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MiR-328-5p inhibits the adipogenic differentiation of hMSCs by targeting fatty acid synthase

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Abstract

Introduction. Adipogenesis, a highly coordinated process regulated by numerous effectors, is largely responsible for the quantity and size of adipocytes. Attenuation of adipocyte differentiation has been proposed as a viable technique for reducing obesity and its associated diseases. MicroRNAs play an important role in human bone marrow mesenchymal stem cells (hMSCs) adipogenic differentiation. However, there is a lack of clarity regarding the role of miR-328-5p in adipogenesis.

Material and methods. Using the lentiviral vectors to overexpress fatty acid synthase (FASN) and miR-328-5p, RT--qPCR and Western blotting were carried out to assess RNA expression and protein levels of FASN and adipogenic marker factors. Meanwhile, Oil red O staining and lipid quantification was performed to evaluate the accumulation of intracellular lipid droplets. Additionally, the validity of FASN as a potential target gene for miR-328-5p was carried out using a luciferase reporter assay.

Results. Our data showed that hMSCs adipogenic differentiation was associaed with the reduced miR-328-5p expression, while an elevated expression of the underlined miRNA attenuated adipogenesis and the expression of adipogenic marker genes. Luciferase reporter assay validated FASN as a target gene of miR-328-5p, and an elevated FASN expression reversed the anti-adipogenic effects of miR-328-5p.

Conclusions. The results revealed that miR-328-5p inhibits hMSCs adipogenic differentiation by targeting FASN. These findings contribute to our understanding of obesity-related disease development. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 4, 292–300)*

Keywords: miR-328-5p; hMSCs; adipogenesis; FASN

Introduction

Obesity has emerged as a global health concern that leads to the onset of a variety of major disorders, including cardiovascular diseases, and type 2 diabetes [1, 2]. Adipocytes are the distinguishing cell type of adipose tissue [3]. The quantity and size of adipocytes are mostly determined by adipogenesis, a highly coordinated process regulated by several effectors, including miRNAs [4]. Attenuation of adipocyte differentiation has been proposed as a viable technique for reducing obesity and its related disorders.

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There are several cellular models of adipogenesis, however, the murine-derived 3T3-L1 cell line, initially isolated in 1974, is the most often utilized in *in vitro* studies of adipogenesis [5]. Human bone mesenchymal stem cells (hMSCs) can also differentiate into adipocytes and are another useful model of adipogenesis [6]. hMSCs derived from human bone marrow are multipotent stem cells capable of self-renewal and multilineage differentiation, including chondrogenesis, osteogenesis, and adipogenesis [7]. *In vitro*, hMSCs can differentiate into adipocytes in the presence of a hormone cocktail. As a result, these cells serve as a suitable model for studying adipogenesis.

MiRNAs are short (20–22 nt) transcripts that do not have the potential to encode proteins. MiRNAs interact with the complementary 'seed' sequences in the 3′--UTR of target genes, inhibiting the mRNA and protein level of the gene [8]. MiRNAs have a considerable role in embryonic development, cancer, and cell differen-

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tiation [9–11]. Adipogenesis has been linked to several miRNAs, including miR125b-5p [12], miR-206-3p [13], miR-130a [14], and microRNA-27a/b-3p [15]. Even though multiple studies have revealed the crucial regulatory roles played by miRNAs during adipogenic differentiation [4], the significance of miR-328-5p in hMSCs adipogenic differentiation remains unknown.

Adipogenesis is an intricate process involving numerous transcription factors that affect cell differentiation [16, 17], which in turn upregulates genes involved in adipogenesis [18], including fatty acid synthase (FASN). FASN activity regulates the expression of key adipogenic transcription factors such as Peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT enhancer-binding proteins α (C/EBPα) [19]. Moreover, a FASN-derived signal is necessary for the functional differentiation of 3T3-L1 cells.

During the preliminary experiments on adipogenic differentiation of hMSCs, we found that miR-328-5p was downregulated and FASN was upregulated. Furthermore, bioinformatics analysis revealed that miR-328-5p could be a potential factor in adipogenic regulation by targeting the FASN gene. In our study, we investigated that miR-328-5p regulates hMSC adipogenic development *via* targeting FASN. These findings contribute to our understanding of the progression of obesity-related diseases.

