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Original Article

Effect of miR-195 on proliferation and adipogenic differentiation of hADSCs

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Abstract: We investigated the effects and mechanism of microRNA-195 (miR-195) action on proliferation and adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells (hADSCs). Adipogenic differentiation of hADSCs was induced with 3-Isobutyl-1-Methylxanthine (IBMX), insulin (INS), dexamethasone (DEX) and neuropeptide Y (NPY). Oil Red O staining and triglyceride accumulation determination were used to identify the formation of lipid droplets. MiR-195 expression level during adipogenic differentiation of hADSCs was examined by real-time PCR. We constructed the lentivirus expression vector, LV-hsa-miR-195-inhibitor, which was introduced into hADSCs, followed by induction of lipoblast differentiation. MTT was performed to evaluate cell proliferation. Real-time PCR and western blotting were used to examine the mRNA and protein expression of fatty acid synthase (FASN) during transfected hADSC adipogenic differentiation. An in vitro model of adipogenic differentiation of hADSCs was successfully constructed. The expression of miR-195 decreased after hADSCs were induced to differentiate into adipocytes. LV-hsa-miR-195-inhibitor significantly downregulated miR-195 expression in hADSCs cells. Moreover, the miR-195-downregulated group significantly increased adipogenesis and grew slowly, compared with the negative control group. FASN expression increased during adipogenic differentiation, and the miR-195-downregulated group elevated the mRNA and protein expression of FAS in hADSCs adipogenic differentiation, compared with the negative control group. In conclusion, IBMX, INS, DEX and NPY induced hADSC differentiation into mature adipocytes. Downregulation of miR-195 promoted hADSC adipogenic differentiation and suppressed cellular proliferation by regulating the expression of FASN, implying that miR-195 may be a potential therapeutic target for treatment of obesity and lipid metabolism disorders.

Keywords: MicroRNA-195, hADSC, adipogenic differentiation, proliferation, fatty acid synthetase complex, lentiviral expression vector

Introduction

Obesity is an energy imbalance in our body, leading to excessive accumulation of white adipose tissues, which are important characteristics of a metabolic disorder. High dietary fat and sugars, lack of physical activity, and genetic susceptibility make its prevalence grow rapidly. Obesity has become a global health issue and is one of the major risk factors for diabetes, dyslipidemia, and cardio-cerebro-vascular diseases. Thus, it is a serious threat to human health. The pathological process is closely related to adipogenesis and lipid metabolism. Therefore, studying its molecular mechanisms contributes to the prevention and treatment of these diseases.

MicroRNA (miRNA) is a single-stranded, small non-coding RNA of 19-25 nucleotides, which can selectively regulate gene expression through binding to mRNA [1]. MiRNAs can inhibit mRNA transcription by incomplete complementary binding to 3,-UTRs of the target mRNA or by mediating the degradation of mRNA by complementary binding [2, 3]. MiRNAs have been involved in diverse biological processes including early development [4], cell prolifera-

tion [5], apoptosis [6], cell death and differentiation [7]. Recently more and more studies have shown that many miRNAs play an important role in adipocyte differentiation and lipid metabolism, such as, miR-143 [8], miR-122 [9], miR-370 [9], miR-27a [10] and miR-320 [11]. MiR-195 was initially shown to be highly and specifically expressed in mouse lung tissue, and then its homolog was found in human cells, encoded by the intergenic spacer sequence of chromosome 17, and its mature sequence is: UAGCAGCACAGAAAUAUUGGC. However, Lee et al. [12] have shown that the expression of miR-195 decreased in differentiated adipocytes, and Ortega et al. [13] have demonstrated that it was downregulated in obese mature adipocytes compared with cells from lean subjects. Based on these findings, miR-195 may play a role in adipocyte differentiation.

