each concentration of berberine used was regarded as one treatment group, while no berberine was added in the control group. Each treated or control group contained three parallel wells. The culture plates were incubated for 0, 24, 48 and 72 h. The 3T3-L1 cells were subsequently treated with CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), and the absorbance at 450 nm was measured for the supernatant of each well with a Multiskan EX plate reader (Thermo LabSystems, Helsinki, Finland).

Oil-Red O staining. On day 10 of adipocyte differentiation induction, the 3T3-L1 cells were stained with 1 mg/ml Oil-Red O dye (Abcam, Cambridge, MA, USA). The cells were fixed with 70% ethanol and dehydrated with 100% propylene glycol, and were subsequently stained with Oil-Red O. The cells were observed under a microscope (CX41RF; Olympus Corporation, Tokyo, Japan) and fat droplets in the adipocytes were stained red.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for miRNA-27a and miRNA-27b. The total RNA was extracted using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific, Inc.). RT-qPCR was performed to detect the mRNA expression levels of miRNA-27a and miRNA-27b, using a One-Step SYBR PrimeScript RT-PCR kit II (Takara Biotechnology Co., Ltd., Dalian, China) and data collection was conducted using an ABI 7500 (Thermo Fisher Scientific, Inc.). The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 45 sec, and a final extension step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. U6 small nuclear RNA served as an internal control. The gene expression was calculated using

the 2^{- $\Delta\Delta$ C_q} method. The primers used were as follows: Forward, 5'-ACACTCCAGCTGGGAGGGCTTAGCTGCTTG-3' and reverse, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTTCAGTTGAGTGCTCA-3' for miRNA-27a; forward, 5'-ACACTCCAGCTGGGAGAGCTTAGCTGATTG-3' and reverse, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTTCYAGTTGAGGTTTCAC-3' for miRNA-27b; forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3' for U6.

miRNA transfection. The 3T3-L1 cells were transfected with 40 μ M negative control (NC), miRNA-27a and miRNA-27b, miRNA-27a and miRNA-27b mimics or miRNA-27a and miRNA-27b inhibitors (anti-miRNA-27a and anti-miRNA-27b) obtained from Beyotime Institute of Biotechnology (Shanghai, China), which knockdown miRNA-27a and miRNA-27b, using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The 3T3-L1 cells were prepared for further analysis 48 h after transfection.

Triglyceride assay. The content of triglycerides (TGs) were analyzed using the Triglyceride Quantification assay kit (Abcam), according to the manufacturer's protocol. Briefly, the 3T3-L1 cells were collected and resuspended in 0.1 M phosphate-buffered saline. Following centrifugation for 10 min at 400 x g and 4°C, the cells were lysed in 1-2% Triton X-100 for 30 min for each assay. The samples were measured at 546 nm in a plate reader (Multiskan EX; LabSystems, Helsinki, Finland).

Luciferase reporter assays. PPAR- γ was predicted to interact with miRNA 27a by bioinformatics analysis using TargetScan, which predicts biological targets of miRNAs by searching for the presence of 8mer, 7mer, and 6mer sites that match the seed region of each miRNA (21). The 3'-untranslated region (UTR) of human PPAR- γ predicted to interact with miRNA-27a was synthesized and immediately inserted downstream of the *Renilla* luciferase reporter gene in the pGL3 vector (Promega Corporation, Madison, WI, USA), yielding pGL3-PPAR- γ . The 3T3-L1 cells were co-transfected with miRNA-27a mimics or NC using the Lipofectamine 2000. After 24 h, the luciferase activities were examined using the Dual-Luciferase Reporter assay system (cat. no. E1960; Promega Corporation). Firefly luciferase activity was normalized against that of *Renilla* luciferase activity.