Materials and methods

Cell culture and adipogenesis. After obtaining informed consent the hMSCs were grown from posterior iliac crest aspirates taken from healthy donors according to a previously described with slight modification [20, 21]. To verify isolation and enrichment of hMSCs, after reaching passage 5, the obtained cells were evaluated by flow cytometry to confirm that ≥ 95% of the cells were positive for CD90, CD73, and CD105, and negative for CD11b, CD19, CD45, CD34, and CD HLA-DR surface antigens [21, 22]. OriCell hMSC Growth Medium (HUXMA-90011, Cyagen Biosciences, China) supplemented with glutamine, penicillin, streptomycin, and 10% fetal bovine serum, was utilized for cell culturing. 5×10^4 cells were suspended in the indicated culture medium, incubated in a humidified environment at 37°C and continuous CO_2 supply (5%). Adipogenesis was induced by using adipogenic cocktails, comprising dexamethasone and 3-isobutyl-1-methylxanthine (0.5 mM each), insulin (10 μg per mL), and FBS (10%). All of these chemicals were purchased from Gibco, USA. The differentiation was allowed to continue for 3 to 7 days, with media being switched to fresh media every third day. Oil red O staining was employed to identify the development of lipid droplets in cells on day 7th of this process. The levels of mRNA and protein in the underlined cells were measured on days 3 and 7.

Design and assembling of FASN-specific sgRNAs. FASN overexpressing hMSCs cells were established by the dCas9-SAM system [23]. Single-guide RNAs (sgRNAs) were designed and assembled into lentivirus vectors by Shanghai Genechem Co., LTD, (Shanghai, China). Three guide sequence oligos are 5′-GCGCACGAGCATCACCCCAC-3′, 5′-AGCCCCGACGCTCATTGGCC-3′, and 5′-ACAGCTTGGCTGCGCCGCCC-3′.

Lentiviral transduction. Shanghai Genechem Co., Ltd. provided the lentiviral vectors (for FASN and miR-328-5p overexpression) and appropriate control vectors. A green fluorescence protein (GFP, encoded by lentiviruses) was used to assess the transduction potency. Furthermore, serial dilution was carried out to examine lentiviral titers. In 6-well plates, the hMSCs were grown to reach 30% to 40% confluence, followed by adding 1×10^8 TU/mL of lentivirus (10 μL) and polybrene (5 μg/mL) into each well in complete media for transduction. Cells were incubated for 10 h, followed by providing fresh media to the cultured cells and the incubation time was further extended to 72 h. It took two days (48 h) after transduction for cells to become stable, subsequently subjected to puromycin 0.5 g/mL puromycin (dissolved in media) for 6 days, during which time the media were replaced 1–2 days to sustain selection.

Oil red O staining and lipid quantification. Cell washing was carried out with phosphate-buffered saline (PBS) before being fixed in formalin (4%) for 30 min at 25°C. Saturated oil Red O (60%) was used for 30 min to treat the cells after being rewashed twice using PBS. Olympus IX73 microscope (Tokyo, Japan) was used to study the cells stained with Oil Red O. A microplate reader (Bio-rad, Hercules, CA, USA) was employed for the measurement of optical density at 490 nm to evaluate the accumulation of intracellular lipid droplets upon imaging.

qRT-PCR assays. Trizol (Invitrogen, Waltham, MA, USA) was utilized to isolate cellular RNA, followed by using Reverse Transcription System and Oligo (dT) (Thermo Scientific, Waltham, MA, USA) for synthesizing cDNA. A real-time PCR miRNA kit (Ribobio, Guangzhou, China) was used to measure miR-328-5p expression, with the U6 small RNA serving as a normalizing control using Ribobio's suitable primers. Comparatively, β-actin mRNA expression was used as a normalization control for mRNA expression using the primers listed in Supplementary Table 1. For qRT-PCR procedures, a TOYOBO SYBR Premix Ex Taq kit (TOYOBO, Osaka, Japan) was utilized with an ABI 7500 Real-Time PCR System (ABI, Los Angeles, CA, USA), and the $2^{-\Delta\Delta CT}$ technique was employed to analyze the obtained data.

Western blotting. Cells were lysed on ice in RIPA buffer, followed by boiling of lysates in 5 × SDS sample buffer for 5 min. Moreover, an SDS-PAGE was employed to separate protein, followed by transferring onto PVDF membranes (Millipore, Burlingon, MA, USA), that were blocked with skimmed milk. The membrane was incubated with primary rabbit antibodies specific for FASN, PPARγ, Fatty Acid Binding Protein 4 (FABP4), and β-actin (Cat. no. 10624-2-AP Proteintech, USA; Cat. no. AF6284 Affinity, USA; Cat. no. ab92501 Abcam

Table 1. Primers used in this study for β-actin, PPARγ, FABP4, and FASN

Gene symbol	Forward primer	Reverse primer
PPARγ	5'- GGGATGTCTCATAATGCCATCAG- 3'	5'-GCCCTCGCCTTTGCTTTG-3'
FABP4	5-'GGATGATAAACTGGTGGTGGAATG-3'	5'- CAGAATGTTGTAGAGTTCAATGCGA -3'
FASN	5-'CACAGGGACAACCTGGAGTT-3'	5-' ACTCCACAGGTGGGAACAAG-3'
β-actin	5'-GCGAGAAGATGACCCAGATCATGT-3'	5'-TACCCCTCGTAGATGGGCACA-3'

USA; Cat. no. 20536-1-AP Proteintech, USA; accordingly). Each antibody was diluted at a 1:1000 dilution unless otherwise specified. Furthermore, the membrane was incubated with secondary antibodies *i.e.,* anti-rabbit IgG (1:10000; Cat. no. SA00001-2 Proteintech, USA) conjugated with HRP before chemiluminescence was performed to detect protein bands.