Fatty acid synthase (FASN) is the key enzyme in de novo lipogenesis that catalyzes all the biosynthesis steps of long-chain fatty acids from acetyl-CoA precursors [14]. High concentrations of FASN are found in adipose tissue, liver and lactating mammary glands. Many studies have found FASN is closely associated with obesity [15, 16]. However, Mao et al. [17] have proven that miR-195 suppresses osteosarcoma cell invasion and migration in vitro by targeting FASN. Based on these findings, we hypothesized that miR-195 may regulate adipogenesis by targeting FASN in adipocytes.

Neuropeptide Y (NPY) is a 36-amino acid peptide, which is synthesized in neural tissue of the central and peripheral nervous systems. In recent years, the number of studies on the involvement of NPY in the occurrence and development of obesity and metabolic syndrome has increased [18-20]. Zofia et al. [21] have confirmed that NPY promotes lipid droplet accumulation, lipid synthesis, proliferation and differentiation of adipocytes, leading to obesity, and that the function of NPY is similar to insulin in adipocyte differentiation. Combined with the results of previous studies [22, 23], we used 100 nM NPY to promote adipogenic differentiation.

Human adipose tissue-derived mesenchymal stem cells (hADSCs) have many characteristics including extensive proliferative potential and the ability to differentiate into adipogenic, osteogenic, chondrogenic and myogenic lineages [24]. In this study, we used in vitro cultured hADSCs. We constructed the lentivirus expression vector, LV-hsa-miR-195-inhibitor, to establish a stable hADSC line with downregulated miR-195 expression, to examine the role of miR-195 downregulation in proliferation and adipogenic differentiation of hADSCs, and the potential mechanism. Our data indicated that miR-195, which was downregulated during adipogenic differentiation of hADSCs, inhibits proliferation and enhances differentiation by regulating FASN mRNA. This finding suggests that miR-195 may be a potential novel molecular target for obesity therapy.

Materials and methods

Cell culture

Human primary adipose tissue-derived stem cell strain was purchased from SXBIO Biotechnology (Shanghai, China). Cell lines were cultured in Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12; HyClone, MA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37°C with 5% CO_2 in a humidified atmosphere. Culture medium was replaced every 48 h until the cells reached 80% confluence. Cells were digested with 0.25% trypsin. Third passage cells were used in experiments.

Adipogenic differentiation

Cells were seeded in 6-well plates at a density of 2×10^5 cells/well. When cell confluence reached complete fusion and contact inhibition for 2 days, adipogenic differentiation was induced by replacing the plating media (DMEM/F12 basic media with 10% FBS) with the induction media [DMEM/F12 basic media containing 0.5 mM IBMX, 1 μM DEX and 10 μM INS]. After 48 h the induction media was replaced with an NPY-containing medium (DMEM/F12 basic media containing 100 nM NPY; Sigma-Aldrich, St. Louis, MO, USA) for an additional 12 days.

Cell transfection

The lentivirus expression vector, LV-hsa-miR-195-inhibitor, and a negative control (LV-hsa-miR-195-NC) with a green fluorescent marker were purchased from GeneChem Biotechnology (Shanghai, China). Cells were inoculated into 60-mm cell culture dishes (5 ml/well) at 1×10^5

cells/ml. When the cells reached 80-90% confluence, Lipofectamine 2000 was used to transfect the vectors into hADSCs according to the manufacturer's instructions. After 48 h of transfection, fluorescence microscopy and real-time PCR were performed to check transfection efficiency.

Cell proliferation assay

After culturing in plating media for 24 h, cells were cultured in basic media (DMEM/F12) for 24 h. Cells were then seeded in 96-well plates at 5×10^3 cells/well. Each group had five repeats. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5 mg/ml] was added to the cells followed by addition of 150 μl of DMSO (4 h after MTT application) at 24, 48 and 72 h. Then, the OD value that was measured with a plate reader (Thermo Multiskan MK3 spectrophotometer; Thermo Fisher Scientific, Waltham, MA, USA) was tested at 570 nm, to produce a growth curve indicative of the ability of the cells to proliferate.