Statistical analysis. The data are presented as the mean \pm standard deviation. One-way analysis of variance, followed by Dunnett's test was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Berberine inhibits the cell viability of 3T3-L1 cells. Following treatment with concentrations of berberine (1, 10, 20, 40 and 80 μ M) for 72 h, the growth of the 3T3-L1 cells was significantly reduced compared with that of the control

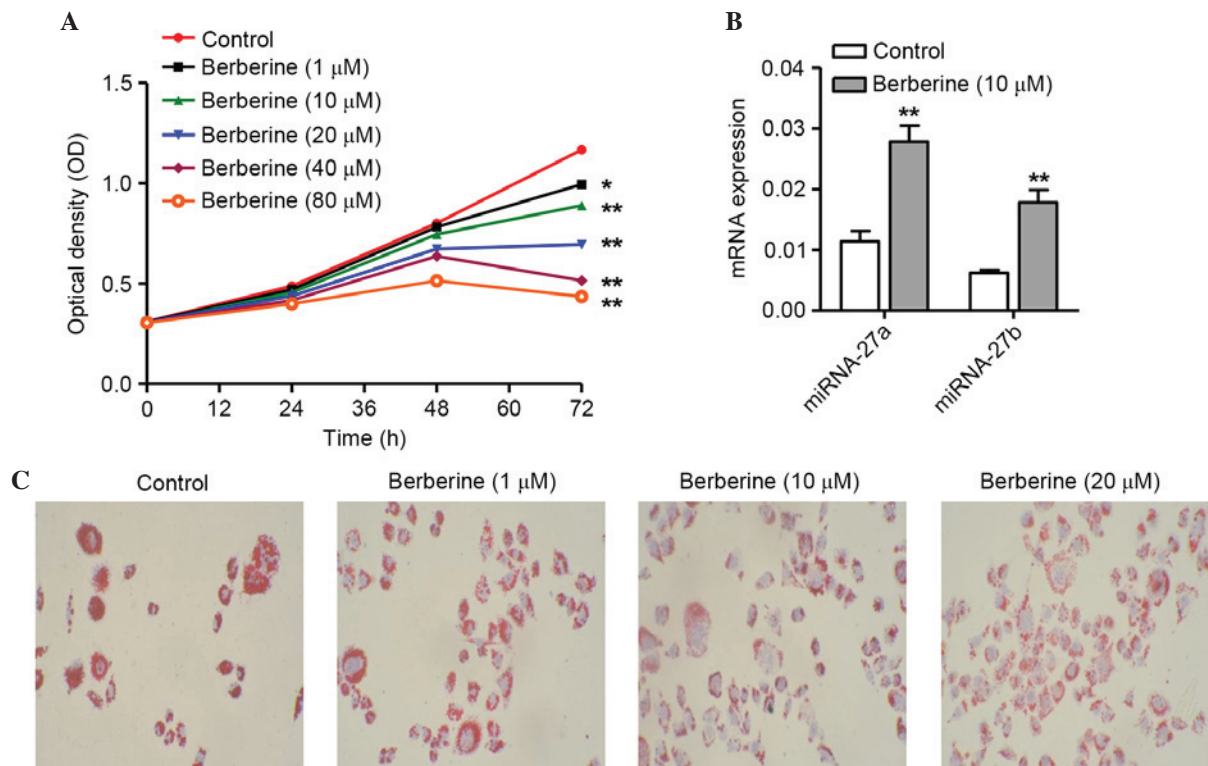


Figure 1. Berberine inhibits cell viability, differentiation and upregulates miRNA-27a and miRNA-27b in 3T3-L1 cells. (A) Berberine (1, 10, 20, 40 and 80 μ M) significantly inhibited the viability of 3T3-L1 cells in a time- and dose-dependent manner when compared with the control group. (B) miRNA-27a and miRNA-27b were upregulated in 3T3-L1 cells following treatment with berberine. (C) Berberine (1, 10 and 20 μ M) significantly inhibited the differentiation of 3T3-L1 cells in a dose-dependent manner when compared with the control group (magnification, x200). The data are presented as the mean \pm standard deviation (n=3; *P<0.05 and **P<0.01 compared with the control). miRNA, microRNA.

group (P<0.05). The cell viability of 3T3-L1 cells was reduced by berberine treatment in a dose- and time-dependent manner (Fig. 1A).