Prediction of miRNA target genes. Predictions of miR-328-5p target genes were carried out *via* online algorithms (http://www.targetscan.org/) *i.e.,* TargetScan 6.2, miRBase 21, and PicTar.

Luciferase reporter assay. The pGL3 Luciferase Reporter Vectors (Promega, Madison, WI, USA) were employed to determine the FASN's validity as a putative miR-328-5p target gene. The putative miR-328-5p interacting site was found to be present in both the mutant type (MUT) and wild type (WT) of the FASN 3'-UTR, which was used to make the luciferase gene cloned in the pGL3 vector. Before transfecting 293T cells with miR-328-5p mimics, the vectors were sequenced to ensure they were constructed correctly. Using the Renilla luciferase activity, data were collected 48 h after transfection, followed by normalizing the data. The findings were then compared to those levels found in cells transfected with a control miRNA.

Statistical analysis. The statistical evaluations were carried out by SPSS ver. 16.0 (Chicago, IL, USA) while the applied statistical method was Student's *t*-tests. The obtained data of triplicate experiments have been presented as means ± SD. A P-value less than 0.05 was found to be statistically significant.

Results

MiR-328-5p expression decreased during hMSCs adipogenesis

An adipogenic medium was used to culture hMSCs for 3–7 days before the miR-328-5p expression was evaluated during differentiation. On days 3 and 7 of adipogenesis, the expression levels of miR-328-5p were found to decrease as compared with the starting point (Fig. 1).

Preparation of hMSCs with altered miR-328-5p expression

We transduced lentiviral vectors into cells to overexpress miR-328-5p. GFP expression was observed in transduced cells, indicating successful transduction,

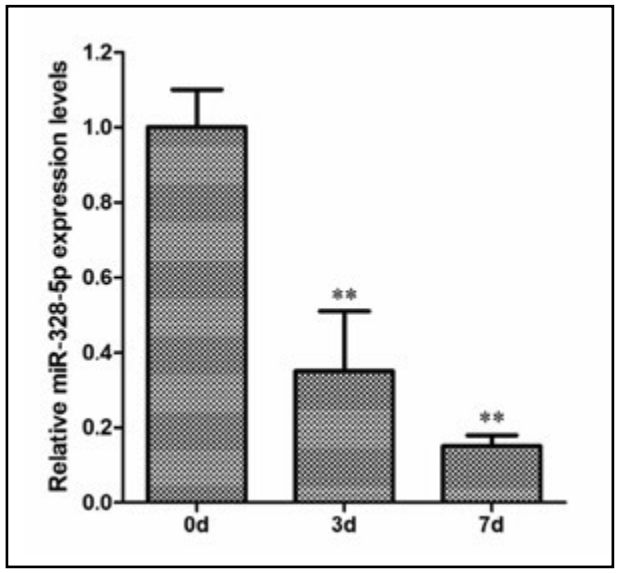

Figure 1. The expression level of miR-328-5p over the course of adipogenic differentiation of hMSCs was assessed with qPCR at indicated time points. Data are expressed as the mean ± SD (n = 3). $^{**}P < 0.01$ *vs*. day 0.

as shown in Fig. 2A. Subsequently, the miR-328-5p expression in the underlined cells was then examined using qPCR, as depicted in Fig. 2B.

Impact of miR-328-5p on hMSCs adipogenesis

The role of miR-328-5p in adipogenesis was further investigated by measuring the levels of the adipogenic markers *i.e.,* PPARγ and FABP4 at the transcriptional and translational level *via* qPCR and Western blotting. The findings revealed that an elevated expression of miR-328-5p was associated with lower levels of the PPARγ and FABP4 biomarkers (Fig. 3A, and B). The Oil Red O staining indicated that elevated miR-328-5p expression reduced lipid accumulation on the 7th day after differentiation, as indicated in Fig. 3C. Moreover, the quantitative analysis demonstrated a statistically substantial reduction in intracellular lipid accumulation, as shown in Fig. 3D.