Real-time PCR

Primer 5.0 software was used to design primers. The FASN sense strand was: 5'-TCTCTGGTGGTGTCTACATTTCG-3', and the antisense strand was: 5'-GCAGGATAGCACTCTCAGACAG-3', the β-actin sense strand was: 5'-AGGGGCCGGACTCGTCATACT-3', and the antisense strand was: 5'-GGCGGCACCACCATGTACCCT-3'. Both miR-195 (HmiRQP0283) and U6 (HmiRQP9001) primers were purchased from Guangzhou Fulengen Co., Ltd. (Guangzhou, China). Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse-transcribed cDNA was synthesized using a reverse transcription synthesis system (Toyobo, Osaka, Japan). Real-time PCR was performed using a Power SYBR Green PCR Master Mix on the ABI 7300 Instrument. β-actin was used as an internal reference for FASN mRNA expression level, U6 was used as an internal reference for miR-195 expression level, and the relative expression level was calculated via the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Samples were homogenized in RIPA buffer (Auragene, Changsha, China), and protein quantification was performed with a BCA protein assay kit. Proteins were separated by SDS-PAGE and electrotransferred to PVDF membranes (Millipore, Billerica, MA, USA). Rabbit anti-human FAS polyclonal primary antibody (Ab82419, Abcam, Cambridge, UK) was added and incubated in a shaker at 4°C overnight, and goat anti-rabbit secondary antibody (111-035-003, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was added for incubation at 37°C for 1 h. X-ray film was exposed, developed and fixed. β-Actin was used as an internal reference, and the primary and secondary antibodies used were mouse anti-human β-actin monoclonal antibody (Ab-130094, Abcam) and goat anti-mouse secondary antibody (111-035-008, Jackson ImmunoResearch Laboratories Inc.), respectively.

Oil Red O staining and determination of triglyceride content

The medium was removed, the cells were rinsed twice with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) and fixed in 4% (w/v) polyoxymethylene in PBS for 30 min at room temperature. Oil Red O (0.5 g; Sigma-Aldrich) was dissolved in isopropanol (100 ml), diluted with water (6:4) and filtered. The fixed cells were then stained with the filtered Oil Red O solution for 30 min at room temperature, washed in water and photographed. Water was evaporated at 32°C, and 1 ml/well isopropanol was added. After 20 min, the extraction liquid was removed to 96-well plates, and the OD value was measured with a plate reader at 490 nm.

Statistical analysis

All experiments were repeated independently at least three times, and the results are presented as mean ± SEM. Data were evaluated using Student's t-test, and differences between groups were considered statistically significant at $P < 0.05$. All statistical analysis was performed with SPSS 18.0 software.

Results

MiR-195 is downregulated during adipogenic differentiation of hADSCs

After 48 h of induction, media was replaced with 100 nM NPY-containing media. After incu-

