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Original Article

miR-135b-5p regulates human mesenchymal stem cell osteogenic differentiation by facilitating the Hippo signaling pathway

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Abstract: Background: A multifunctional titanium surface with osteogeneic, angiogeneic and antibacterial properties is needed to improve the osseointegration and long-term survival of dental implants. Particularly, the switch between the differentiation of mesenchymal stem cells (MSCs) into osteogenic and adipogenic lineages is regulated by numerous miRNAs. However, the association between miR-135b-5p and the Hippo signaling pathway during osteogenesis has not been elucidated. In the present study, we demonstrate that miR-135b-5p facilitates the in vitro osteogenesis of human mesenchymal stem cells (hMSCs). Methods: hMSCs and Human Calvarial Osteoblasts (HCO) cells were cultured in vitro, followed by the transfection of the miR-135b-5p mimic or inhibitor using Lipofectamine 2000. The target of miR-135b-5p was determined by bioinformatics analysis and luciferase assay. Cell viability was tested using the MTT assay. The osteogenesis level was evaluated by alizarin red staining. miRNA and mRNA expression levels were detected by real-time PCR. The protein levels were assessed by western blotting. Results: miR-135b-5p was shown to be highly expressed in osteoblasts compared with that in hMSCs. The overexpression of miR-135b-5p promotes hMSC proliferation and osteogenesis, whereas its knockdown causes the inhibition of these processes. Furthermore, aberrant expression of miR-135b-5p promotes both osteogenic and proliferation factors. We next showed that the Hippo signaling pathway was activated by miR-135b-5p transfection. Next, we found that large tumor suppressor 1 (LATS1) and MOB kinase activator 1B (MOB1B), key negative regulators of the HIPPO signaling pathway, are direct targets of miR-135b-5p. In addition, the knockdown of LATS1 or MOB1B led to an increase in TEAD activity. Conclusion: miR-135b-5p regulates osteogenesis by controlling LATS1 and MOB1B expression and subsequently activating the HIPPO signaling pathway.

Keywords: miR-135b-5p, mesenchymal stem cell, osteogenic differentiation, HIPPO signaling pathway, LATS1, MOB1B

Introduction

Despite the good biocompatibility of titanium, dental implants still develop delayed osseointegration and complicated bacterial infection due to their insufficient osteogenic activity and antibacterial effect [1]. Human bone marrow-derived mesenchymal stem cells (hMSCs) are a dynamic cell component of the bone marrow that can facilitate the rapid regeneration of bone tissue to improve the bone-implant surface. Generally, hMSCs can be induced to differentiate into multiple lineages in vitro, including osteoblasts, chondrocytes, and adipocytes. A recent study suggested that a specific subset of adult bone marrow cells identified by certain markers could differentiate into osteoblasts in vivo under serial transplantation [2]. However, the precise control of hMSC osteogenic differentiation in vivo remains poorly understood.

MicroRNAs are small non-coding RNAs that can suppress target gene expression or degrade target mRNAs at the post-transcriptional level via binding to the 3'-untranslated region (UTR) [3]. miRNAs have been shown to play key regulatory roles in numerous physiological processes, such as cell proliferation, apoptosis, and differentiation [4]. Several miRNAs have been reported to participate in adipogenesis through regulating different signaling pathways [5]. Researchers adopted a microRNA array and iden-

tified multiple dysregulated miRNAs during hMSC osteogenesis [6]. Particularly, inhibition of miR-222 has been shown to accelerate bone healing with the enhancement of osteogenesis [7], and miR-27a has been reported to attenuate adipogenesis and promote osteogenesis in steroid-induced rat BMSCs by targeting PPARgamma and GREM1 [8]. Some signaling pathways, such as BMP and Wnt, have been confirmed to be involved in regulating osteogenesis. For example, miR-140-5p is found to suppress BMP2-mediated osteogenesis in undifferentiated human mesenchymal stem cells [9]. miR-17 regulates osteogenesis in bone-related disorders through the Wnt/β-catenin signaling pathway [10]. miR-135b-5p is also observed to be dysregulated in exosomes during the osteogenic differentiation of human bone marrow-derived mesenchymal stem cells and involved in osteogenic differentiation of human unrestricted somatic stem cells [6, 11].

