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MiR-374b promotes osteogenic differentiation of MSCs by degrading PTEN and promoting fracture healing

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Abstract. – OBJECTIVE: To investigate whether miR-374b can promote the differentiation of MSCs into osteoblasts by mediating PTEN, thus promoting fracture healing.

MATERIALS AND METHODS: Primary cultured mouse mesenchymal stem cells were obtained for following experiments. Flow cytometry was used to determine the expression of MSCs surface antigens to identify the purity. Alizarin red staining was used to detect whether MSCs could differentiate into osteoblasts. QRT-PCR was used to detect the expression of osteogenic marker genes as well as miR-374b and PTEN in bone marrow-derived MSCs from fractured mice model. ALP activity detection kit was used to detect ALP activity in cells. Changes of osteogenic proteins in cells were evaluated by Western blot. Bioinformatics methods were used to predict the binding sites between miR-374 and target genes. Luciferase reporter assay was used to confirm whether miR-374 could bind to the target gene.

RESULTS: Under normal culture, MSCs grew into a long fusiform shape on the 4th day. After induced in the osteogenic induction medium for seven days, calcified nodules appeared. The results of the detection of MSCs surface antigen markers showed that CD90 was 99.12%, and CD45 was 0.23%. Alizarin red staining showed that MSCs possess the ability to differentiate into osteogenic. The expression level of MiR-374b and PTEN increased significantly in the early stage of fracture in mice, but no significant difference was observed at a later stage. After overexpression of miR-374b, the cell ALP activity, the expression of osteogenesis-related genes and osteogenesis-related proteins was significantly increased, while after knocking out miR-374b, the opposite result was observed. The result of luciferase reporting assay showed that miR-374b can bind to PTEN. As mentioned above, overexpression of miR-374b resulted in upregulation of osteogenic-related genes and proteins, while over-expression of both PTEN and miR-374b could partly reverse the outcomes.

CONCLUSIONS: miR-374b can promote osteogenic differentiation of MSCs by degrading PTEN, thereby promoting fracture healing.

Key Words:
Fracture, miR-374b, PTEN, MSCs.

Introduction

Although the general fracture can be surgically cured, it takes much longer time for complete heal, which makes the simple surgical treatment not ideal. Such long period of healing time for general fracture treatment has been a thorny issue and an enormous challenge for orthopedist clinically. Recently, a large number of studies have been conducted on the molecular mechanism of fracture healing, and many molecules such as cytokines, growth factors, transcription factors and matrix proteins are found involved in the process[1,2], which may provide new ideas for the fractures treatments.

MicroRNAs (miRNAs) are a class of single-stranded small RNAs (ssRNA) that are 19-25 nt in length and processed by stem-loop structured transcriptional precursors[3]. Previous authors[4,5] have shown that miR-374 promotes cell proliferation mainly in the carcinogenesis of gastric cancer, lung cancer, and breast cancer. Additionally, miR-374a and miR-374b can directly target C/EBP-beta 3'UTR region to regulate differentiation and lipid metabolism of porcine preadipocytes[6]. The dietary protein of pregnant sows can affect the lipid metabolism of offspring through the regulation of miR-374b[7]. However, no relevant reports of miR-374b in fractures have been found in the literature.

PTEN (phosphatase and tensin homolog) is a new tumor suppressor gene discovered on human

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chromosome 10 by three independent research groups in the United States in 1997. It is the first type of tumor suppressor genes with bispecific phosphatase activity discovered so far[8]. Some studies have shown that miRNA can regulate the expression of PTEN[9], and their expression levels are negatively correlated[10].

MSCs (mesenchymal stem cells) are a group of non-hematopoietic stem cells derived from mesoderm with a high degree of self-renewal ability and multi-directional differentiation potential[11], which have the potential to differentiate into chondrocytes and possess the characteristics of easy acquisition and *in vitro* amplification[12]. Studies have demonstrated that many miRNAs are involved in the regulation of osteoblast differentiation of MSCs by acting on the corresponding genes[13]. Therefore, in this work, based on the detection of miR-374b and PTEN expression in fracture mice, we used primary bone marrow mesenchymal stem cells as the research object to explore the impact of miR-374b on osteogenic differentiation of MSCs in order to provide new clues for the pathogenesis of fracture.