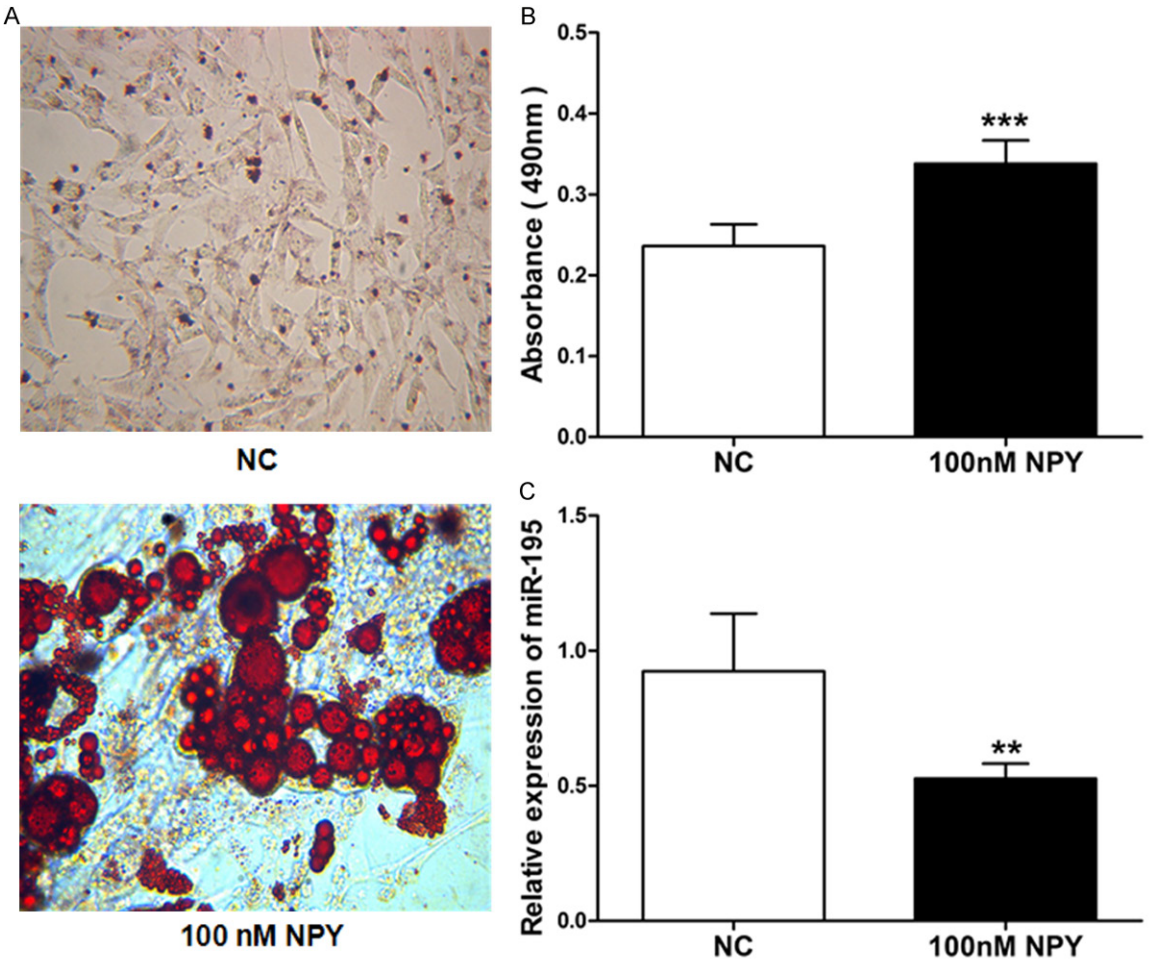

Figure 1. MiR-195 expression during adipogenic differentiation of hADSCs. A. Adipogenic differentiation was induced for 14 days and determined by Oil Red O staining. B. Triglyceride content was measured to determine intracellular lipid accumulation. C. MiR-195 levels were determined in the negative control (NC) or 100 nM NPY-treated hADSCs using real-time RCR. Data represent mean ± SEM. Comparisons were made with the corresponding controls using the Student's t-test for triglyceride content and miR-195 expression in hADSCs, ***P < 0.001, **P < 0.01.

bating hADSCs with adipogenic media for 14 days, the cells exhibited an expanded morphology and differentiated into the adipogenic lineage. The cells contained multiple intracellular red lipid-filled droplets, which accounted for 80-90% of the volume of the cells, and Oil Red O staining was positive in the adipogenesis induction group. However, there were no morphological changes or lipid droplet formation in cells cultured in the control media (**Figure 1A**). Moreover, in the adipogenesis induction group the synthesis of triglycerides was significantly promoted (**Figure 1B**). These results showed that treatment of cells with MIX + 100 nM NPY promoted hADSCs differentiation into mature adipocytes, similar to the findings of Liu et al. [22, 23].

