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Original Article

miR-135a-5p affects adipogenic differentiation of human adipose-derived mesenchymal stem cellsby promoting the Hippo signaling pathway

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Abstract: MicroRNAs (miRNAs) have been extensively studied and play a regulatory role during adipogenesis. Specifically, many miRNAs participate in regulation of mesenchymal stem cell (MSC) differentiation into adipogenic and osteogenic lineages. However, the regulatory mechanisms of miR-135a-5p in the Hippo signaling pathway during adipogenesis need to be explored. In this study, we observed that miR-135a-5p promotes adipogenesis of human adipose-derived MSCs (hADMSCs). miR-135a-5p was overexpressed in adipocytes compared to hADMSCs and further upregulation of miR-135a-5p promoted proliferation and adipogenesis of hADMSCs. In contrast, miR-135a-5p reduction inhibited these processes. Luciferase activity detection and Western blotting confirmed that overexpressed miR-135a-5p had the ability to upregulate the HIPPO signaling pathway. Subsequently, we observed that miR-135a-5p is targeted to key negative regulators in the HIPPO signaling pathway, including MOB kinase activator 1B (MOB1B) and large tumor suppressor 1 (LATS1). Moreover, suppression of LATS1 and MOB1B upregulated the activity of TEAD. In conclusion, we have verified that miR-135a-5p plays an active role in adipogenesis by targeting LATS1 and MOB1B expression, thereby enhancing the HIPPO signaling pathway.

Keywords: Adipose tissue-derived stromal cell, miR-135a-5p, adipogenic differentiation, HIPPO signaling pathway, LATS1, MOB1B

Introduction

The primary reason for obesity is an imbalance between energy intake and consumption. A variety of diseases are associated with obesity, including hypertension, pathoglycemia, dyslipidemia, and even heart disease [1]. Additionally, body fat distribution is particularly important for body aesthetics, and fat cells as a source of stem cells for research and plastic surgery is a topic of emerging importance. Obesity is characterized by adipocyte hypertrophy and hyperplasia at the cellular level. Thus, an in-depth understanding of the mechanisms regulating adipogenic differentiation is of great significance [2].

As oligonucleotides of non-coding RNA, microRNAs play an inhibitory role in gene expression by suppressing or degrading target mRNAs at the post-transcriptional level [3]. miRNAs are involved in regulating various physiological processes, such as cell apoptosis, proliferation, and cell differentiation [4].

It has been reported that multiple miRNAs are involved in regulatory processes duringadipogenesis via various signaling pathways [5]. Researchers have identified multiple dysregulated miRNAs during adipogenesis in 3T3-L1 cells [6]. Specifically, miR-199 has been shown to inhibit the adipogenic transdifferentiation of myoblasts [7], and miR-27a has been shownto shift mesenchymal stem cells from osteogenic differentiation to adipogenic differentiation in postmenopausal osteoporosis [8].

The regulatory mechanism of adipogenesis involves different types of signaling pathways.

For instance, miR-540 has beenis a novel adipogenic inhibitor that blocks adipogenesis via targeting PPARγ [9]. miR-204-5p is targeted to DVL3 and inhibits the Wnt/β-catenin pathway, which promotes the adipogenic differentiation of human adipose-derived mesenchymal stem cells (hADMSCs) [10]. miR-135a-5p has also been shown to inhibit 3T3-L1 adipogenesis through activation of the canonical Wnt signaling pathway [11].

The Hippo signaling pathway is an evolutionarily conserved pathway that regulates the growth of tissue via integrin-dependent adhesion [12-15]. Driven by extracellular mechanical signals or cell-cell contacts, the Hippo pathway functions by activating transcriptional co-activators with PDZ-binding motifs (TAZ) and Yes-associated protein (YAP), two major downstream effectors of the Hippo pathway. Both TAZ and YAP are transcriptional co-factors that are responsible for controlling the target genes of the Hippo pathway, including connective tissue growth factor (CTGF) [16] and survivin [17].