Identification of miR-328-5p target genes

TargetScan 6.2 (http://www.targe tscan.org/), PicTar (http://picta r.mdc-berlin.de/), and miRBase 21

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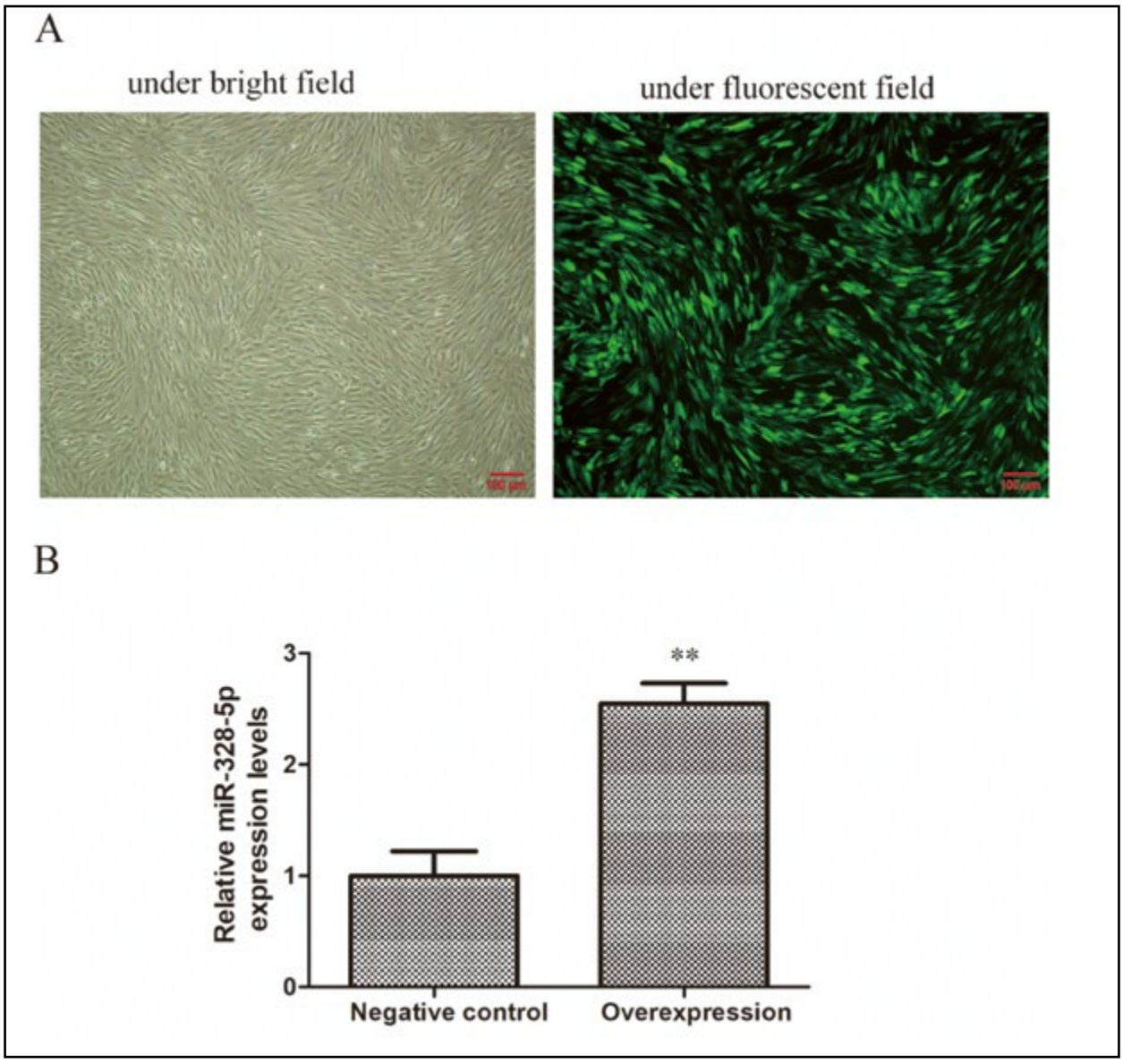

Figure 2. Screening of stably transduced hMSCs. Control or miR-328-5p overexpression lentiviral vectors were used to transfect hMSCs. **A.** Lentivirally transduced cells were assessed *via* light and fluorescent microscopy (40×); scale bar, 100 μm, with representative images being shown. Fluorescence distribution of GFP (green fluorescent protein) on cells indicating transfection efficiency; **B.** qPCR was used to measure miR-328-5p overexpression. Data are expressed as the mean ± SD (n = 3). $^{**}P < 0.01$ *vs*. negative control.