The Hippo signaling pathway, an evolutionarily conserved pathway that controls tissue growth by the regulation of cell proliferation, differentiation, and death, has been connected to integrin-dependent adhesion [12-15]. Stimulated by extracellular mechanical signals such as ECM rigidity or cell-cell contacts, the Hippo pathway mediates its signaling by regulating the expression and activity of the two major downstream effectors Yes-associated protein (YAP) and transcriptional co-activator with the PDZ-binding motif (TAZ). Both act as transcriptional co-factors controlling the expression of Hippo pathway target genes such as connective tissue growth factor (CTGF) [16] and survivin [17]. Recently, it was demonstrated that TAZ mediates Wnt3a-stimulated osteogenic differentiation through PP1A, suggesting that the Wnt signal regulates the Hippo pathway in osteogenesis [18]. In addition, the Snail/Slug-YAP/TAZ axis activates a series of YAP/TAZ/TEAD and Runx2 downstream targets that control skeletal stem cell homeostasis and osteogenesis [19].

Although miR-135b-5p has been shown to be involved in osteogenesis, the effects of the specific mechanism of miR-135b-5p on regulating osteogenesis and its impact on Hippo signaling remain unclear. In the present study, we show that miR-135b-5p promotes hMSC differentiation into mature osteoblasts by positively regulating the Hippo signaling pathway.

Materials and methods

Cell culture

hMSCs and human osteoblast HCO cells were purchased from ScienCell (Shanghai, China). Cells were maintained as previously described [20]. Cells in passages 3-6 were used in following experiments.

For osteogenic induction, nearly confluent cells were first cultured in serum-free medium for 24 h to synchronize the cell cycle for better differentiation followed by culture in osteogenic medium (Lifetech, Shanghai, China) for an additional 7 days. Cells were then fixed with 10% formaldehyde and stained with 1% alizarin red (Sigma, Beijing, China).

Transfection of hMSCs

The miR-135b-5p mimic and inhibitor were prepared by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The miR-135b-5p target site was predicted using online software and was found to be highly conserved among vertebrates. siRNA-YAP, siRNA-TAZ, siRNA-LATS1, siRNA-MOB1B, and siRNA-scramble were synthesized by Invitrogen (Carlsbad, CA, USA). The hMSCs were transfected with the miR-135b-5p mimic, miR-135b-5p inhibitor, siRNA-YAP, siRNA-TAZ, siRNA-LATS1, siRNA-MOB1B, and siRNA-scramble using Lipofectamine 2000 (Invitrogen) according to the manual. After 2 days of transfection, cell differentiation was induced as previously mentioned. hMSCs were maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin G, and 250 μg/ml streptomycin at 37°C and 5% CO_2.

Bioinformatics analysis

The miRNA targets were predicted using PicTar (http://pictar.org/), TargetScan (http://www.targetscan.org/vert_42/), and miRanda (http://www.microrna.org/microrna/).

MTT assay

Cell viability was assessed by the MTT assay. Cells were cultured in 96-well plates at 2×10^3 cells/well, and the media were changed to fresh media every other day. The cells were then transfected with the miR-135b-5p mimic or miR-135b-5p inhibitor as described above for 48 h. During the last 4 h of each day of cul-

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG
OSterix	CTCAGCTCTCTCCATCTGCC	GGGACTGGAGCCATAGTGAA
DSPP	GCCACTTTCAGTCTTCAAAGAGA	GCCCAAATGCAAAAATATGTAA
NANOG	GATTTGTGGGCCTGAAGAAA	ATGGAGGAGGGAAGAGGAGA
OCT4	GTGGAGGAAGCTGACAACAA	GGTTCTCGATACTGGTTCGC
MYC	TCAAGAGGCGAACACACAAC	GGCCTTTTCATTGTTTTCCA
SOX2	AACCCCAAGATGCACAACT	GCTTAGCCTCGTCGATGAAC

ture, the cells were treated with MTT at 50 mg/well (Sigma, St. Louis, MO). Dimethyl sulfoxide was applied to dissolve the formazan, and the absorbance was measured at 450 nm using an ELISA plate reader (Bio-tek, Winooski, VT).