Recently, it was observed that PPARγ agonists promote stem cell differentiation by suppressing YAP transcriptional activity, suggesting a relationship between the Hippo signaling pathway and PPARγ [18]. In addition, knockdown of integrins that specifically regulate the Hippo pathway mediator TAZ promoted adipogenic differentiation [19].

Although miR-135a-5p has been verified to be involved in adipogenesis, and severalfindings have demonstrated the potential role of the Hippo pathway in adipogenic differentiation, the impact of miR-135a-5p on Hippo signaling and adipocytic differentiation remains unclear. Accordingly, in our study, we have confirmed that miR-135a-5p plays a positive role in the differentiation of hADMSCs into mature adipocytes by activating the Hippo signaling pathway.

Materials and methods

Isolation and differentiation of hADMSCs

Subcutaneous fat biopsies (approximately 2-4 g) obtained from volunteers were carefully minced with surgical scalpels and later incubated with 2% collagenase type H (Roche, Mannheim, Germany) at 37°C for 90 min. After the collagen was completely digested, these tissues were centrifuged at 400 g for 5 min and washed with PBS three times. They were thenpassed through a 70-μm cell strainer (BD Biosciences, San Jose, CA, USA) to obtain single cells.

The cells were cultured in Advanced MEM supplemented with 5% PLT max (Mill Creek Life Sciences, Rochester, MN, USA) and 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA) in an atmosphere of 37°C and 5% CO_2. 3T3-L1 preadipocytes and HPA-adipocytes were obtained from ATCC and maintained in DMEM containing 10% FBS at 37°C and 5% CO_2.

Transfection of hADMSCs

A miR-135a-5p mimic and inhibitor were prepared by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). siRNA-LATS1, siRNA-YAP, siRNA-TAZ, siRNA-MOB1B, and siRNA-scramble were synthesized by Invitrogen (Carlsbad, CA,USA). The hADMSCs were transfected with miR-135a-5p mimic, miR-135a-5p inhibitor, siRNA-LATS1, siRNA-TAZ, siRNA-YAP, siRNA-MOB1B, or siRNA-scramble using Lipofectamine 2000 (Invitrogen) based on the manufacturer's instructions. Cell differentiation was induced as previously described 2 days after transfection. hADMSCs were maintained in DMEM supplemented with 10% FBS in an atmosphere at 37°C and 5% CO_2.

Bioinformatics analysis

miR-135a-5p targets were analyzed and predicted using PicTar (http://pictar.org/), Target Scan (http://www.targetscan.org/vert_42/), and miRanda (http://www.microrna.org/microrna/).

Cell viability assays

MTT assays were conducted to analyze cell viability. In brief, cells were cultured in a 96-well plate with 2×10^3 cells/well, and the media was changed every other day. After transfection with miR-135a-5p mimic or miR-135a-5p inhibitor, 50 mg/well MTT was added to the cells (Sigma, St. Louis, MO). The absorbance of formazan dissolved in dimethyl sulfoxide was measured at 450 nm on an ELISA plate reader (Bio-tek, Winooski, VT).