Berberine inhibits the differentiation of 3T3-L1 cells. The present study first examined the antiobesity potential of berberine by determining pre-adipocyte differentiation into adipocytes. Cultured 3T3-L1 cells were exposed to berberine at different doses and cell differentiation was induced using the differentiation medium. Following reculturing in DMEM with 10% FBS for 10 days, cell differentiation was terminated and fat droplets were detected using Oil-Red O staining. As shown Fig. 1B, the 3T3-L1 cells in the control group exhibited normal differentiation, as indicated by the appearance of numerous intracellular lipid droplets. However, treatment of 3T3-L1 cells with berberine at different concentrations (10 and 20 μ M) caused a dramatic reduction in lipid droplet accumulation dose-dependently. These results indicated that berberine efficiently inhibited adipocyte differentiation and may exhibit antiobesity effects in 3T3-L1 cells.

Berberine upregulates the expression levels of miRNA-27a and miRNA-27b in 3T3-L1 cells. Based on the observed effect of berberine treatment on cell viability and differentiation, the present study selected the 10 μ M berberine conditions for further mechanistic studies on miRNA-27a and miRNA-27b changes. RT-qPCR was used to confirm the expression levels of miRNA-27a and miRNA-27b following treatment with berberine. Treatment with berberine was observed

to upregulate the expression levels of miRNA-27a and miRNA-27b (1.44- and 1.87-fold increase; P<0.01; Fig. 1C). Overall, the present findings provided evidence suggesting that berberine treatment upregulates the mRNA expression levels of miRNA-27a and miRNA-27b in 3T3-L1 cells.

miRNA-27a and miRNA-27b regulate the berberine-mediated inhibition of differentiation in 3T3-L1 cells. After determining that miRNA-27a and miRNA-27b were upregulated by berberine in 3T3-L1 cells, the present study investigated whether the expression levels of miRNA-27a and miRNA-27b regulated the differentiation of 3T3-L1 cells after treatment with berberine. To demonstrate the association between cell differentiation and miRNA-27a, as well as miRNA-27b, miRNA-27a and miRNA-27b mimics were transfected into 3T3-L1 cells. The mRNA expression levels of miRNA-27a and miRNA-27b were subsequently assessed by RT-qPCR. As shown in Fig. 2A and B, transfecting 40 nM miRNA-27a or miRNA-27b mimics into 3T3-L1 cells resulted in a 1.71- and 1.70-fold increase in the mRNA expression levels of miRNA-27a and miRNA-27b, respectively. By contrast, knockdown of miRNA-27a or miRNA-27b by transfecting anti-miRNA-27a and anti-miRNA-27b into the 3T3-L1 cells resulted in a 72.2 and 53.8% decrease in the mRNA expression levels of miRNA-27a and miRNA-27b, respectively (P<0.01; Fig. 2C).

Furthermore, 3T3-L1 cell differentiation was significantly decreased following treatment with miRNA-27a and miRNA-27b mimics, combined with berberine treatment