To test the miR-195 expression during adipogenic differentiation of hADSCs, we used real-time PCR to examine the miR-195 levels in the cells. As shown in **Figure 1C**, the expression of miR-195 was significantly downregulated during adipogenic differentiation of hADSCs, which is consistent with the findings by Lee et al. [12].

Low expression of miR-195 inhibits proliferation of hADSCs

To examine the role of miR-195 in hADSCs proliferation, we transfected LV-hsa-miR-195-inhibitor into hADSCs to knockdown the expression of miR-195. The transfection efficiency was evaluated by fluorescent microscopy (**Figure 2A**) and real-time PCR (**Figure 2B**). Low expression of miR-195 was confirmed by real-

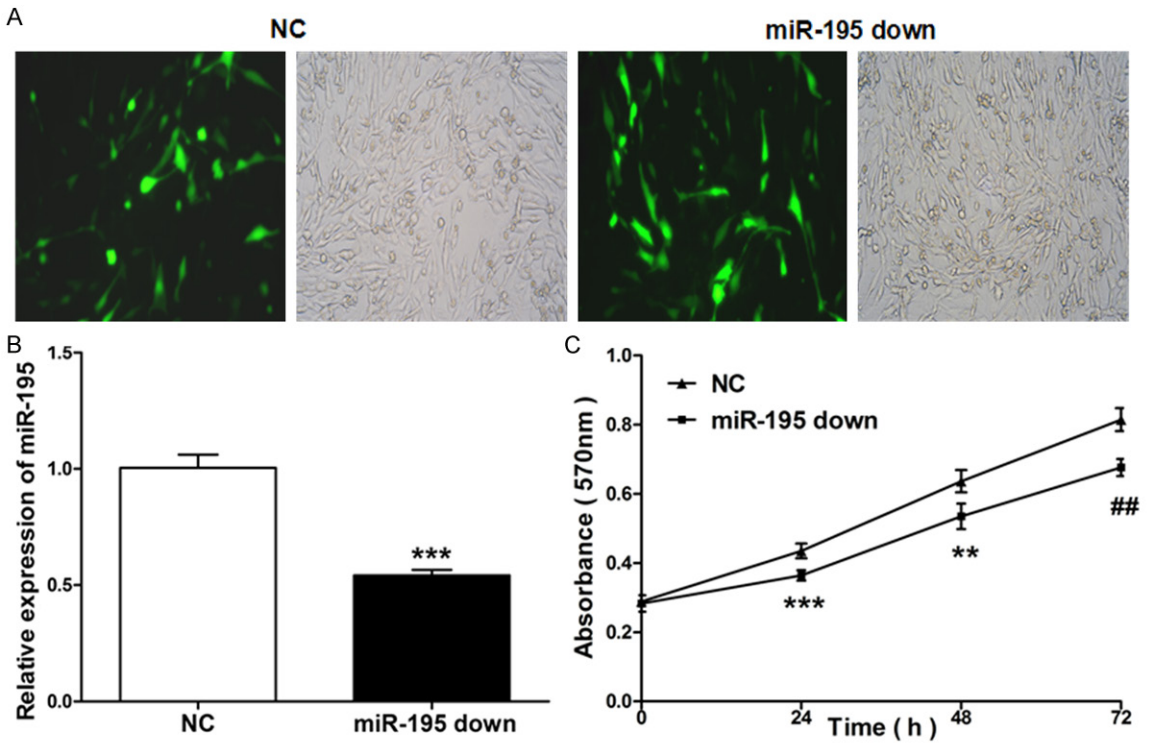

Figure 2. Effects of low expression of miR-195 on the proliferation of hADSCs. hADSCs were transfected with LV-hsa-miR-195-inhibitor or NC. A. Fluorescence microscopy pictures (magnification, ×100, left) and representative bright field microscopy (magnification, ×100, right) are shown in the top panel. B. Real-time PCR was used to detect the miR-195 expression level in transfected hADSCs. ***P < 0.001. C. MTT assays were performed to examine hADSCs proliferation at the indicated time points. Data were assessed by the Student's t-test, ***P < 0.001, **P < 0.01, ##P < 0.01.

time PCR. To test the effect of miR-195 downregulation on proliferation of hADSCs, cells transfected with LV-hsa-miR-195-inhibitor were examined at 24, 48 and 72 h after transfection. The transfected cells showed a slower growth rate than the control cells (**Figure 2C**).