Materials and Methods

Animal Model

Twenty male SD (Sprague Dawley, SD) mice of 6-8 weeks old (18.0-24.6 g) were injected intraperitoneally with 1% sodium pentobarbital (Thermo Fisher Scientific, Waltham, MA, USA) at a dose of 1 mL/kg. After the mice were completely anesthetized, they were skinned and their right lower limbs disinfected. Then, 0.5 cm longitudinal incision was cut on their right knee joint, muscle fascia was separated, and the tibia was traversed in the upper 1/3 of the tibia with ophthalmic scissors. The incision was at last sutured layer by layer, then the closed fracture model in right tibial plateau of mice was completed, and the left tibia of their own was sham operated. We took the left tibia of mice as a control group and the right fracture as the experimental group. After 4 d, 8 d, 12 d, 16 d, 20 d and 24 d, the bone tissues of the left and right tibia of mice were collected and stored in liquid nitrogen at -80°C for further studies. This study was approved by the Animal Ethics Committee of Yantaishan Hospital Animal Center.

Isolation and Culture of Mouse Bone Marrow MSCs

The mice were sacrificed by cervical dislocation and soaked in iodophor for 15 min. Bilateral femoral bones were removed under aseptic conditions. L-DMEM (Dulbecco's Modified Eagle Medium) (Thermo Fisher Scientific, Waltham, MA, USA) was aspirated into the medullary cavity repeatedly with a syringe, so that the bone marrow cells were washed out and dispersed sufficiently to form a single cell suspension. The L-DMEM medium containing 10% fetal bovine serum was added to the single cell suspension. Then 1×10^6 cells/mL were plated into a cell culture dish of 10 cm in diameter. MSCs were cultured in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO_2 at 37°C. After primary culture for 24 or 48 h, the medium was changed once to remove cells that were not attached to the wall. When the cells confluence was up to 80% to 90%, trypsin (Thermo Fisher Scientific, Waltham, MA, USA) was used to passage the cells with a subculture ratio of 1:3.

Osteogenesis Induction of MSCs

MSCs were grown in a 6-well plate at a certain density. On the following day, the medium of the experimental group was replaced by osteogenic medium (DMEM high glucose medium) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin and 50 μg/mL streptomycin, 10 mmol/L glycerophosphate, 10^{-7} mol/L dexamethasone, 50 μg/mL ascorbic acid and 1% HEPES (hydroxyethyl piperazine ethanesulfonic acid) (Thermo Fisher Scientific, Waltham, MA, USA). The medium was replaced every three days to maintain the cell culture.

Flow Cytometry Identification of MSCs

Approximately 4×10^5 MSCs were collected and washed three times by phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA, USA). The cells were then divided equally into four 1.5 mL Eppendorf tube (EP) tubes and resuspended with 500 μL of PBS in each EP tube. CD45-FITC and CD90-FITC (BD Biosciences, San Jose, CA, USA) antibodies were added to each tube respectively and incubated in the dark at room temperature for 30 minutes. The unbound antibody was washed away by PBS. Lastly, the surface antigen expression of MSCs was measured by flow cytometry.

Alizarin Red Staining

After osteogenic induction for 14 days, the medium was discarded and the MSCs were washed by PBS for two to three times. Next, the cells were

fixed in anhydrous ethanol for 30 minutes, washed with double distilled water for two to three times. Then, cells were stained with 10% alizarin red stain for ten minutes by two to three times. The staining result was observed under light microscope.