Luciferase reporter constructs and assay

The 3'-UTR sequences of human LATS1 and MOB1B containing the seed target sequence of miR-135b-5p were amplified by PCR and were cloned into the pmiR-RB-REPORT™ (Guangzhou RiboBio Co., Ltd.) dual luciferase plasmid. The XhoI and NotI (Invitrogen) restriction sites were underlined above. The mutation of these sequences from AAGCCAU to AAGCGUA and from AAAAGCCAUA to AAAAGCGUAA was performed using the Quick Change Site-Directed Mutagenesis kit (Agilent Technologies, Edinburgh, UK).

The pmiR-RB-REPORT vector (50 ng) containing either wildtype or mutated human LATS1 or MOB1B 3'UTR was co-transfected into 293T cells (obtained from the Xiangya Cells Center of Central South University, Changsha, China) together with the miR-135b-5p mimic (100 nM) or miR-135b-5p inhibitor using Lipofectamine 2000 (Invitrogen). A non-target control vector was used as a transfection control. Three replicates were prepared for each transfection experiment. Firefly and Renilla luciferase activities were measured using the Dual-Glo Luciferase assay system (Promega, *Madison, WI, USA).*

Alizarin red staining (ARS)

Calcium deposits were detected by alizarin red S staining after 28 days of osteogenic induction. Cells were incubated with 2% alizarin red (pH 4.2) (Sigma-Aldrich) for 10 min and were washed with distilled water. Cells were detected by phase-contrast microscopy at 28 days to verify the presence of mineralized nodules.

Real-time PCR

TRIzol reagent (Invitrogen) was used to extract total RNA according to the manufacturer's instructions. RNA was eluted in 50 mL of RNase-free water (Promega) and was stored at -80°C. To analyze gene expression, the qRT-PCR mixture system containing cDNA templates, primers, and SYBR Green qPCR Master Mix were subjected to qRT-PCR according to standard methods. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH used as an internal control. The primer sequences were listed in **Table 1**.

Western blotting

Cells were lysed with loading lysis buffer that was diluted from 5 × loading lysis buffer (2.5 mL of 0.5 mol/L Tris-HCl, 0.39 g of dithiothreitol, 0.5 g of sodium dodecyl sulfate (SDS), 0.025 g of bromophenol blue, and 2.5 mL of glycerin). An equal amount of protein was transferred onto the polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated with primary antibodies against YAP, TAZ, LATS1, MOB1B, and GAPDH (Cell Signaling Technology, Danvers, MA) at 4°C for 1 h. Next, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) for 1 h at room temperature. The binding signals were visualized by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, UK).

Statistical analysis

All data were presented as the mean ± standard deviation and were analyzed using SPSS 16.0 software. All experiments were repeated at least three times. The differences between groups were determined using two-tail unpaired Student's t-test or ANOVA. A P-value < 0.05 was considered to indicate a significant difference.

Results

miR-135b-5p promotes hMSC osteogenic differentiation

To identify the expression of miR-135b-5p in osteoblasts, we selected hMSCs and osteoblast HCO cells to test the miR-135b-5p level. It was shown that miR-135b-5p expression in