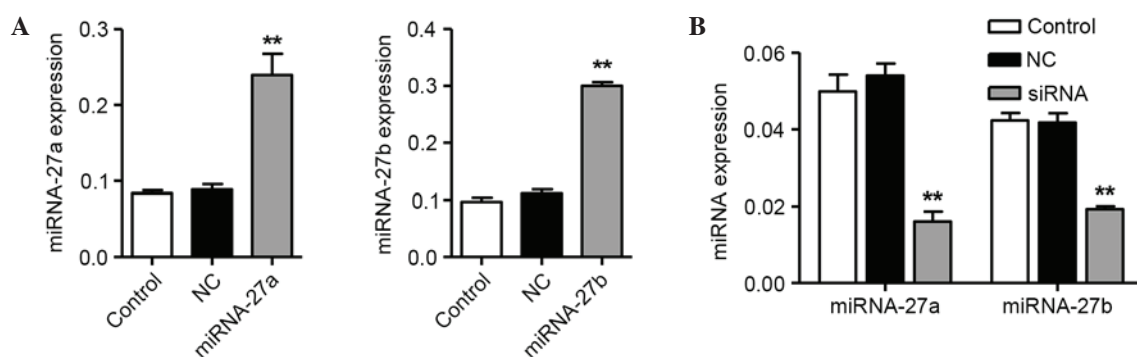


Figure 2. mRNA expression levels of miRNA-27a and miRNA-27b in 3T3-L1 cells. (A) RT-qPCR analysis showing the upregulation of miRNA-27a and miRNA-27b in 3T3-L1 cells transfected miRNA-27a and miRNA-27b mimics, respectively. (B) RT-qPCR analysis showing the downregulation of miRNA-27a and miRNA-27b in 3T3-L1 cells transfected with siRNA targeting anti-miRNA-27a and anti-miRNA-27b. The data are presented as the mean \pm standard deviation (n=3; **P<0.01 vs. NC). NC, negative control; siRNA, small interfering RNA; miRNA, micro RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

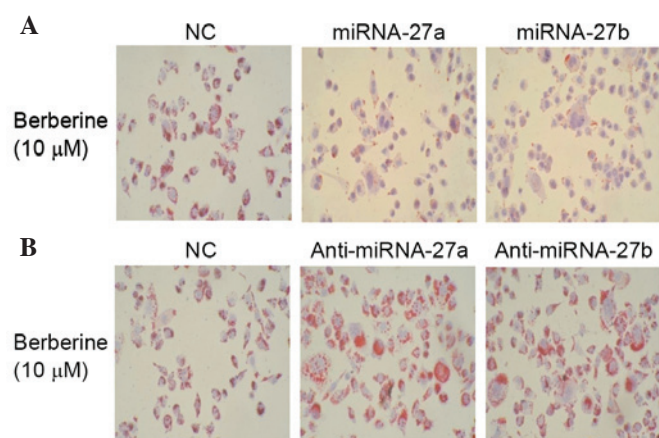


Figure 3. miRNA-27a and miRNA-27b regulate the berberine-mediated inhibition of differentiation in 3T3-L1 cells. (A) Overexpression of miRNA-27a and miRNA-27b enhanced berberine-mediated inhibition of differentiation in 3T3-L1 cells. (B) Inhibition of miRNA-27a and miRNA-27b attenuated the berberine-mediated inhibition of differentiation in 3T3-L1 cells. Magnification, x200. NC, negative control; miRNA, microRNA.

alone (Fig. 3A). However, knockdown of miRNA-27a and miRNA-27b increased the differentiation of 3T3-L1 cells following treatment with berberine compared with NC (Fig. 3B). These results suggested that the overexpression of miRNA-27a and miRNA-27b increased the berberine-mediated inhibition of 3T3-L1 cell differentiation, and that knockdown of miRNA-27a and miRNA-27b attenuated the inhibition of cell differentiation.

miRNA-27a and miRNA-27b regulate TG contents in 3T3-L1 cells following treatment with berberine. After determining that the differentiation of 3T3-L1 cells was regulated by miRNA-27a and miRNA-27b, the present study analyzed the intracellular TG contents in the 3T3-L1 cells following transfection with miRNA-27a or miRNA-27b mimics, as well as anti-miRNA-27a and anti-miRNA-27b, and NC for 48 h. As shown in Fig. 4A, the intracellular TG contents were decreased by 44.8 and 37.9% by the miRNA-27a and miRNA-27b mimics transfection in berberine treated 3T3-L1 cells, respectively (P<0.01). However, as