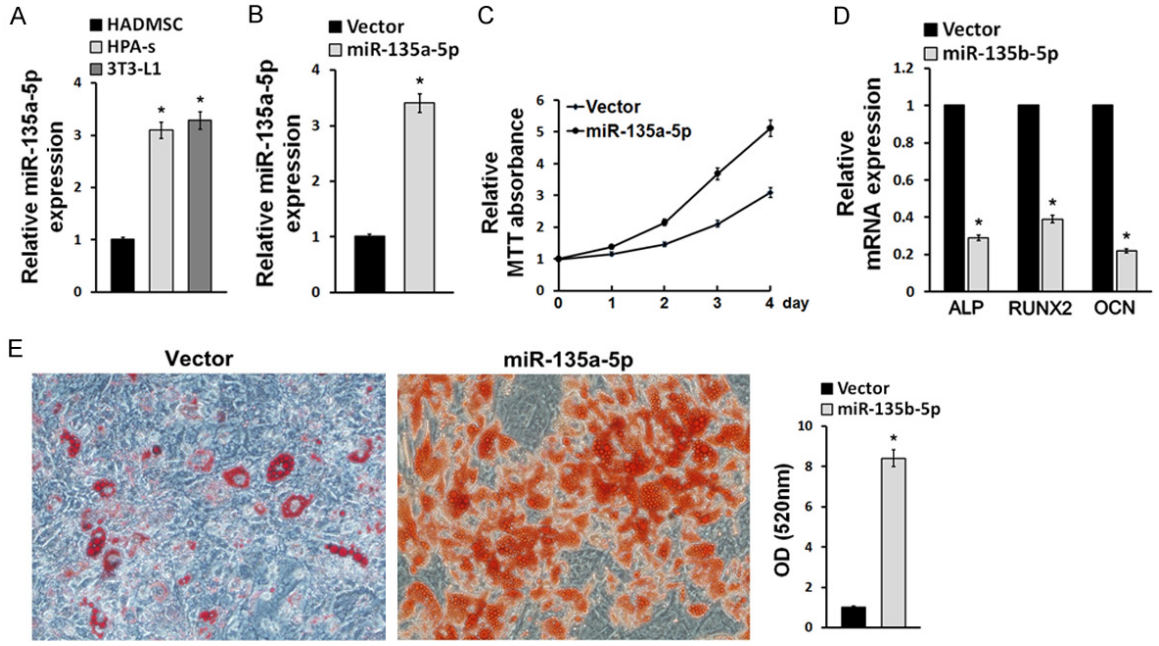

Figure 1. miR-135a-5p facilitates hADMSC adipogenic differentiation. A. MiR-135a-5pexpression in hADMSC, HPA-s and 3T3-L1 cells. B. MiR-135a-5p expression in hADMSCs. C. hADMSC cell viability tested by the MTT assay. D. Osteogenic factor mRNA expression in hADMSCs. E. Oil red O staining in hADMSCs. *P < 0.05 compared with the control.

Luciferase assays

To construct the pmiR-RB-REPORT vector, LATS1, and MOB1B sequences were amplified and cloned into the pmiR-RB-REPORT™ (Guangzhou RiboBio Co., Ltd.) dual luciferase plasmid. Additionally, the Quick Change Site-Directed Mutagenesis kit was used to perform single nucleotide site-directed mutagenesis of LATS1 and MOB1B, specifically changing the sequence from AAGCCAU to AAGCGUA and transforming AAAAGCCAUA to AAAAGCGUAA. The pmiR-RB-REPORT vectors (50 ng) containing either wild-type or mutant LATS1 or MOB1B 3'UTR were transfected into 293T cells (purchased from Xiangya Cells Center of Central South University, Changsha, China), which were co-transfected with the miR-135a-5p mimic (100 nM) or the miR-135a-5p inhibitor using Lipofectamine 2000 (Invitrogen). A control vector without the target sequence was selected as a negative control. These processes were conducted inthree replicates. Luciferase activities were analyzed by the Dual-Glo Luciferase assay system (Promega, Madison, WI, USA).

Oil Red O staining

The cells were fixed in 4% paraformaldehyde (Beyotime) for 30 minthen stained at room temperature with freshly prepared Oil Red O (Beyotime) working solution for 20 min. The Oil Red O working solution was composed of 60% Oil Red O stock solution and 40% H_2O. After washing twice with water, the fixed cells were observed under a microscope (TE-2000-E; Nikon, Tokyo, Japan).

Real-time PCR

Total RNA was extracted withTrizol reagent (Invitrogen) and stored in 50 mL of RNase-free water at -80°C. Total RNA was reverse transcribed into cDNA. The cDNA was added to the qRT-PCR reaction system containing cDNA template, primers, and SYBRGreen qPCR Master Mix. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative expression levels with 18S rRNA as an internal control.