RNA Extraction and qRT-PCR Detection

After MSCs were induced for seven days, the total cellular RNA was extracted with TRIzol. Expression of osteoblast marker genes ALP, Runx2 (Runt-related transcription factor 2), Bglap (bone gamma-carboxyglutamate (gla) protein) in the cells and relative expression of miR-374b and PTEN in tibia of the fractured mice were detected using micro-RNA PCR assay kit (TaKaRa, Otsu, Shiga, Japan)). (Primer of above mentioned genes (Invitrogen, Carlsbad, CA, USA): ALP (F: 5'-ACACCTTGACTGTGGTTACTGCTGA-3', R: 5'-CCTTGTAGCCAGGCCCGTTA-3'); Runx2 (F: 5'-TTCTCCAACCCACGAATGCAC-3', R: 5'-CAGGTACGTGTGGTAGTGAGT-3'); Bglap (F: 5'-GGCAGCGAGGTAGTGAAGA-3', R: 5'-CCTGAAAGCCGATGTGGT-3'); miR-374b (F: 5'-TCAGCGGATATAATACAAC-CTGC-3', R: 5'-TATCGTTGT-TCTCCACTCCTTCAC-3'); PTEN(F:5'-ATACCAGGACCAGAGGAAACC-3', R: 5'-TTGTCATTATCCGCACGCTC-3'); GAPDH (F: 5'-AGGTCGGTGTGAACGGATTTG-3', R: 5'-TGTAGACCATGTAGTTGAGGT-3').

Lentiviral Vector Construction and Cell Transfection

A retroviral transduction system using shuttle vector was established for the miR-374b knockout in MSCs. The sequence of miR-374b inhibitor was cloned into the shuttle vector, which was then transfected into MSCs to reduce the expression of miR-374b. Low-density MSCs were seeded into DMEM medium containing 10% fetal bovine serum. After 24 hours, complete medium and fresh virus particles containing 6 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA) were added to MSCs for three consecutive days to complete the virus transduction.

ALP Vitality Testing

The MSCs incubated for seven days were collected and lysed. After centrifugation, the supernatant was aspirated and assayed for ALP (alkaline phosphatase) activity according to the kit instruction. Subsequently, after the medium was discarded, ALP staining was performed using 250 μL mixture of 0.1 g/L Naphthol AS-MX alkaline phosphatase solution and 0.6 g/L Fast Blue RR Salt. Then, after ten minutes in the dark, the vitality assay was performed.

Detection of Protein by Western Blot Assay

The MSCs culture medium was discarded and the cells were washed with PBS and lysed on ice. The supernatant was collected after centrifugation and bicinchoninic acid (BCA) (Beyotime, Shanghai, China) method used for the determination of protein concentration. 40 mg of each protein sample was added in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel in electrophoresis system. After the gel was transferred into polyvinylidene fluoride (PVDF) (Thermo Fisher Scientific, Waltham, MA, USA) membrane, the membrane was blocked, incubated with primary antibody overnight, and incubated with secondary antibodies. Finally, image exposure was performed to observe the protein expression.

Luciferase Reporting Assay

The 3' UTR of PTEN and the CMV promoter were amplified and cloned into the pGL3-luciferase basic vector (Invitrogen, Carlsbad, CA, USA). Sequences of primers and cloning strategy are available on request. For the luciferase assays, 50 nM of miR-374b mimics or scrambled RNA were co-transfected with the reporter vector and the Renilla control vector into the cells. 24 h post transfection, the measurements were performed using the Dual-luciferase reporter assay kit (Promega, Madison, WI, USA).

Statistical Analysis

The data were statistically analyzed by statistical product and service solutions (SPSS16.0, SPSS Inc., Chicago, IL, USA) software. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$), *t*-test was used to compare the differences between two groups, and one-way analysis of variance (ANOVA) followed by Least Significant Difference as its Post-Hoc test was used to compare multiple groups. $p<0.05$ indicated the difference was statistically significant.