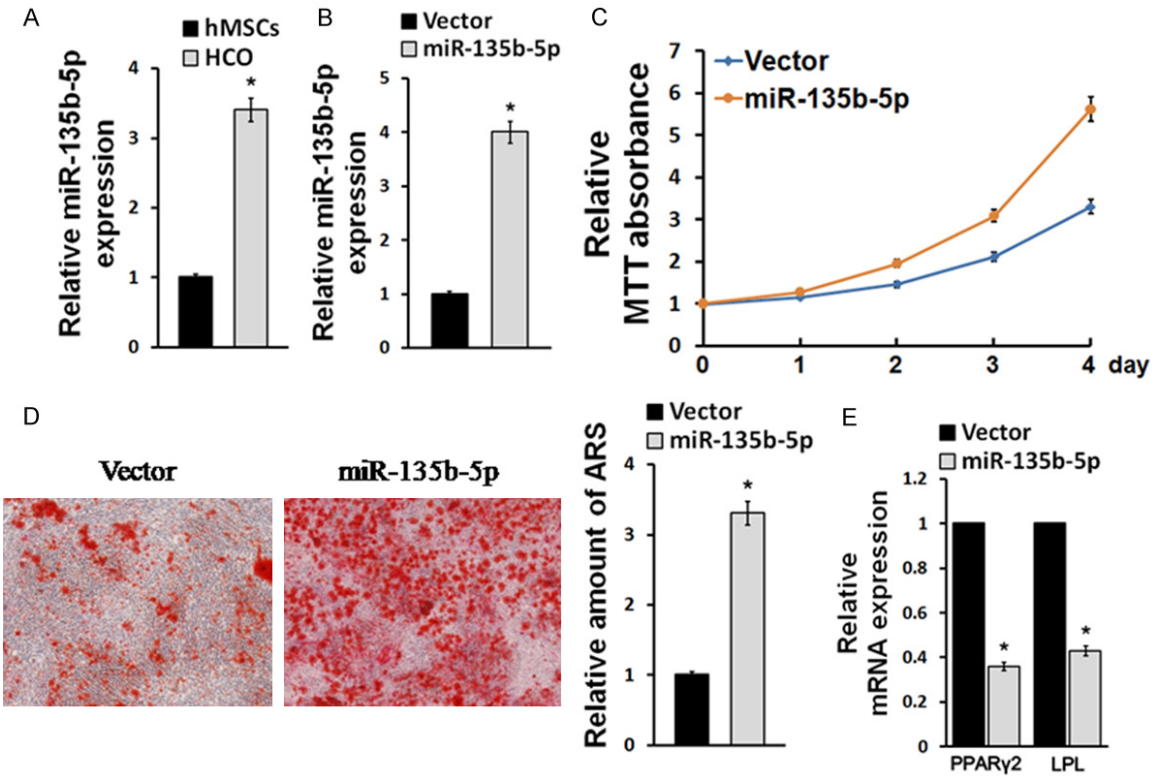

Figure 1. miR-135b-5p facilitates hMSCs osteogenic differentiation. A. miR-135b-5p expression in hMSC and HCO cells. B. miR-135b-5p expression in hMSCs after miR-135b-5p mimic transfection. C. hMSC cell viability as detected by the MTT assay after miR-135b-5p mimic transfection. D. Alizarin red staining in hMSCs after miR-135b-5p mimic transfection. E. Adipogenic factor mRNA expression in hMSCs after miR-135b-5p mimic transfection. *P < 0.05, compared with the control.

HCO cells was significantly higher than that in hMSCs, suggesting its overexpression in osteoblasts (P < 0.05, **Figure 1A**). Next, we transfected the miR-135b-5p mimic into hMSCs and found it obviously facilitated cell proliferation (P < 0.05, **Figure 1B**, **1C**). Moreover, miR-135b-5p significantly enhanced ARS compared with the control, indicating its promotion role in hMSC osteogenic differentiation (P < 0.05, **Figure 1E**). Conversely, miR-135b-5p mimic transfection markedly suppressed the expression of adipogenesis factors, such as PPARγ2 and LPL (P < 0.05, **Figure 1D**).

Knockdown of miR-135b-5p blocks osteogenic differentiation in hMSCs

Since miR-135b-5p exhibited a promotion function in hMSC osteogenesis, we further transfected the miR-135b-5p inhibitor to observe the inhibition effect. Transfection of the miR-135b-5p inhibitor effectively downregulated the miR-135b-5p level in hMSCs and significantly restrained hMSC proliferation (P < 0.05, **Figure 2A**, **2B**). In addition, ARS was obviously reduced in hMSCs after miR-135b-5p inhibitor transfection (P < 0.05, **Figure 2D**). More importantly, the miR-135b-5p inhibitor apparently elevated adipogenesis-related factor expression in hMSCs (P < 0.05, **Figure 2C**). Taken together, miR-135b-5p may facilitate hMSC osteogenesis and restrain adipogenesis.