(http://www.mirba se.org/) were utilized to search for probable direct miR-328-5p target mRNAs. FASN has been identified as a possible target gene for miR-328-5p, which is likely to be a direct adipogenic target. According to TargetScan, there were two miR-328-5p binding sites within the 3′-UTR of FASN (Fig. 4A). Standard luciferase reporter experiments were used to further validate the expected interaction of miR-328-5p with FASN 3'UTRs. When the FASN 3'UTR was co-transfected with miR-328-5p mimic, luciferase activity decreased by 62 percent ($P < 0.01$) in luciferase reporter experiments, but not in cells transfected with mutant FASN 3'UTR (Fig. 4B).

To further verify the targeted relationship between miR-328-5p and FASN, the transcriptional and translational levels of FASN were evaluated in miR-328-5p overexpressed hMSCs adipogenic differentiation (Fig. 4C, D). The FASN expression was found to be decreased in miR-328-5p overexpressing hMSCs. The underlined data indicated that miR-328-5p targets FASN.

FASN overexpression rescues the adipogenic differentiation effect of hMSCs by miR-328-5p overexpression

To validate the binding between FASN and miR-328-5p during adipogenic differentiation of hMSCs,

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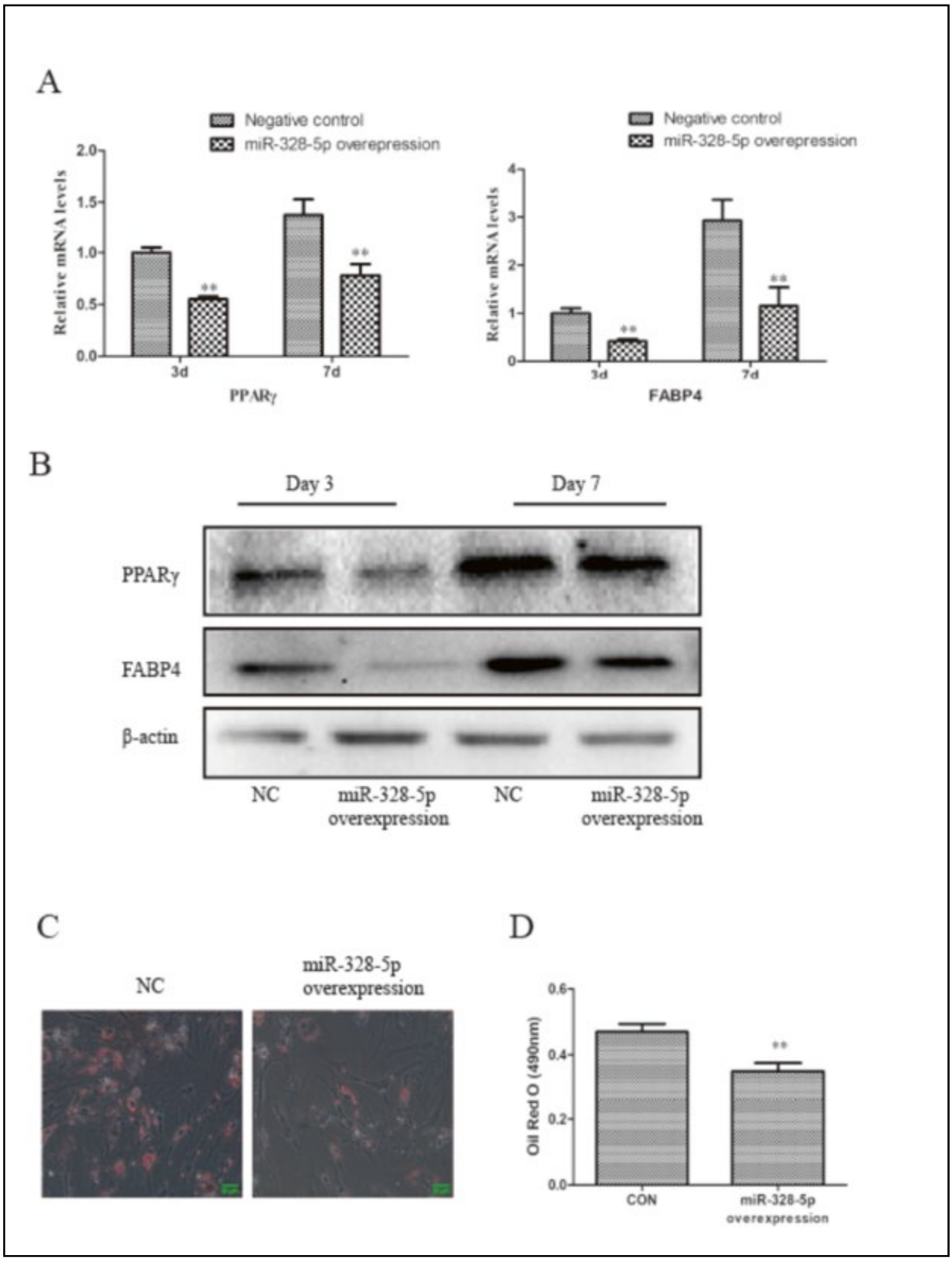

