**microRNA-324-3p Promotes Osteoblasts Differentiation via Suppressing SMAD7**

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Abstract: Fracture healing is a complex dynamic process that involves the balance between osteoblasts and osteoclasts. Several microRNAs (miRNAs) have been shown to participate in fracture healing. In this study, we investigated the role of miR-324-3p in osteoblast differentiation. MC3T3-E1 cell differentiation was induced by icariin, and miR-324-3p expression levels during cell differentiation were measured using qRT-PCR. Cell proliferation and differentiation were assessed to evaluate the function of miR-324-3p. Luciferase activity was used for target gene verification. During MC3T3-E1 differentiation, miR-324-3p levels gradually increased over time. Further experiments showed that miR-324-3p overexpression significantly promoted cell viability, whereas miR-324-3p downregulation showed the opposite effect. For cells with miR-324-3p mimic, the levels of bone sialoprotein, Runx2, osteocalcin, and alkaline phosphatase activity were significantly elevated. SMAD7 is the target gene of miR-324-3p, and its level is gradually downregulated during MC3T3-E1 cell differentiation. MiR-324-3p may promote MC3T3-E1 cell differentiation by targeting SMAD7.

Key words: Cell differentiation, MC3T3-E1, microRNA-324-3p, SMAD7

**Introduction**

The fragility fracture is a fracture injury caused by a fall at or below the center of gravity and is more common in older people. Fragility fractures are always caused by osteoporosis and are often referred to as osteoporotic fractures. Fragility fracture is a major public health problem worldwide and has a profound impact on social health and the economy. Fracture healing requires the synergistic action of multiple factors to regulate bone cell proliferation and differentiation of bone cells. Osteoblasts are mesenchymal cells involved in bone formation during fracture healing by participating in the formation of new bone. The recovery process of fracture healing, including cell migration, differentiation, tissue healing, and release of cytokines and growth factors, depends on the activity of osteoblasts. MicroRNAs (miRNAs) are non-coding single-stranded small RNAs that are specifically expressed during cell proliferation, differentiation, and tissue growth and development. It plays a significant regulatory role in life processes. At present, many miRNAs have been found to be aberrantly expressed in osteoporotic patients, such as miR-16-5p, miR-34a, miR-151a-3p and miR-155. Moreover, miRNAs can be involved in the biological behavior of different types of osteoblasts and further regulate fracture healing. A recent study showed that miR-324-3p is expressed at low levels in osteoporosis patients and is correlated with lumbar bone density. MiR-324-3p is closely associated with bone microstructure and histomorphometry. Another study also reported downregulation of miR-324-3p in cases of type 2 diabetes, which is associated with defects in osteoblast formation. However, the role of miR-324-3p in fracture healing has not been reported.

In this study, MC3T3-E1 cells were selected for cell experiments and treated with icariin (ICA) to induce osteoblast differentiation. The expression of miR-324-3p was measured during osteoblast differentiation. To further determine the effect of miR-324-3p on osteoblast differentiation, gain- and loss-of-function experiments were conducted. The underlying mechanism was preliminarily investigated.

**Materials and Methods**

**Cell lines and reagents**

Mouse osteoblast MC3T3-E1 cells were purchased from the Yingwan Biotechnology Company (Shanghai, China). α-MEM, fetal bovine serum (FBS; 10%), penicillin, and streptomycin were obtained from Beyotime (Beyotime, Shanghai, China). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kyushu, Japan). TRIZOL, miRNA cDNA synthesis Kit, miRNA qPCR assay Kit, HiFiScript cDNA synthesis Kit, and Lipofectamine 2000 were prepared by CWBiotech (Beijing, China).

**Cell culture**

The MC3T3-E1 cells were cultured in α-MEM under sterile conditions. Cell culture was performed at 37 °C and 5% CO₂, with 95% humidity.