miR-135b-5p activated the Hippo signaling pathway

The Hippo signaling pathway played an important role in cell proliferation and differentiation and was found to be related to osteogenesis. Thus, we intended to investigate whether miR-135b-5p may affect Hippo signaling pathway activity. The luciferase assay reported that TEAD activity was enhanced by miR-135b-5p mimic intervention and was declined after miR-135b-5p inhibitor transfection (P < 0.05, **Figure 3C**). We further tested osteogenesis-related and proliferative factor levels in hMSCs after miR-135b-5p dysregulation. It was shown that various osteogenesis-related factors, such as ALP, Osterix, RUNX2, OPN, DSPP, and PHEX,

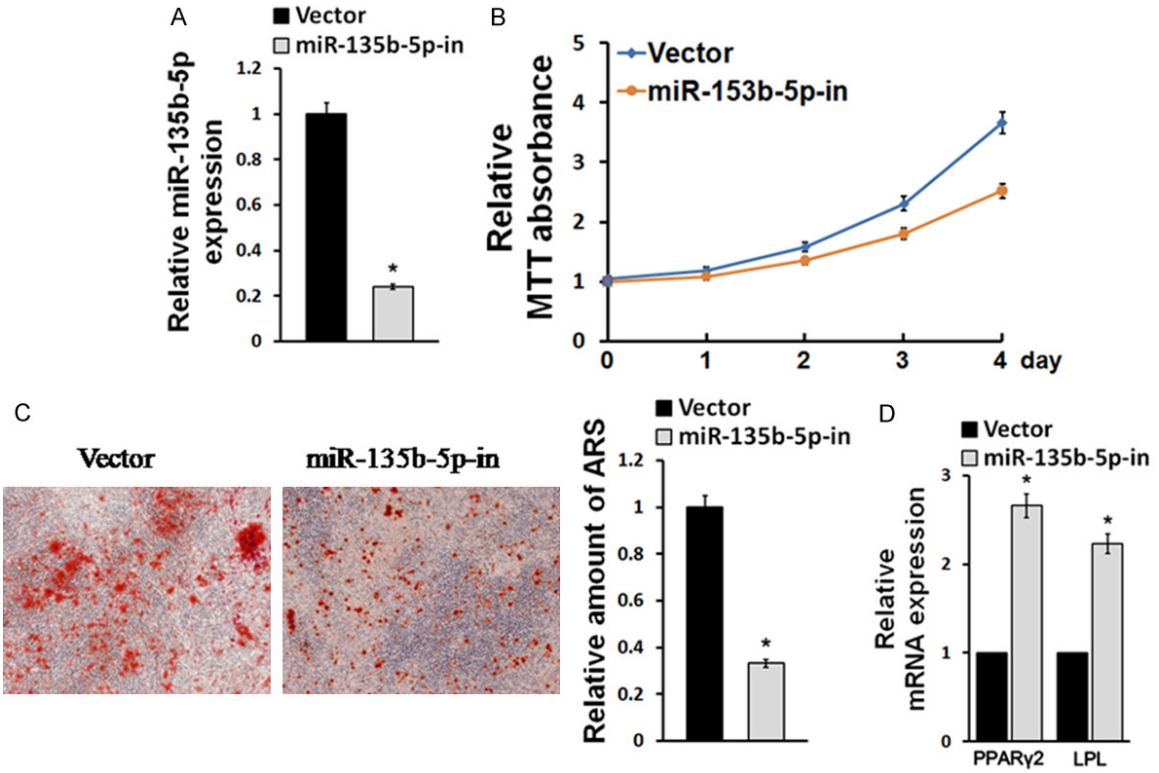

Figure 2. The miR-135b-5p inhibitor suppresses hMSC osteogenic differentiation. A. miR-135b-5p expression in hMSCs after miR-135b-5p inhibitor transfection. B. hMSC cell viability as detected by the MTT assay after miR-135b-5p inhibitor transfection. C. Alizarin Red staining in hMSCs after miR-135b-5p inhibitor transfection. D. Adipogenic factor mRNA expression in hMSCs after miR-135b-5p inhibitor transfection. *P < 0.05, compared with the control.