Results

Cultivation and Identification of MSCs

Under normal culture conditions, MSCs grew into the long fusiform form on the fourth day (Figure 1Aa). When cultured for seven days

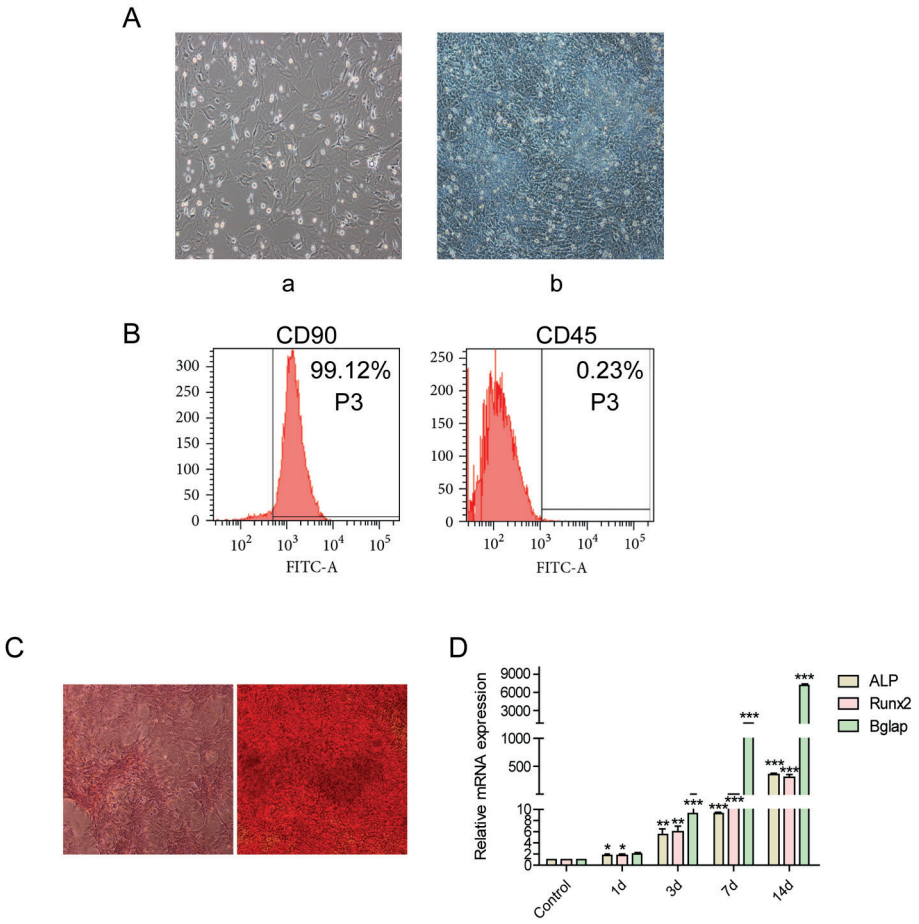

Figure 1. Phenotypic characterization of bone marrow mesenchymal stem cells. ***A,*** (a) MSCs grow into a fusiform shape on the fourth day under normal culture conditions; (b) Cell morphology of MSCs cultured for seven days in osteogenic induction medium is shown. ***B,*** Specific surface antigens of MSCs, including positive CD90 and negative CD45, were identified by flow cytometry. ***C,*** In alizarin red staining, MSCs cultured in osteogenic induction medium for 14 days show significant calcified nodules, while the control group does not appear. ***D,*** The expression levels of osteoblast marker gene ALP, Runx2 and Bglap at different days of induction are shown. On the third day, the expression levels increased significantly, and more markedly on the seventh day.

in the osteogenic induction medium the MSCs changed to round shape and the nuclei significantly shrunk (Figure 1Ab). Additionally, the number of osteoblasts increased as well. As to the surface markers of MSCs, CD90 was 99.12% and CD45 was 0.23%, indicating the purity of MSCs met the requirements (Figure 1B). Alizarin red staining results showed that MSCs cultured in osteogenic induction medium for 14 days appeared obvious calcified nodules (Figure 1C). Osteoblast marker genes ALP, Runx2, Bglap were upregulated significantly at day three and day seven of induction (Figure 1D). The above results indicated that MSCs were well cultured and had the ability to differentiate into osteoblasts.