**Osteoblast differentiation and transfection**

When MC3T3-E1 cells were in the logarithmic growth phase with a density of 70-80% confluence, they were re-suspended and seeded into 6-well plates. To induce cell differentiation, the cells were treated with 5 μmol/l icariin (ICA) and incubated for 12 days. The phenol red-free α-MEM culture medium was changed daily during cell incubation. Lipolectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect 50 nM miR-324-3p mimics (5'-ACUGGCCCCGGUGCGUGCG-3'), miR-324-3p inhibitor (5'-CCACGACAGCUGGGCCGCU-3'), or their negative controls (mimic NC (5'-UUCUCGAACUGUCAGCUU-3') or...
were collected, washed twice with phosphate buffer saline (PBS), and cells/well. After incubation for 24, 48, and 72 h, the cells in each group CCK-8 assay of Wisconsin, USA), with GAPDH as the internal standard. three independent experiments using ImageJ software (LOCI, Universitition; Beyotime, China) for 2 h. The band intensity was quantified in horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilu- ti-Ocn antibody (1:1,000 dilution, ab93876, Abcam, Cambridge, MA, PA5-79424, Thermo Fisher Scientific, Carlsbad, California), anti-Runx2 antibodies overnight at 4°C as follows: anti-Bsp antibody (1:1,000 dilution, ab76956, Abcam, Cambridge, MA), and an SMAD7 forward 5ʹ-CATCACCTTAGCCGACTCTG-3ʹ, reverse 5ʹ-GTCT GAGAAGCATAA-3ʹ, reverse 5ʹ-AGGGCAATAAGGTAGTGAA-3ʹ; and osteocalcin (Ocn) forward 5ʹ-GAGGCTCT GGTCCGAGG-3ʹ, reverse 5ʹ-GCGGCGGCCCACTGCCCCAGG-3ʹ; bone sialoprotein (Bsp), forward 5ʹ-TGAGTTTTTTTAGGAGAAAT- GAAAG-3ʹ, reverse 5ʹ-CTAAACTCTAAATCATCCCCTCTC-3ʹ; Runx2 forward 5ʹ-CCTCTGAACCTCTGCACCAAGT-3ʹ, reverse 5ʹ-TGGAGTGG- ATGGGATGGGGAT-3ʹ; osteocalcin (Ocn) forward 5ʹ-GAGGCTCTG TGGAGTGAA-3ʹ, reverse 5ʹ-CTAAACTCTAAATCATCCCCTCTC-3ʹ; and SMAD7 forward 5ʹ-CATCACCTTAGCCGACTCTG-3ʹ, reverse 5ʹ-GTCT- TCTCCCTCCAGTATGC-3ʹ. The PCR conditions were as follows: 95°C for 5 min, 95°C denaturation for 30 s, 60°C annealing for 30 s, followed by 20 cycles of 72°C for 30 s, and 72°C for a further 5 min. 

Western blotting 
Total protein was isolated using RIPA lysis buffer and evaluated using the BCA protein assay kit (Thermo-Fisher, Waltham, MA, USA). Protein samples were loaded onto SDS-PAGE followed by transferring onto PVDF membranes. PVDF membranes were incubated with antibodies overnight at 4°C as follows: anti-Bsp antibody (1:1,000 dilution, PA5-79424, Thermo Fisher Scientific, Carlsbad, California), anti-Runx2 antibody (1:1,000 dilution, ab76956, Abcam, Cambridge, MA), and anti-Ocn antibody (1:1,000 dilution, ab93876, Abcam, Cambridge, MA, USA), washed with TBST, and further incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilu- tion; Beyotime, China) for 2 h. The band intensity was quantified in three independent experiments using ImageJ software (LOCI, University of Wisconsin, USA), with GAPDH as the internal standard.

CCK-8 assay 
MC3T3-E1 cells were seeded in 96-well plates at a density of 4×10⁴ cells/well. After incubation for 24, 48, and 72 h, the cells in each group were collected, washed twice with phosphate buffer saline (PBS), and resuspended in culture medium. Then, 10 µl CCK-8 reagent and 100 µl medium were added. After incubation for one hour, the change in the OD at 450 nm was measured.

Alkaline phosphatase (ALP) activity detection 
After transfection, the cells were collected from each group and lysed using RIPA buffer. The cell supernatants were collected to measure ALP activity. Cells were seeded into 6-well plates at a density of 5×10³ cells/well. The alkaline phosphatase assay kit was purchased from Beyotime (Shanghai, China), and ALP values were detected. Absorbance was measured at a wavelength of 405 nm. ALP activity (U/ml) = pNP/ volume of samples for each well/reaction time. The results are repre- sented as relative activity relative to the control.

Luciferase reporter assay 
By using TargetScan7.1 online tools (http://www.targetscan.org/vert/71), The target gene of miR-324-3p was predicted. The wild-type (Wt, the binding sites) or mutant (Mut, the mutant sites) binding sites were cloned into psiCHECK-2 (Promega Corporation, Madison, USA) to construct the Wt vector or Mut vector. The Wt vector or Mut vector and miR-324-3p mimic or inhibitor were then co-transfected into MC3T3-E1 cells. Cell transfection was performed using Lipofectamine 2000 (Invitrogen). After incubation for 48 h, relative luciferase activity was detected using a VersaMax tunable microplate reader (Molecular Devices, LLC).