were obviously upregulated in hMSCs after miR-135b-5p transfection, whereas they were reduced by the miR-135b-5p inhibitor (P < 0.05, **Figure 3A**). Proliferative factors, including Oct-4, Nanog, CD44, SOX2, AXIN2, and MYC, were found to be enhanced in hMSCs from the miR-135b-5p mimic transfection group but declined after miR-135b-5p inhibitor transfection (P < 0.05, **Figure 3B**). Moreover, we extracted nuclear protein and evaluated the YAP and TAZ levels. Western blotting revealed that the miR-135b-5p mimic elevated YAP and TAZ protein expression in hMSCs, while the miR-135b-5p inhibitor produced the opposite effect (**Figure 3D**). Knockdown of YAP or TAZ apparently reduced TEAD activity in hMSCs, confirming their central roles in the Hippo signaling pathway (P < 0.05, **Figure 3D**).

miR-135b-5p targets LATS1 and MOB1B to trigger the Hippo signaling pathway

Bioinformatics analysis revealed that two negative regulatory factors of Hippo signaling pathway, LATS1 and MOB1B, exhibited complementary binding sites for miR-135b-5p on the sequences of their 3'-UTR (**Figure 4A**). It was revealed that the luciferase activity of LATS1 and MOB1B 3'UTR was declined in miR-135b-5p-transfected hMSCs and was enhanced after miR-135b-5p inhibitor transfection (**Figure 4B**). Furthermore, Western blot demonstrated that LATS1 and MOB1B expression was upregulated in the miR-135b-5p inhibitor group (**Figure 4C**). Meanwhile, the suppression of LATS1 or MOB1B expression markedly increased TEAD activity, demonstrating their negative regulatory roles in the Hippo signaling pathway (P < 0.05, **Figure 4D**).

Discussion

The lineage commitments must be tightly regulated during stem cell maintenance to achieve the fine balance between proliferation and differentiation. Stem-cell therapy has proven to be effective clinically and could potentially lead to robust treatment outcomes for dental tissue