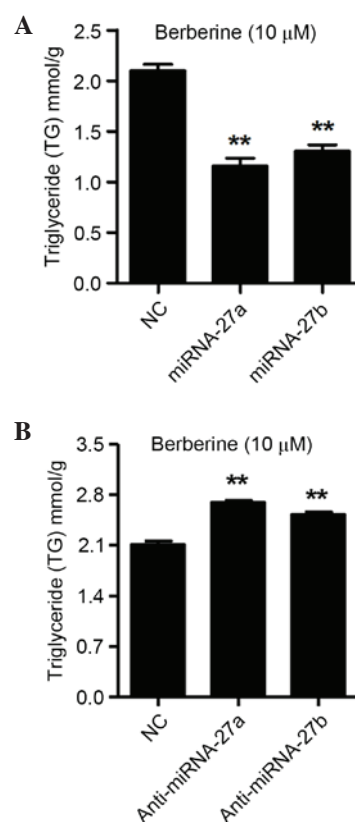


Figure 4. miRNA-27a and miRNA-27b regulate triglyceride contents in 3T3-L1 cells. (A) Overexpression of miRNA-27a and miRNA-27b reduced triglyceride contents in 3T3-L1 cells in response to treatment with berberine. (B) Inhibition of miRNA-27a and miRNA-27b increased triglyceride contents in 3T3-L1 cells in response to treatment with berberine. The data are presented as the mean \pm standard deviation (n=3; **P<0.01 compared with the NC). NC, negative control; miRNA, microRNA.

shown in Fig. 4B, the intracellular TG contents were increased by 27.8 and 19.7% by anti-miRNA-27a and anti-miRNA-27b transfection in berberine treated 3T3-L1 cells, respectively (P<0.01). These results indicated that miRNA-27a and miRNA-27b negatively regulate the intracellular TG contents, which have been demonstrated to impair the differentiation of 3T3-L1 cells.