Western blot analysis

Western blotting analysis was performed as described in previous studies. Briefly, the cells were lysed according to the RIPA buffer manufacturer's protocol.Equal amounts of protein as measured by a BCA Protein Assay were separated by SDS/PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 10% non-fat dry milk in TBST,

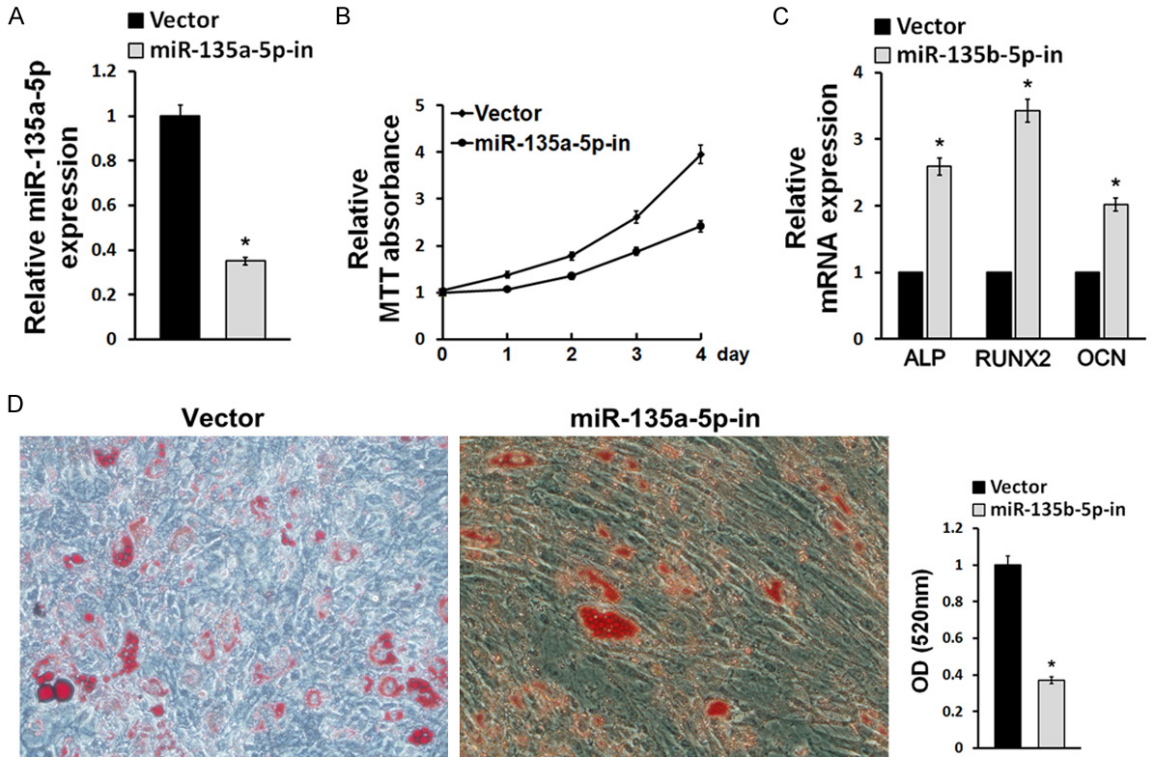

Figure 2. miR-135a-5p inhibitor suppresses hADMSC adipogenic differentiation. A. miR-135a-5p expression in hADMSCs. B. hADMSC cell viability determined by the MTT assay. C. Osteogenic factor mRNA expression in hADMSCs. D. Oil red O staining in hADMSCs. *P < 0.05 compared with the control.

the membranes were then incubated with the indicated primary antibodies at 4°C for 1 h, including antibodies against YAP, LATS1, TAZ, p84, MOB1B and GAPDH (Cell Signaling Technology, Danvers, MA). The HRP-conjugated secondary antibodies (Sigma) were added for 1 h, and bands were visualized using enhanced chemiluminescence (ECL) reagents (ECL; Thermo FisherScientific, UK).

Data analysis

Data were processed and analyzed by SPSS 18.0 and presented as the mean ± standard deviation. All data were obtained from at least three independent experiments. Statistical significance was calculated usinga two-tailed unpaired Student's *t*-test or ANOVA. A *P*-value of < 0.05 was considered to bestatistically significant.