Low expression of miR-195 promotes adipogenic differentiation of hADSCs

The expression of miR-195 decreased during adipogenic differentiation of hADSCs, hence, we hypothesized that miR-195 may be involved in the regulation of adipocyte differentiation. In contrast to the NC group, the accumulation of lipid droplets was obvious and the triglyceride synthesis increased in the miR-195-downregulated cells (**Figure 3A** and **3B**), indicating that adipogenic differentiation was induced in the transfected cells at day 14 post-differentiation. The results indicate that low expression of miR-195 in hADSCs significantly enhanced adipogenic differentiation.

miR-195 downregulation enhances the expression of FASN during adipogenic differentiation of hADSCs

To examine the effect of low expression of miR-195 on FASN during adipogenic differentiation of hADSCs, we transfected LV-miR-195-inhibitor or NC into hADSCs and induced the transfected cells to differentiate into adipocytes. We analyzed FASN expression by real-time PCR and western blot analysis. LV-miR-195-inhibitor-transfected cells had increased mRNA and protein levels of FASN compared with the control cells (**Figure 4A** and **4B**), suggesting that the expression of FASN increased during adipocyte differentiation. These results showed that low expression of miR-195 upregulated the expression of FASN during adipogenic differentiation of hADSCs.

Discussion

In this study, we investigated whether miR-195 is involved in the adipogenic differentiation of