Relative Expression of miR-374b and PTEN in Fractured Mice

In fractured mice, compared with the control group (left tibia), expression of miR-374b and PTEN in the experimental group (right tibia) was significantly increased on the 4th, 8th, 12th and 16th days, but no significant difference was observed on the 20th and 24th days (Figure 2A-B). This result illustrated that the expression of miR-374b and PTEN was upregulated in the early stage of fracture.

miR-374b Promotes Osteogenic Differentiation of MSCs

In MSCs, overexpression and knockdown of miR-374b was achieved by transfection (Figure

3A). After overexpression of miR-374b, the cell ALP activity, as well as the expression of osteogenesis-related genes, including ALP, Bglap, and Runx2, were significantly enhanced. On the contrary, reverse results were observed after knockdown of miR-374b (Figure 3B-C). In addition, overexpression of miR-374b enhanced the expression of osteoblast-associated proteins ALP, Runx2, OCN and OPN, while knockdown of miR-374b brought down the expression of above proteins (Figure 3 D-E). All the above results suggested that miR-374b promotes osteogenic differentiation of MSCs.

miR-374b Regulates PTEN Expression

The binding site of miR-374b and PTEN was predicted by bioinformatics analysis (microRNA.org), which was also further confirmed by luciferase reporting assay. The results showed that miR-374b can bind to PTEN, as shown in Figure 4A. After overexpression of miR-374b, gene and protein expression of PTEN significantly declined, while knockdown of miR-374b enhanced the expression conversely (Figure 4B&C). The results demonstrated that miR-374b can negatively regulate the expression of PTEN.

miR-374b Promotes Osteogenic Differentiation of MSCs by Degrading PTEN

After overexpression of miR-374b, the expression of osteoblast-associated genes ALP, Bglap, and Runx2 increased significantly, while overexpression of PTEN and miR-374b in MSCs reversed this result (Figure 4D). What's more, overexpression of miR-374b improved the expression of osteoblast-associated protein Runx2 and OCN, but double overexpression of PTEN and miR-374b in MSCs at the same time brought down the expression of above proteins (Figure 4E). In conclusion, miR-374b can regulate the expression of PTEN, and then, affect the osteogenic differentiation of MSCs.

Discussion

The process of bone formation mainly includes the secretion of osteoblasts matrix, fiber surrounding, formation of osteoid and the deposition of calcium salts[14], which makes osteoblasts functional cells in the process of bone formation. MSCs are another type of stem cells with the potential of self-renewal and multi-directional differentiation in the bone marrow. Under certain inducing conditions, MSCs can differentiate into three germ layer cells (mesoderm such as osteoblasts, adipocytes, and myocytes; ectodermal neurons; endodermal hepatocytes)[15]. The mechanism by which MSCs differentiate into osteoblasts is not well understood, but it is clear that their differentiation is regulated by many regulatory factors such as transcription factors and environmental regulatory factors such as secretion factors, extracellular matrix, chemical factors, and interactions between cells[16].

MiRNAs can regulate osteoblast formation by rapidly and efficiently binding to target sites to degrade target genes or inhibit the translation of target proteins[12,15]. The role of miRNAs related to the regulation of osteoblast differentiation has been gradually discovered. It has been reported that miRNA-210 promotes the differentiation of MSCs into osteoblasts in mouse[17], which is