Results

miR-135a-5p promotes hADMSC adipogenic differentiation

To determine miR-135a-5p expression in adipocytes, we selected hADMSC, adipocyte HPA-s, and preadipocyte 3T3-L1 cells to measure their miR-135a-5p levels. As shown in **Figure 1A**, miR-135a-5p expression in HPA-s and 3T3-L1 cells was higher than that in hADMSCs, indicating its upregulation in adipocytes (P < 0.05). Subsequently, hADMSCs were transfected with the miR-135a-5p mimic to explore its impact on cell proliferation and it was observed that expression of the miR-135a-5p mimic in hADMSCs significantly facilitated cell proliferation (P < 0.05, **Figure 1B**, **1C**). Furthermore, we observed that miR-135a-5p mimic transfection inhibited expression of osteogenesis factors, including ALP, RUNX2 and OCN (P < 0.05, **Figure 1D**). Conversely, miR-135a-5p significantly enhanced Oil Red O staining compared to the control, suggesting that miR-135a-5p has a positive effect on hADMSC adipogenic differentiation (P < 0.05, **Figure 1E**).

miR-135a-5p knockdown blocks adipogenic differentiation of hADMSCs

Because miR-135a-5p promoted hADMSC adipogenesis, we transfected the cells with a miR-135a-5p inhibitor to investigate whether the