Figure 3. miR-328-5p overexpression impairs hMSC adipogenesis. **A–B.** The expression levels of PPARγ and FABP4 in hMSCs treated with adipogenic medium for 3 and 7 days were determined by qPCR and Western blotting. Staining **(C)** and quantitation **(D)** of lipid droplets by Oil Red O among these groups of cells on day 7 after adipogenic induction. (200×); scale bar, 20 μm, with representative images being shown. Data are expressed as the mean ± SD (n = 3). $^{**}P < 0.01$ *vs*. negative control. Abbreviation: NC — negative control.

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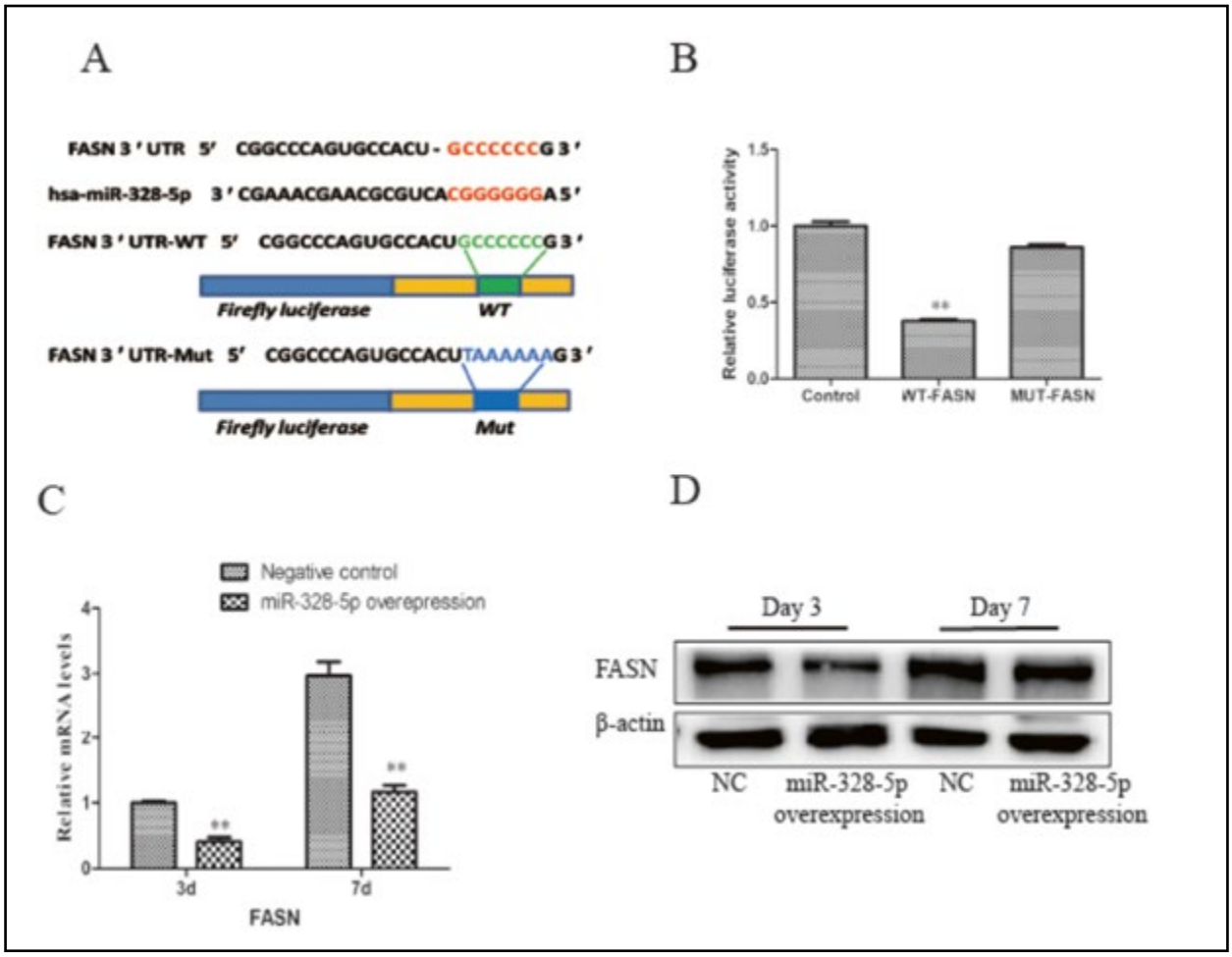