Statistical analysis 
All data are expressed as means and standard deviations, and all experiments were performed at least three times. GraphPad software was used for the data analysis. Differences between groups were analyzed using one-way ANOVA (SPSS Inc., USA) and Tukey’s post hoc test. Statistical significance was set at P value < 0.05.

Results 
miR-324-3p was upregulated during cell differentiation of MC3T3-E1 
Differentiation of MC3T3-E1 cells was induced by ICA, and cell differentiation was performed for 12 days. To detect the differentiation of MC3T3-E1 cells, the levels of osteoblast differentiation markers were measured. As shown in Fig. 1A, the mRNA levels of Bsp, Runx2, and Ocn, as well as the activity of ALP, increased over time, indicating that osteoblast differentiation was successfully induced. The levels of miR-324-3p during MC3T3-E1 cell differentiation were also measured. The data suggested that the miR-324-3p level increased gradually over time (Fig. 1B).
The effect of miR-324-3p overexpression on MC3T3-E1 cell proliferation

To explore the effect of miR-324-3p on MC3T3-E1 cell proliferation, gain- and loss-of-function experiments were performed. Cell transfection was performed to regulate miR-324-3p levels in MC3T3-E1 cells. As illustrated in Fig. 2A, miR-324-3p expression was significantly elevated after miR-324-3p mimic transfection ($P < 0.001$). In contrast, miR-324-3p inhibitor transfection significantly inhibited miR-324-3p expression in MC3T3-E1 cells ($P < 0.001$; Fig. 2A). Cell proliferation was detected at different levels of miR-324-3p expression. The results of the CCK-8 assay demonstrated that miR-324-3p overexpression promoted cell proliferation, whereas miR-324-3p downregulation showed the opposite effect (Fig. 2B).

The effect of miR-324-3p overexpression on MC3T3-E1 cell differentiation

The levels of osteoblast differentiation markers were measured to investigate the role of miR-324-3p in MC3T3-E1 cell differentiation. In cells transfected with the miR-324-3p mimic, the mRNA and protein levels of Bsp, Runx2, and Ocn, as well as the enzymatic activity of ALP, were significantly elevated ($P < 0.05$, Fig. 3A-G). Conversely, the mRNA and protein levels of Bsp, Runx2, Ocn, and ALP activity were remarkably reduced by miR-324-3p downregulation ($P < 0.05$, Fig. 3A-G).

Figure 2. The promoting influence of miR-324-3p overexpression on MC3T3-E1 cell proliferation. A. The levels of miR-324-3p in different MC3T3-E1 cell groups. B. Cell proliferation of MC3T3-E1 cell after different treatment. *** $P < 0.001$, in comparison with the control group.

Figure 3. The promoting influence of miR-324-3p overexpression on MC3T3-E1 cell differentiation. A-G. The mRNA and protein levels of Bsp (A-B), Runx2 (C-D), Ocn (E-F), and ALP activity (G) in different MC3T3-E1 cell groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, in comparison with the control group.
SMAD7 is a target gene of miR-324-3p

The targeting sequences of miR-324-3p and SMAD7 were predicted using TargetScan, as shown in Fig. 4A. The relationship between miR-324-3p and SMAD7 was confirmed using a luciferase reporter assay. The results showed that the luciferase activity of MC3T3-E1 cells transfected with miR-324-3p mimic was significantly decreased compared to that of untreated cells (P < 0.001, Fig. 4B). However, miR-324-3p downregulation observably enhanced the luciferase activity of MC3T3-E1 cells (P < 0.001, Fig. 4B). Consistently, the overexpression of miR-324-3p dramatically suppressed the expression of SMAD7 at the mRNA and protein levels, which was enhanced by miR-324-3p knockdown (P < 0.001, Fig. 4C, D). SMAD7 expression was gradually downregulated during MC3T3-E1 cell differentiation (Fig. 4E, F).

Discussion

Fracture healing is a complex dynamic process regulated by many types of cells and cytokines. Osteoblasts and osteoclasts play important roles in bone remodeling. Osteoblasts are mesenchymal cells that play a major role in bone formation during fracture healing by participating in the formation of new bones. The recovery process of tissue healing and growth factor release after fracture mainly depend on osteoblast activity. miRNAs can regulate protein expression by binding to non-coding regions of targeted genes. miRNAs play an important role in many physiological processes in cells, including cell proliferation, differentiation, apoptosis, and autophagy. A series of miRNAs have been identified to participate in the fracture healing, such as miR-126 and miR-214. In this study, the role of miR-324-3p in osteoblast differentiation was investigated.