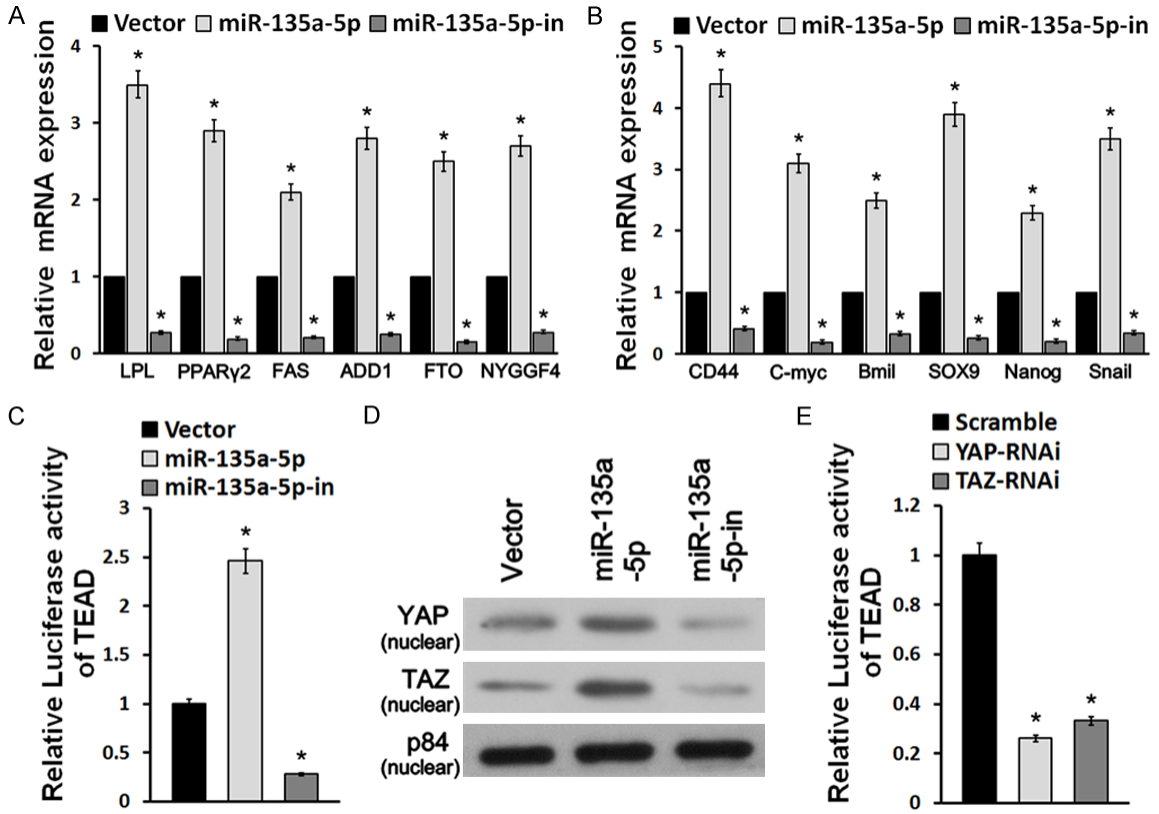

Figure 3. miR-135a-5p activatesthe Hippo signaling pathway. A. The mRNA levels of adipogenesis-related factors. B. The mRNA levels of proliferation-relatedfactors. C. Luciferase reporter assay detection of TEAD activity. D. Protein expression of YAP and TAZ in the cell nucleus detected by Western blotting. E. Luciferase reporter assay detection of TEAD activity. *P < 0.05 compared with the control.

miR-135a-5p inhibitor could inhibit adipogenesis in hADMSCs. The miR-135a-5p inhibitor efficiently reduced the level of miR-135a-5p in hADMSCs, which could cause significant inhibition of hADMSC proliferation (P < 0.05, **Figure 2A**, **2B**). Moreover, the miR-135a-5p inhibitor increased osteogenesis-related factor expression in hADMSCs (P < 0.05, **Figure 2C**). More importantly, Oil Red O staining was attenuated in hADMSCs after miR-135a-5p inhibitor transfection (P < 0.05, **Figure 2D**). Taken together, these findings indicate that miR-135a-5p may promote hADMSC adipogenesis and block osteogenesis.

miR-370-5p activated the Hippo signaling pathway

We also tested adipogenesis-related factors and proliferative factor expression in hADMSCs during dysregulation of miR-135a-5p. As shown in **Figure 3A**, expression of multiple related factors involved in adipogenesis, including LPL, PPARγ2, FAS, ADD1, FTO and NYGG4, showed a significant increasing trend in hADMSCs after miR-135a-5p transfection. Consistent with this finding, the levels of lipogenic factorsdeclined when the cells were transfected with the miR-135a-5p inhibitor (P < 0.05). Proliferative factors, such as CD44, C-MYC, BMIL, SOX9, NANOG and SNAL1, were found to be upregulated in hADMSCs after treatment withthe miR-135a-5p mimicbut were reduced after miR-135a-5p inhibition (P < 0.05, **Figure 3B**).

The Hippo signaling pathway is essential factor to the processes of cell proliferation and differentiation and has been shown to be involved in adipogenesis. Therefore, we explored whether miR-135a-5p can affect Hipposignaling pathway activity. We observed that the miR-135a-5p mimic enhanced TEAD activity inluciferase assays (P < 0.05, **Figure 3C**). Furthermore, we extracted nuclear proteins and measured YAP and TAZ expression levels. We-