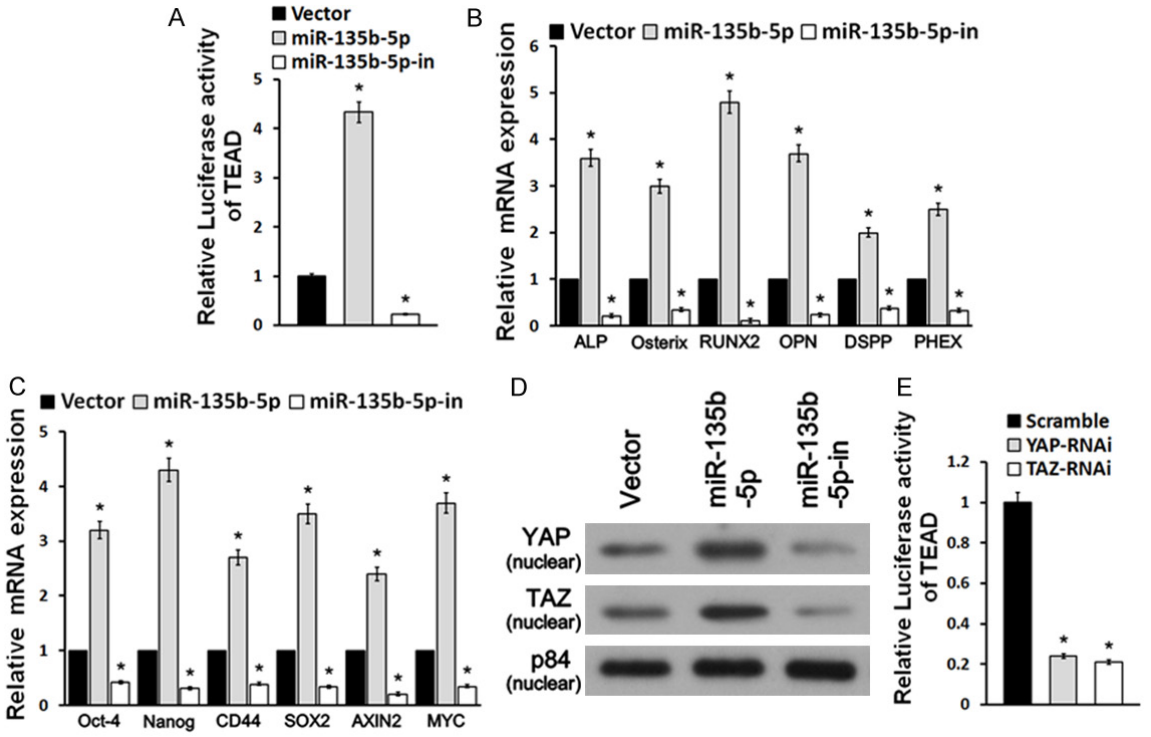

Figure 3. miR-135b-5p activates the Hippo signaling pathway. A. Luciferase assay detection of TEAD activity in miR-135b-5p mimic- or inhibitor-transfected hMSCs. B. Osteogenesis-related factor mRNA expression as detected by real-time PCR in miR-135b-5p mimic- or inhibitor-transfected hMSCs. C. Cell proliferation-related factor mRNA expression as tested by real-time PCR in miR-135b-5p mimic- or inhibitor-transfected hMSCs. D. Western blot detection of YAP and TAZ expression in the nucleus from miR-135b-5p mimics- or inhibitor-transfected hMSCs. E. Luciferase assay detection of TEAD activity in YAP siRNA- or TAZ siRNA-transfected hMSCs. *$P < 0.05$, compared with the control.

regeneration. Several studies have demonstrated that miRNAs are critical regulators of osteoblast differentiation [21]. In this study, we identified miR-135b-5p as an enriched miRNA in hMSCs that positively regulates osteogenic lineage commitment. Recent miRNA profiling studies have demonstrated that miR-135b-5p is one of the miRNAs dysregulated in various differentiated cell types compared with that in undifferentiated hMSCs [6, 22], but its functional roles were not investigated in these earlier reports.

In the present study, we first examined miR-135b-5p expression in osteoblasts and found that its expression is obviously upregulated in osteoblasts compared with that in MSCs. This result is opposite to the impact of miR-135b-5p on the osteogenic differentiation of mesenchymal stem cells derived from multiple myeloma patients [22]. We then demonstrated that the overexpression of miR-135b-5p enhanced hMSC osteogenesis, as illustrated by the significant increase in ARS and in the mRNA expression of ALP, Osterix, RUNX2, OPN, DSPP, and PHEX. Meanwhile, its upregulation also restrained the adipogenesis process of hMSCs, as confirmed by the obvious enhancement in the mRNA expression of PPARγ2 and LPL. Conversely, the downregulation of miR-135b-5p suppressed osteogenic differentiation. Our data strongly suggest that miR-135b-5p plays a critical physiological role in regulating the osteogenesis of hMSCs.

The pathogenic role of the activation of the Hippo pathway in osteogenesis may be regulated by canonical Wnt signaling. YAP binds to β-catenin and JUP, which might lead to the membrane localization of pYAP. These molecular interactions could sequester β-catenin and YAP, thus restraining the canonical Wnt and Hippo pathways and shifting the cell fate toward an osteogenic lineage [23]. The physiological output of the kinase cascade, including MST1/2 phosphorylation and LATS1 activation,