In the present study, MC3T3-E1 cells were recruited as the research objective and ICA was used to induce osteoblast differentiation. Changes in miR-324-3p expression during osteoblast differentiation were detected. With the induction of cell differentiation, miR-324-3p levels increased significantly over time. Thus, miR-324-3p may play a key role in osteoblast differentiation. MiR-324-3p has been reported to be involved in the bone metabolism and osteoblast formation. MiR-324-3p has been demonstrated to be expressed at low levels in osteoporosis patients, and a high correlation has been detected between the level of miR-324-3p and bone density. In type 2 diabetes, low miR-324-3p levels have been shown to inhibit the progress of osteoblast formation and are associated with defects in osteoblast formation. These findings confirm our hypothesis regarding the potential effect of miR-324-3p on osteoblast differentiation.

Gain- and loss-of-function experiments were performed to investigate the effect of miR-324-3p on osteoblast differentiation and proliferation. After regulation of miR-324-3p levels in MC3T3-E1 cells, osteoblast differentiation was measured by detecting the levels of osteoclast differentiation-related markers, including Bsp, Runx2, Ocn, and ALP. Bsp is a member of the N-terminal binding glycoprotein family (SIBLING) of small integrin-binding ligands, is involved in many aspects of bone remodeling, and is closely related to osteoclast differentiation. Runx2 is a key regulator of osteoblast and osteoclast differentiation, and promotes bone formation. Ocn is a specific gene involved in bone for-
mation that promotes the formation of osteocalcin, which is a specific protein in bone tissue. ALP promotes osteoblast maturation and calcification, and its quantitative measurement represents the degree of osteoblasts. The gain- and loss-of-function experiments in the present study revealed that miR-324-3p overexpression promoted the production of Bsp, Runx2, Ocn, and ALP, indicating that miR-324-3p could enhance osteoblast differentiation. Additionally, miR-324-3p overexpression promotes the proliferation of MC3T3-E1 cells, indicating its involvement in osteoblast growth.

Direct binding of target genes is the main mechanism by which miRNAs exert regulatory effects, and its molecular mechanism is the binding of miRNA to the 3’-untranslated region (UTR) region of target genes. Therefore, identification of target genes is crucial for studying the mechanisms of miRNAs. In this study, SMAD family member 7 (SMAD7) was predicted to be a potential gene of miR-324-3p via TargetScan. The relationship between them was subsequently confirmed via a luciferase activity assay. SMAD7 is a member of the Drosophila mothers against decapentaplegic protein family (SMAD) and negatively regulates the activation of the TGF-β/BMP signaling pathway, which serves as an important factor for osteogenic differentiation and bone formation.

Downregulation of SMAD7 is involved in the function of Zingerone in osteoblast differentiation. According to the present findings, SMAD7 was gradually downregulated during MC3T3-E1 cell differentiation, which is in accordance with previous reports. And it had been proved that miR-324-3p overexpression observably inhibited the luciferase activity of SMAD7, and the opposite effect was observed in the presence of miR-324-3p knockdown in MC3T3-E1 cells. Thus, it was inferred that miR-324-3p might enhance MC3T3-E1 cell differentiation and proliferation by targeting SMAD7; however, this requires further validation. Moreover, the lack of a signaling pathway assessment is a limitation of the present study. Previously, TGF-β and BMP signaling have been reported as canonical signals and mineralization of mouse osteoblasts. Downregulation of SMAD7 is also involved in the promotion of Zingerone in osteoblast differentiation. According to the present findings, SMAD7 was gradually downregulated during MC3T3-E1 cell differentiation, which is in accordance with previous reports. And it had been proved that miR-324-3p overexpression observably inhibited the luciferase activity of SMAD7, and the opposite effect was observed in the presence of miR-324-3p knockdown in MC3T3-E1 cells. Thus, it was inferred that miR-324-3p might enhance MC3T3-E1 cell differentiation and proliferation by targeting SMAD7; however, this requires further validation. Moreover, the lack of a signaling pathway assessment is a limitation of the present study. Previously, TGF-β and BMP signaling have been reported as canonical signals and mineralization of mouse osteoblasts. Downregulation of SMAD7 is also involved in the promotion of Zingerone in osteoblast differentiation.

In conclusion, this study provides a new insight into the role of the miR-324-3p/Smad7 axis in osteogenic differentiation and provides a new idea for the clinical treatment of osteoporosis, bone fracture, and other related diseases.

Conflicts of Interest
The authors have declared no conflict of interest.

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