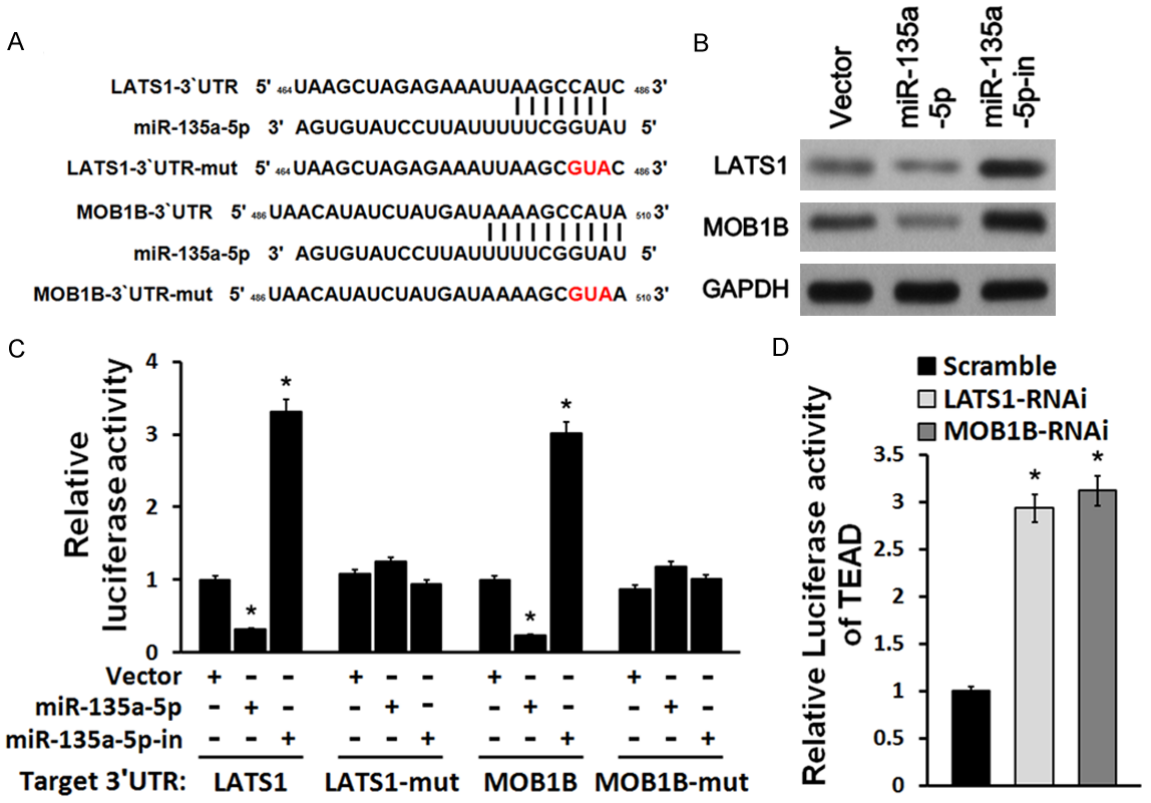

Figure 4. miR-135a-5p targets LATS1 and MOB1B to activate the Hippo signaling pathway. A. Predicted miR-135a-5p target sequences in the 3'UTR of LATS1 and MOB1B. B. Protein expression of LATS1 and MOB1B in hADMSCs transfected with miR-135a-5p mimic or inhibitor. C. Dual-luciferase reporter assay of the hADMSCs transfected with the LATS1 or MOB1B 3'UTR reporter and the miR-135a-5p mimic or inhibitor. D. Luciferase reporter assay detection of TEAD activity in hADMSCs transfected by LATS1 or MOB1B siRNA. *P < 0.05, compared with the control.

stern blots demonstrated that the miR-135a-5p mimic enhanced YAP and TAZ protein expression in hADMSCs, while the miR-135a-5p inhibitor had a diametric influence (**Figure 3D**). TEAD activity was markedly weakened by downregulation of YAP or TAZ in hADMSCs, indicating their essential roles in the Hippo signaling pathway (P < 0.05, **Figure 3D**).

miR-135a-5p triggers the Hippo signaling pathway by targeting LATS1 and MOB1B

Bioinformatics analysis determined that LATS1 and MOB1B have complementary binding sites in their 3'UTR sequences for miR-135a-5p (**Figure 4A**). Since these are negative regulatory factors in the Hippo pathway, Western blotting was performed. LATS1 and MOB1B expression was elevated in cells treated with the miR-135a-5p inhibitor (**Figure 4B**). Furthermore, the luciferase activity ofreporters containing the MOB1B and LATS1 3'UTRs was attenuated after treatment with the miR-135a-5p mimic and enhanced after miR-135a-5p inhibitor transfection (**Figure 4C**). Meanwhile, inhibition of LATS1 and MOB1B expression obviously increased TEAD activity, revealing their negative regulatory impacton the Hippo pathway (P < 0.05, **Figure 4D**).