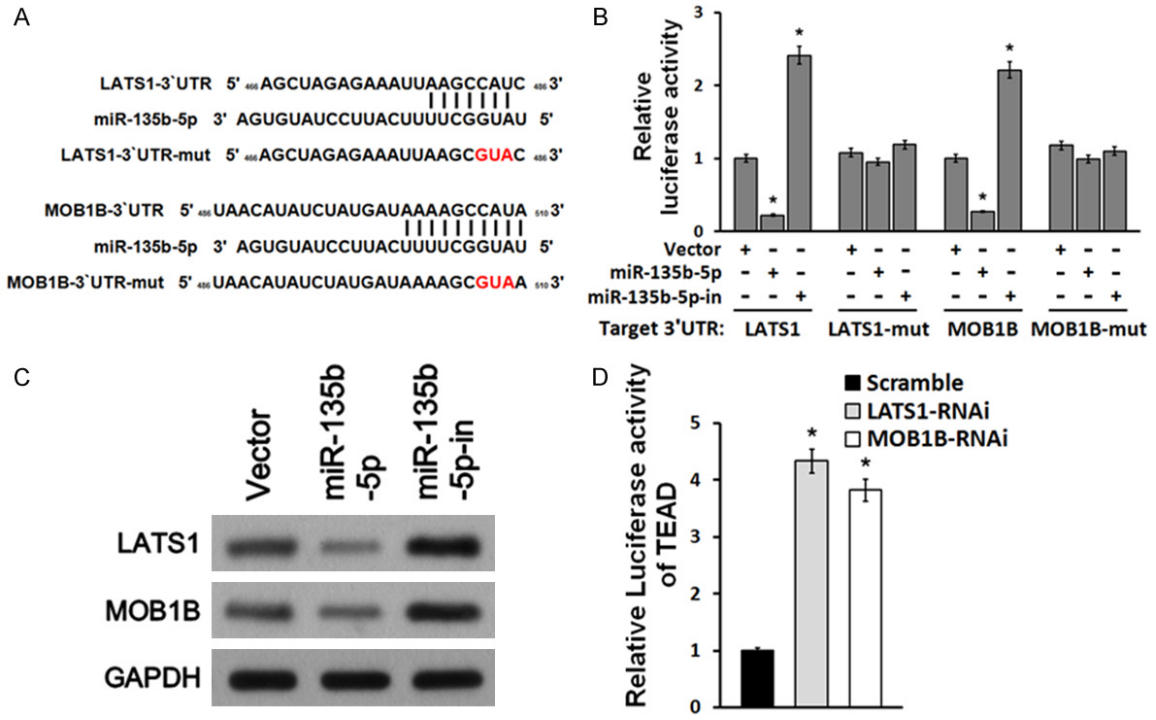

Figure 4. miR-135b-5p targets LATS1 and MOB1B to activate the Hippo signaling pathway. A. Predicted miR-135b-5p target sequence in the 3'UTR of LATS1 and MOB1B. B. Dual-luciferase reporter assay of the hMSCs transfected with the LATS1 or MOB1B 3'UTR reporter and the miR-135b-5p mimic or inhibitor. C.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

is to restrict the activities of two transcriptional coactivators, YAP and TAZ. When YAP and TAZ are active, they translocate into the nucleus to bind TEAD and induce the expression of a wide range of genes that are involved in cell proliferation, differentiation, and migration [24]. In the present study, we demonstrated that YAP and TAZ nuclear expression was obviously elevated upon miR-135b-5p mimic transfection, suggesting that miR-135b-5p may be a positive regulator of the Hippo signaling pathway and osteogenesis.

To further explore the mechanisms underlying the effects of miR-135a-5p on osteogenic differentiation, we adopted bioinformatics analysis and the dual luciferase reporter assay and verified that LATS1 and MOB1B are direct targets of this miRNA. We then examined the direct role of LATS1 and MOB1B in regulating osteogenesis. Active MST1/2 phosphorylates SAV1 and MOB1A/B, two scaffold proteins that assist MST1/2 in the recruitment and phosphorylation of LATS1/2 at their hydrophobic motifs [25, 26]. In this study, we demonstrated that the knockdown of LATS1 and MOB1B led to an enhancement of TEAD activity, indicating that LATS1 and MOB1B regulate the extent of osteogenesis by modulating the Hippo signaling pathway. These findings were further corroborated by inducing the overexpression or knockdown of miR-135b-5p, which led to alterations in the protein level of LATS1 and MOB1B. To demonstrate the effect of miR-135b-5p on the activity of the Hippo signaling pathway, we analyzed the expression of downstream effectors and targets of this pathway in response to the overexpression or knockdown of miR-135b-5p in the hMSCs. We demonstrated that YAP, TAZ, and TEAD activities were upregulated and downregulated in response to transfection with the miR-135b-5p mimic and inhibitor, respectively.

In conclusion, the present study provided potential evidence that miR-135b-5p induces the post-transcriptional silencing of LATS1 and MOB1B, important negative regulators of the

Hippo signaling pathway. We also demonstrate that miR-135b-5p facilitates osteogenesis by regulating the activity of the canonical Hippo signaling pathway in hMSCs. These findings suggest that miR-135b-5p, together with LATS1 and MOB1B, may serve as potential therapeutic targets for the management of dental implantation.

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Disclosure of conflict of interest

None.

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