Figure 4. miR-328-5p targets the 3'-UTR of FASN. **A.** The binding sites of miR-328-5p and FASN mRNA 3'-UTR. **B.** Luciferase activity assay. **C–D.** The mRNA and protein levels of FASN were monitored after miR-328-5p overexpression lentivirus was transfected. Data are expressed as the mean ± SD (n =3). $^{**}P < 0.01$ *vs*. negative control. Abbreviation: NC — negative control.

we co-transfected miR-328-5p and FASN lentiviral vectors to overexpress miR-328-5p and FASN in hMSCs. It has been revealed that an elevated expression of FASN may reduce the effect of overexpressed miR-328-5p on adipogenic marker genes, as depicted in Figs. 5A and 5B. In addition, according to Oil Red O staining, accumulated lipid droplets were found to be decreased in overexpressed miR-328-5p and FASN co-transfected cells, as highlighted in Fig. 5C–D. The obtained results suggested that an elevated FASN expression significantly decreased the function of overexpressed miR-328-5p on hMSCs adipogenic differentiation.

Discussion

Obesity is becoming a more prevalent cause of mortality worldwide, prompting researchers to explore the mechanisms underlying adipogenesis [24, 25]. Adipocyte differentiation is a complex process controlled at multiple levels. Extensive studies on the regulation of adipogenesis have shown that transcription factors including PPARγ and C/EBPα, as well as signaling pathways like PI3K/Akt, insulin, and Wnt/β-catenin are involved in the regulation of adipogenesis [17, 26]. Furthermore, a growing number of miRNAs associated with adipogenesis have also been identified.

In this study, a considerably decreased miR-328-5p expression has been observed during hMSCs adipogenic differentiation. We constructed a lentivirus that overexpressed miR-328-5p and revealed that overexpressed miR-328-5p inhibits adipogenic differentiation by reducing adipogenic marker genes expression at the mRNAs and protein levels. The underlined data showed that miR-328-5p is a negative regulator in hMSCs differentiating into white adipocytes. According to the reported study, miR-328 positively regulates brown adipose tissue (BAT) differentiation [27]. Reduced miR-328 expression inhibited preadipocyte differentiation, whereas enhanced