Discussion

The formation of mature adipocytes from ADMSCs involves multipotent MSCs that differentiate into committed hADMSCs and preadipocytes before maturing into adipocytes, and the process is mediated by C/EBP and PPAR [20]. miR-135a-3p has been described as an inhibitory molecule that controls adipogenesis via regulation of the Wnt/β-catenin pathway [11]. However, there is still a lack of information regarding the role of its opposite strand, miR-135a-5p, in adipocytic differentiation. A previous study showed an upregula-

tion of miR-135a-5p levels in severe intrauterine adhesion [21]. Another study showed that the miR-135a-5p level was significantly dysregulated in lymphangiogenesis of human gastric cancer [22]. A recent study reported that HDL-carried miR-135a-5p isassociated with intake of trans fatty acid, indicating its role in adipogenesis and fat metabolism [23]. Unfortunately, aspecific regulatory mechanism of miR-135a-5p's involvement in various biological activities has not been thoroughly elucidated to date.

Our experiments examined miR-135a-5p expression in adipocytes at different developmental stages, including hADMSCs, preadipocytes, and mature adipocytes, and we observed its overexpression during adipocyte differentiation and maturation. This result is opposite tothe miR-135a-5p expression trend in 3T3-L1 adipogenesis. We also found that upregulated miR-135a-5p promoted adipogenesis of hADMSCs, which was verified by a significant increase in the number of lipid droplets and increases in LPL, PPARγ2, FAS, ADD1, FTO and NYGGF4 mRNA expression. In addition, its upregulation also blocked osteogenesis in hADMSCs as indicated by significant upregulation of the mRNA expression of ALP, RUNX2 and OCN. Consistent with this, the downregulation of miR-135a-5p restrained adipogenic differentiation. From our data, it seems that miR-135a-5p plays a central physiological role in mediating the adipogenesis of hADMSCs.

The pathogenic role of the Hippo signaling pathway in adipogenesis may be associated with its inhibitory effects on the canonical Wnt pathway. Blocking the canonical Wnt and Hippo pathways to shift the cell fate towards an adipocyte depends on YAP binding to β-catenin and JUP to sequester β-catenin and YAP [24]. Activation of the Hippo signaling pathway has also been implicated in PKA-induced adipogenesis [25]. Consistent with our results, YAP has been reported to inactivate PTEN, which is known to block the IGF1-mTORC1 pathway and adipogenesis [26, 27]. The activities of YAP and TAZ are restricted by phosphorylation of MST1/2 and activation of LATS1 at physiological conditions. When YAP and TAZ are active, they translocate into the nucleus to bind the TEAD and induce various gene expressions that are involved in cell migration, proliferation, and differentiation [28]. Recent studies found that nuclear expression of YAP and TAZ was significantly enhanced after miR-135a-5p mimic transfection, suggesting that miR-135a-5p may play an active role in the Hippo signaling pathway and adipogenesis.

To further understand the mechanisms of miR-135a-5p in the differentiation of adipocytes, bioinformatics analyses, and dual luciferase reporter assays were conducted to confirm that direct targets of miR-135a-5p were LATS1 and MOB1B. Subsequently, the function of LATS1 and MOB1B in regulating adipogenesis was investigated. Active MST1/2 phosphorylates SAV1 and MOB1A/B, which can be conducive to recruiting and phosphorylating LATS1/2 at their hydrophobic motifs [25, 29]. In the present study, TEAD activity was greatly enhanced by the knockdown of LATS1 and MOB1B, revealing that LATS1 and MOB1B regulate adipogenesis by modulating the Hippo signaling pathway. These findings were further corroborated by dysregulating miR-135a-5p, which led to alterations of LATS1 and MOB1B expression. The impact of miR-135a-5p on the Hippo signaling pathway was also explored in our study, which revealed that expression of downstream effectors and targets changed with the overexpression or knockdown of miR-135a-5p in the hADMSCs. We confirmed that the activity of YAP, TAZ and TEAD increased or decreased in response to transfection with the miR-135a-5p mimic or inhibitor, respectively.

In conclusion, our experiments provide evidence that miR-135a-5p may silence LATS1 and MOB1B at the post-transcriptional level. Furthermore, miR-135a-5p could facilitate adipogenesis through the Hippo signaling pathway in hADMSCs. Our results demonstrate that miR-135a-5p along with LATS1 and MOB1B may be therapeutic targets for the treatment of obesity and related metabolic disorders.

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Disclosure of conflict of interest

None.

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