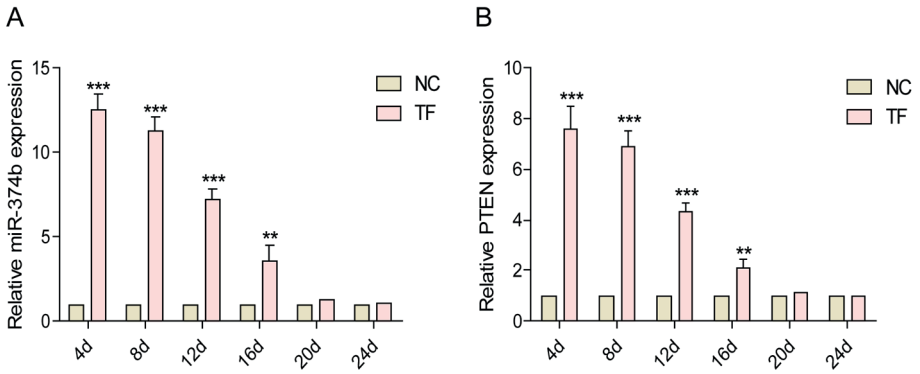

Figure 2. Relative expression levels of miR-374b and PTEN in mouse bone marrow mesenchymal stem cells. MiR-374b and PTEN in the experimental group (right tibia) of fractured mice significantly increased on the 4th, 8th, 12th, and 16th days, compared with control group (left tibia), but no significant difference was observed on the 20th and 24th days.