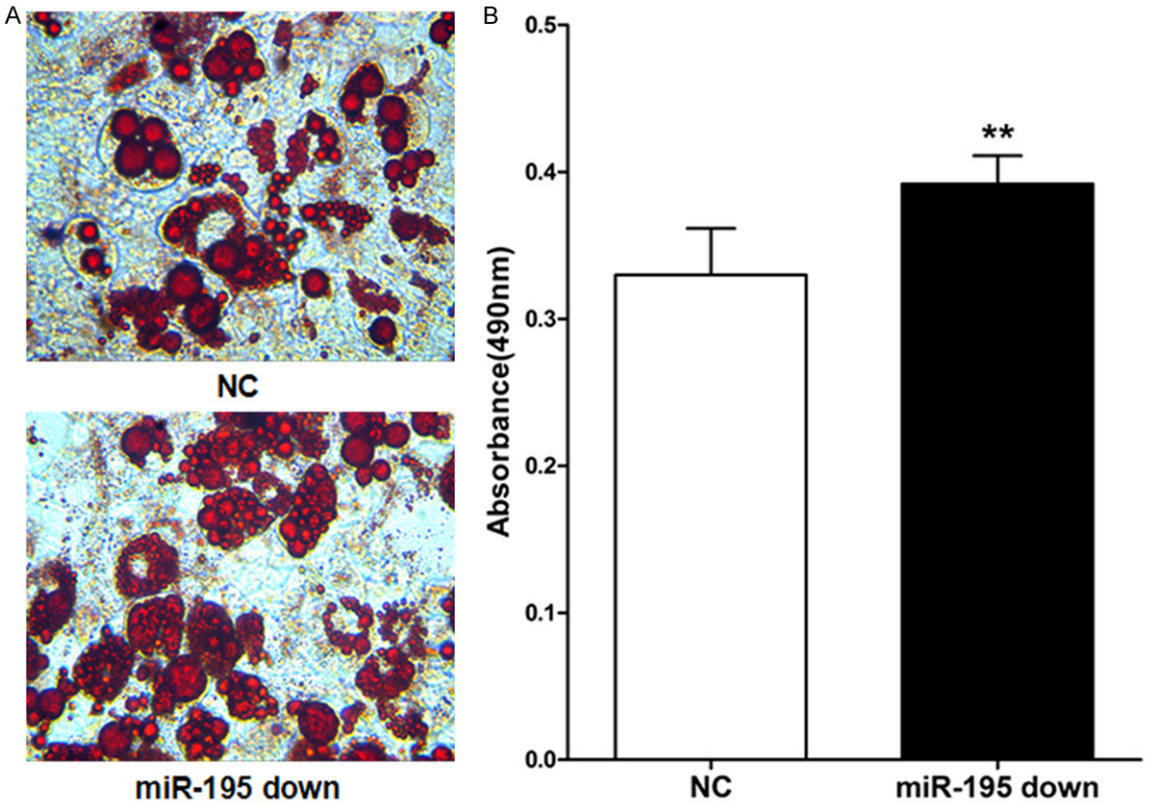

Figure 3. Effects of low expression of miR-195 on the adipogenic differentiation of hADSCs. hADSCs were transfected with LV-hsa-miR-195-inhibitor or NC. A. Adipogenic differentiation was induced for 14 days, and then Oil Red O staining was performed. B. Triglyceride content was determined after adipogenesis. **P < 0.01.

hADSCs. The levels of miR-195 expression decreased during the adipogenic differentiation of hADSCs, and knockdown of miR-195 with LV-hsa-miR-195-inhibitor in hADSCs inhibited proliferation and enhanced adipogenic differentiation. Furthermore, low expression of miR-195 increased FASN expression at the transcription and translation levels. Taken together, these results indicate that miR-195 negatively regulates adipogenic differentiation.

MiR-195, a miR-16/15/195/424/497 family member, plays different roles in multiple cancer types [25-27], as it is involved in tumor cellular proliferation, invasion, apoptosis, angiogenesis and metastasis. Recently, miR-195 has been reported to influence the insulin signaling pathway and insulin resistance in type 2 diabetes [28]. Furthermore, saturated fatty acids (SFA) significantly induced the expression of miR-195, which can target the insulin receptor (INSR), thereby, impairing insulin signaling and glycogen metabolism [29]. The roles of miR-195 in the formation and progression of obesity remain to be further investigated. Yet, proliferation, differentiation of preadipocytes and adipogenesis of adipocytes are the core of the development of obesity. In the present study, we found that miR-195 was downregulated during adipogenic differentiation of hADSCs (**Figure 1C**), which is consistent with the findings of Lee et al. [12] and Yun et al. [30]. Based on this, we constructed a lentivirus expression vector targeting miR-195-inhibitor to knockdown miR-195 expression, to verify the effects miR-195 on proliferation and adipogenic differentiation of hADSCs. After transfection with LV-miR-195-inhibitor into hADSCs, treatment with 100 nM NPY during the induction of adipocyte differentiation revealed a negative effect on proliferation and a positive effect on adipogenic differentiation due to the downregulation of miR-195 (**Figures 2C**, **3A** and **3B**). This suggests that miR-195 functions as an anti-adipogenic regulator, which may participate in the formation and progression of obesity.

Regulation of FASN-catalyzed de novo FA biogenesis plays a central role in the pathogenesis