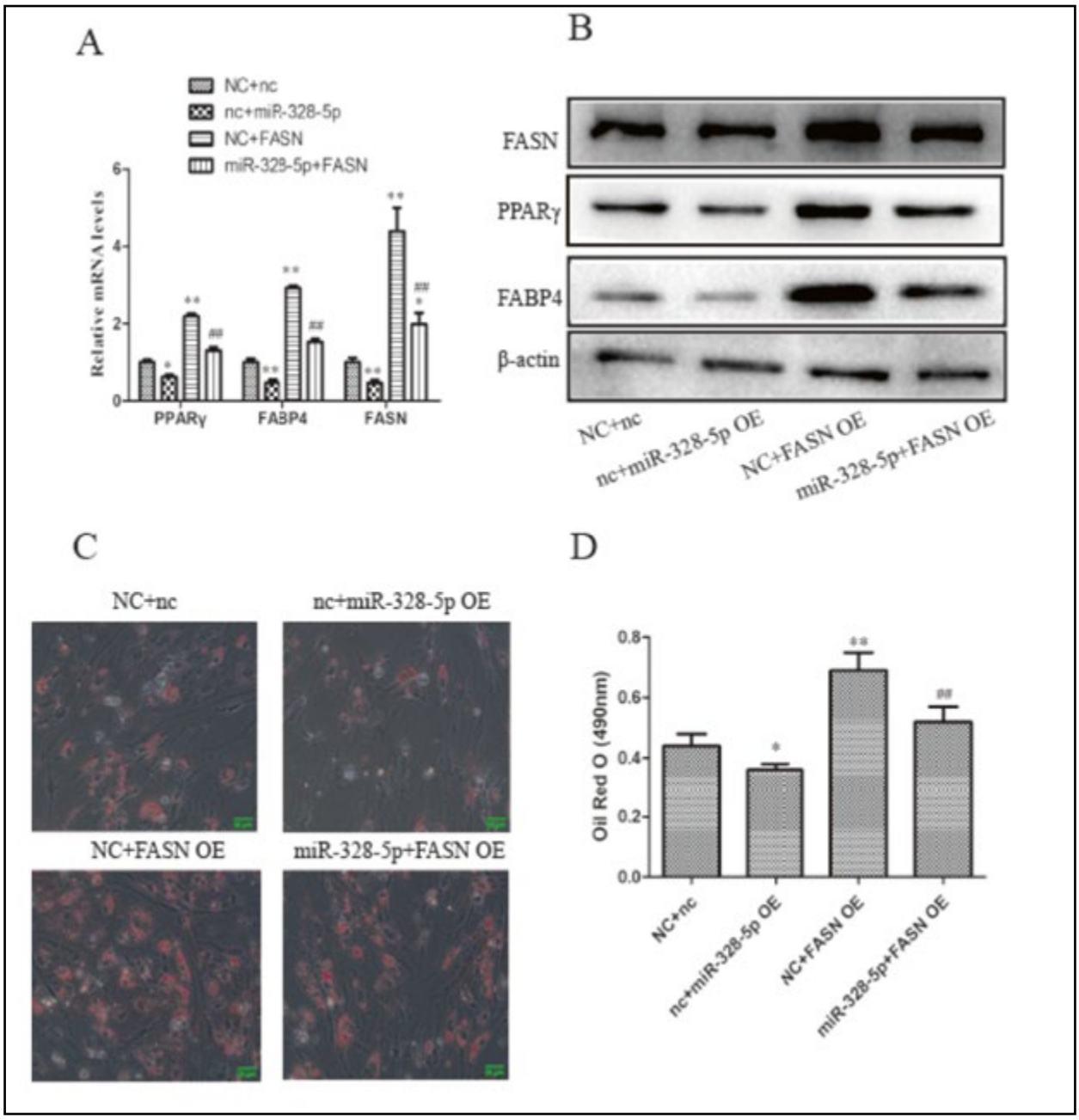

Figure 5. FASN overexpression restored the adipogenic differentiation of hMSCs inhibited by miR-328-5p. hMSCs overexpressed miR-328-5p with FASN overexpression simultaneously. **A–B.** The mRNA and potein levels of FASN, PPARγ and FABP4 were monitored on day 7 after adipogenic induction. Staining **(C)** and quantitation **(D)** of lipid droplets by Oil Red O among these groups of cells on day 7 after adipogenic induction. (200×); scale bar, 20 μm, with representative images being shown. Data are means ± SD (n = 3). $^{*}P < 0.05$ *vs.* negative control, $^{**}P < 0.01$ *vs.* negative control, $^{\#\#}P < 0.01$ *vs.* miR-328-5p overexpression. Abbreviations: FASN OE — FASN overexpression; NC — miR-328-5p overexpression negative control; nc — FASN overexpression negative control; miR-328-5p OE — miR-328-5p overexpression.

miR-328 expression induced BAT differentiation, partly by silencing the β-secretase Bace1 [27]. Our results reveal for the first time that miRNA-328 is involved in regulating the differentiation of MSCs into white adipocytes. Obviously, the role of miR-328 in BAT and WAT is different. Similarly, another microRNA miR-378 also works differently in BAT and WAT. miR-378 increases brown fat mass and suppresses the development of beige adipocytes in subcutaneous WAT [28]. Interestingly, we also found that even in the same tissue, the expression profiles of miRNA in mice and humans were different, that is, the same miRNA played different roles in mice and

humans [29]. Our study enriches and expands the content of miRNA regulation of adipocyte differentiation.

How miRNA-328 regulates the downstream mechanism of MSC adipogenic differentiation? We used predictive bioinformatics algorithms to evaluate FASN as a potential miR-328-5p target gene. FASN is the key enzyme in *de novo* lipogenesis, so it is closely associated with lipids accumulation in adipocytes [30]. However, FASN is not only an endpoint in preadipocyte differentiation but is an active participant. Many studies have shown that palmitate, the main product of FASN, promotes Ob771 preadipocyte differentiation by increasing the terminal differentiation-related markers mRNA and protein levels in a dose-dependent manner [31].

However, whether FASN participates in the adipogenic differentiation of hMSCs is an interesting problem. In this study, it has been revealed that an elevated expression of miR-328-5p significantly reduced FASN expression at the transcriptional and translational levels in hMSCs. Luciferase reporter assays verified the targeted relationship between miR-328-5p and FASN. Consequently, a recovered experiment was extremely important. However, FASN's mRNA is 8.4 kb in length making a full-length clone is challenging. Only a few previous studies have demonstrated FASN overexpression [32] since it is difficult to detect. To up-regulate the expression of FASN in hMSCs, we used the CRISPR/dCas9 Synergistic Activation Mediator (SAM) system which may selectively up-regulate the expression of specific target genes without modifying the endogenous genome [23].

The FASN overexpression during hMSCs adipogenic differentiation was linked with the key regulators of adipogenesis, according to the obtained results which showed that FASN catalyzes the *de novo* lipogenesis and is used to maintain and sustain signaling for the differentiation process [19].

In summary, we found that miR-328-5p suppresses adipogenic differentiation of hMSCs by targeting FASN. Our findings may open up new avenues for developing therapeutic regimens for the treatment of obesity and other associated disorders.

Conflict of interests

The authors declare that they have no conflicts of interest associated with the manuscript.

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