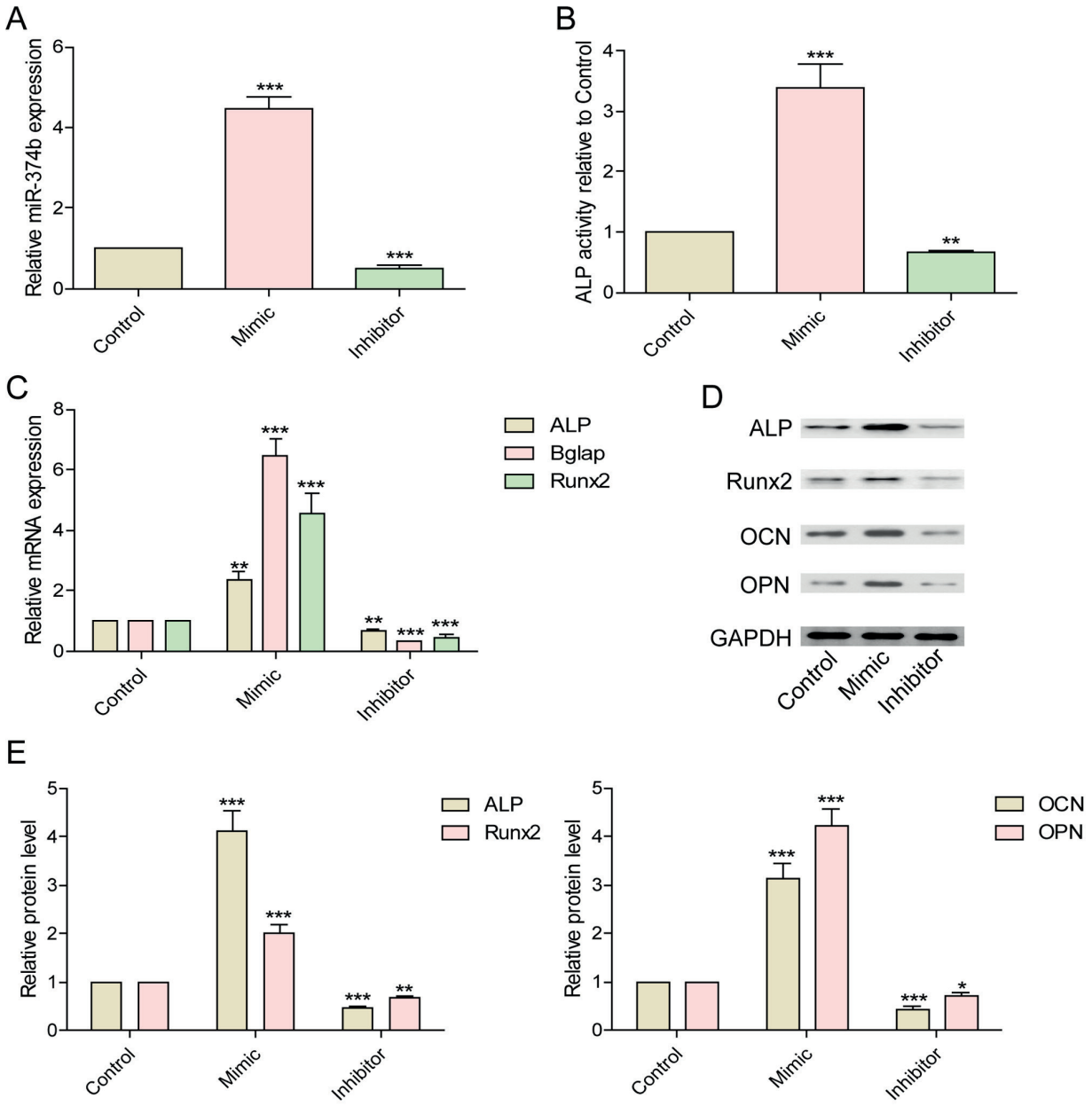

Figure 3. miR-374b promotes osteogenic differentiation of MSCs. ***A,*** After overexpression and knockdown of miR-374b, miR-374b expression in cells significantly increased and decreased, indicating that the transfection effect was good. ***B,*** The cell ALP activity was significantly improved after overexpr ession of miR-374b, while that was significantly reduced after knockdown of miR-374b. ***C,*** Osteoblast-related genes ALP, Bglap, Runx2 significantly increased after overexpression of miR-374b, while knockdown of miR-374b made them significantly decrease. ***D,*** Osteoblast-associated proteins ALP, Runx2, OCN, OPN significantly increased after overexpression of miR-374b, while knockdown of miR-374b made them significantly decrease. ***E,*** Quantitative protein analysis is shown.

similar to the results of our study that miR-374b can promote osteogenic differentiation of MSCs through the degradation of PTEN.

It has been reported in the literature[18] that the change of PTEN level is associated with tumor proliferation and differentiation. Furthermore, the positive rate of PTEN showed a significantly negative correlation with the malignancy of many human tumors. In our study, after overexpression of miR-374b, the gene and protein expression of PTEN were significantly decreased, while osteogenesis-related genes and proteins were upregulated. However, when PTEN and miR-374b were overexpressed in the MSCs at the same time, the trends of above os-

teogenesis-related genes and proteins were reversed and the expression of them decreased. Therefore, according to above results, it is considered that miR-374b can promote osteogenic differentiation of MSCs by decreasing PTEN expression.

In summary, we found that miR-374b can decrease the expression of PTEN and promote osteogenic differentiation of MSCs, which may provide a theoretical basis and research direction for future therapeutic options of fracture healing.

Conclusions

We showed that miR-374b can degrade PTEN by directly targeting its 3'UTR region, so as to