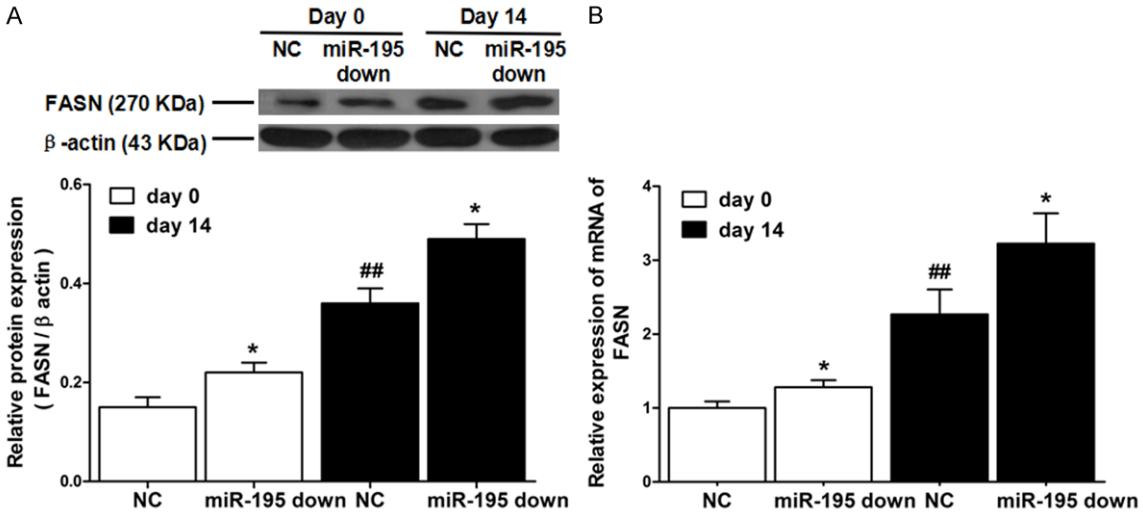

Figure 4. Effects of low expression of miR-195 on FASN expression during adipogenic differentiation of hADSCs. After transfection with LV-hsa-miR-195-inhibitor or NC, cells were induced to differentiate into adipocytes for 14 days. A. Western blotting of FASN protein expression in hADSCs during induction of adipogenic differentiation. $^{\#\#}P < 0.01$ (day 14 vs. day 0 in the control group), $^{*}P < 0.05$ (miR-195 down group vs. NC group on the same day). B. Real-time PCR was used to determine the relative expression of FASN mRNA in identically transfected cells during adipogenic differentiation. The results were analyzed using Student's t-test. $^{\#\#}P < 0.01$ (day 14 vs. day 0 in the control group), $^{*}P < 0.05$ (miR-195 down group vs. NC group on the same day).

of metabolic diseases. Human adipocytes contain substantial levels of FASN activity, which is subject to nutritional and hormonal modulation. In obese animals, FASN levels are elevated. Boizard et al. [31] have reported that overexpression of FASN in obese rat adipocytes was dependent on adipocyte determination and differentiation factor 1/sterol regulatory element-binding protein (ADD1/SREBP). Furthermore, the FASN inhibitor, C75, downregulated peroxisome proliferator-activated receptor g (PPARg), which suppressed adipocyte differentiation [32]. Thus, ADD1, SREBP and PPARg are adipogenic transcription factors involved in adipocyte differentiation. Furthermore, various studies have reported that the inhibition of FASN expression suppressed cancer cell proliferation [33-35]. Also, Mao et al. [17] have shown a direct relationship between miR-195 and FASN. Based on these studies, we postulated that miR-195 may regulate hADSCs proliferation and adipogenic differentiation by targeting FASN. Taken together, the current study indicates that downregulation of miR-195 promotes hADSCs adipogenic differentiation and inhibits proliferation by targeting FASN. However, according to the TargetScan prediction, there are hundreds of predicted miR-195 targets, and a single miRNA can target multiple mRNAs to regulate gene expression. Hence, it is probable that other targets of miR-195 are also involved in hADSCs proliferation and adipogenic differentiation. Therefore, further studies are needed to identify the complete role of miR-195 in hADSCs proliferation and adipogenic differentiation.

In summary, we confirmed that miR-195 plays a role as an anti-adipogenic regulator, which is downregulated during adipogenic differentiation of hADSCs. Low expression of miR-195 led to a high FASN expression at the mRNA and protein level, and in turn enhanced hADSC adipogenic differentiation and suppressed proliferation. These results suggest that miR-195 may serve as a target in the discovery of effective therapies for obesity and lipid metabolism disorders.

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Disclosure of conflict of interest

None.

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