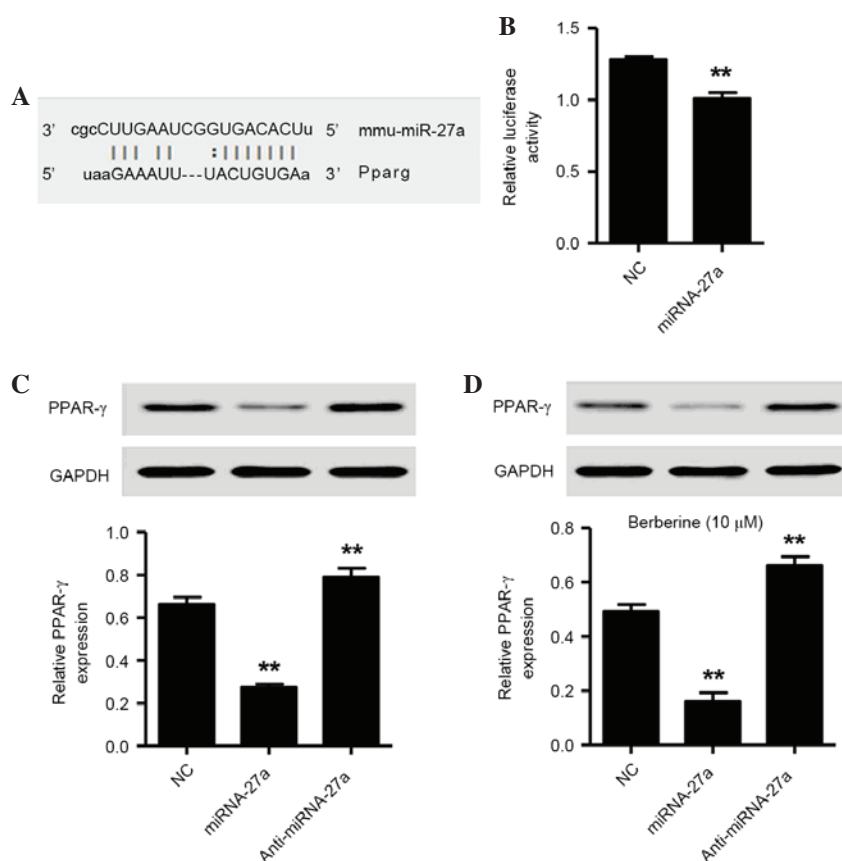


Figure 5. Effects of miRNA-27a on the expression of PPAR- γ in 3T3-L1 cells. (A) The TargetScan predicted base-pairing interaction of miRNA-27a seed sequences and PPAR- γ . (B) PPAR- γ was demonstrated to be a direct target gene of miRNA-27a by assessing the luciferase activity. The PPAR- γ protein levels were examined in 3T3-L1 cells transfected with miRNA-27a mimics, inhibitors or NC in the (C) absence and (D) presence of berberine. The protein expression was quantified and normalized against the expression of GAPDH. The data are presented as the mean \pm standard deviation (n=3; **P<0.01 compared with the NC). NC, negative control; miRNA, microRNA; PPAR, peroxisome proliferator-activated receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

miRNA-27a directly targets the PPAR- γ in 3T3-L1 cells. To investigate the regulatory mechanisms of miRNA-27a, bioinformatics analysis (TargetScan) was used. TargetScan identified that the mRNA sequence of PPAR- γ contained a potential binding site for miRNA-27a (Fig. 5A). To confirm PPAR- γ as a target and that this was regulated by miRNA-27a in 3T3-L1 cells, the PPAR- γ 3'-UTR was cloned and inserted into a luciferase reporter vector. The luciferase assay revealed that miRNA-27a significantly suppressed luciferase activity containing the PPAR- γ 3'-UTR (Fig. 5B). Western blotting analysis demonstrated that miRNA-27a overexpression significantly suppressed endogenous PPAR- γ expression, while inhibition of miRNA-27a significantly increased the protein expression of PPAR- γ in 3T3-L1 cells in the absence and presence of berberine (Fig. 5C and D). Together, these results suggested that PPAR- γ is a target of miRNA-27a and is downregulated by berberine in 3T3-L1 cells.

Discussion

Berberine serves an essential role in regulating numerous important cellular processes, including growth, differentiation, invasion, migration and apoptosis. Previous studies have suggested that berberine inhibits the proliferation of breast cancer cell by inducing cell cycle arrest (22) and promoted osteoblast differentiation by activating Runx2

and p38 mitogen-activated protein kinase (MAPK) (23). By contrast, berberine was observed to suppress Th17 and Th1 T cell differentiation by modulating the activities of extracellular-regulated kinase, p38 MAPK and c-Jun N-terminal kinase (24). In the present study, berberine inhibited the viability (Fig. 1A) and differentiation (Fig. 1B) of 3T3-L1 cells in a dose- and time-dependent manner. Therefore, the effects of berberine on different cell types may not be consistent and comparable.

To investigate the mechanisms by which berberine suppressed the viability and differentiation of 3T3-L1 cells, the mRNA expression levels of miRNA-27a and miRNA-27b were also measured by RT-qPCR. The present data showed that miRNA-27a and miRNA-27b were upregulated in 3T3-L1 cells following treatment with 10 μ M berberine (Fig. 1C), which is in accordance with a recent report by Lo *et al* (25) who demonstrated that berberine treatment upregulated miRNA-21-3p in the HepG2 human hepatoma cell line (25). Potential regulatory miRNAs, which were upregulated or downregulated in 3T3-L1 cells, were recently reported (26,27). No previous study has experimentally defined the direct association between berberine and miRNA-27a, as well as miRNA-27b, although miRNAs have been predicated to be putative targets of berberine. The present findings provided the first evidence, to the best of our knowledge, that berberine directly enhances the expression levels of miRNA-27a and miRNA-27b.