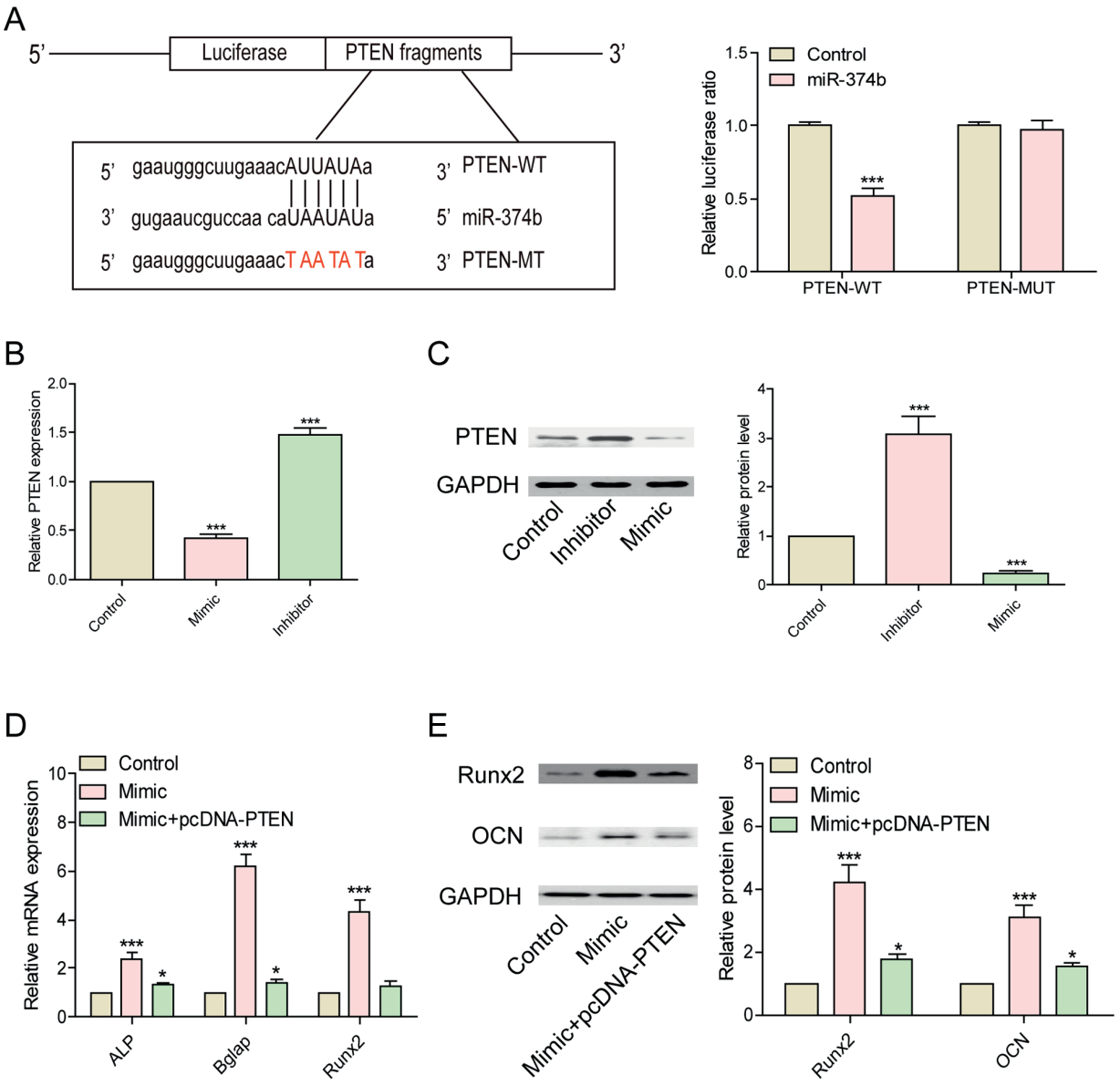

Figure 4. miR-374b promotes osteogenic differentiation of MSCs by degrading PTEN. ***A,*** The binding site of miR-374 to PTEN is shown. ***B,*** PTEN gene expression significantly decreased after overexpression of miR-374b, while knockdown of miR-374b made PTEN gene expression significantly increase. ***C,*** PTEN protein expression significantly decreased after overexpression of miR-374b, while knockdown of miR-374b made PTEN protein expression significantly increase. ***D,*** After overexpression of miR-374b, the expression of osteoblast-associated genes ALP, Bglap, and Runx2 increased obviously, while overexpression of PTEN and miR-374b in MSCs reversed this tendency. ***E,*** Overexpression of miR-374b improved the expression of osteoblast-associated protein Runx2 and OCN, but PTEN and miR-374b overexpressed in MSCs at the same time brought down the expression of above proteins.

promote the osteogenic differentiation of MSCs, which accelerates the healing of fractures.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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