Furthermore, miRNA-27a and miRNA-27b were subsequently overexpressed and knocked down in 3T3-L1 cells (Fig. 2). The overexpression of miRNA-27a and miRNA-27b significantly enhanced the berberine-mediated inhibition of 3T3-L1 cell differentiation (Fig. 3A). However, inhibition of miRNA-27a and miRNA-27b significantly attenuated the berberine-mediated inhibition of 3T3-L1 cell differentiation (Fig. 3B). These data indicated that miRNA-27a and miRNA-27b markedly regulated the differentiation induced by berberine in 3T3-L1 cells and that berberine exerts an antidifferentiation activity that is associated with the upregulation of miRNA-27a and miRNA-27b. Previously, berberine upregulated the expression levels of two C/EBP inhibitors, CCAAT-enhancer-binding protein homologous protein (CHOP) and differentially expressed in chondrocytes-2, while it downregulated C/EBP α , PPAR γ and other adipogenic markers and effectors in differentiating 3T3-L1 pre-adipocytes and mature adipocytes (28). Additionally, the antiadipogenic activity of berberine was notably diminished by knockdown of CHOP expression or by adjusting the differentiation culture media (12). As a result of its antidifferentiation activity in 3T3-L1 cells, miRNA-27a or miRNA-27b may be act as a potential therapeutic target worth further investigation.

An elevated TG level is a major marker of obesity, added to elevated glucose and blood pressure, and reduced high-density lipoprotein. The adipocyte is cell functioning as an energy store for TG and cholesterol esters. It also secretes various adipokines, inducing leptin, adiponectin and resistin, which regulate pathological processes. Previous studies have implied that berberine-treated 3T3-L1 cells exhibited significantly reduced levels of intracellular TGs (29,30). Therefore, the present study further investigated the effects of miRNA-27a and miRNA-27b on berberine-mediated reduction of TG levels in 3T3-L1 cells. As shown in Fig. 4, overexpression of miRNA-27a and miRNA-27b significantly reduced the accumulation of TGs and, in contrast, inhibition of miRNA-27a and miRNA-27b increased the accumulation of TGs in 3T3-L1 cells following treatment with berberine.

It is necessary to identify miRNA targets in order to evaluate the roles of miRNAs abnormally expressed in human cancer and to evolve gene therapies based on miRNAs. miRNA-27a and miRNA-27b are markedly downregulated in adipocytes. They can target and, therefore, can potential regulate a variety of genes associated with adipogenesis. In the present study, PPAR- γ was predicted as a potential target gene of miRNA-27a by bioinformatics using TargetScan (Fig. 5A). A luciferase activity assay indicated that miRNA-27a directly targeted the 3'-UTR of PPAR- γ mRNA (Fig. 5B). PPAR- γ is an important regulator of adipocyte differentiation, functioning in systemic lipid and glucose metabolism (31). It is widely recognized that a number of miRNAs have antidifferentiation activity, which may be via the inhibition of PPAR- γ expression. miRNA-27b targets PPAR- γ to inhibit growth and tumor progression in neuroblastoma cells (32). miRNA-130b reduces fat deposition in adipocytes by inhibiting the expression of PPAR- γ (33). Consistent with this notion, the present study demonstrated that miRNA-27a mimics downregulated the expression of PPAR- γ , however, upregulated the expression of PPAR- γ by anti-miRNA-27a (Fig. 5C). Similar to miRNA-27a mimics, berberine treatment upregulated miRNA-27a and

downregulated PPAR- γ (Fig. 5D). Notably, berberine and miRNA-27a mimics produced almost identical effects on cell differentiation and the accumulation of TGs (Figs. 3 and 4). Therefore, the present study concluded that the antidifferentiation effect of berberine may be performed, at least partly, via the suppression of miRNA-27a/PPAR- γ signaling.

In conclusion, the results of the present study indicated that berberine inhibited cell viability and differentiation, and upregulated the mRNA expression levels of miRNA-27a and miRNA-27b in 3T3-L1 cells. The present data demonstrated that miRNA-27a and miRNA-27b regulate berberine-mediated inhibition of differentiation and TG accumulation. In addition, PPAR- γ , a direct and functional target of miRNA-27a, may mediate miRNA-27a-induced differentiation and TG accumulation. miRNA-27a/PPAR- γ signaling provided novel insight towards understanding the underlying mechanisms of the antidifferentiation activity of berberine and may provide useful information for targeted therapy.

Acknowledgements

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