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miR-374-5p inhibits osteogenesis by targeting PTEN/PI3K/AKT signaling pathway

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Abstract

Purpose This study aims to evaluate the effects of miR-374-5p on osteogenesis in rat osteoblasts, validate its target on PTEN, and explore its role in the PTEN/PI3K/AKT signaling pathway during osteoblast differentiation.

Methods We transfected 293T cells with miR-374-5p mimics and inhibitors, followed by Western blot and qRT-PCR analyses to assess protein and mRNA expression levels. A dual-luciferase assay was performed to confirm direct targeting. Markers of osteoblast function, such as Runx2, OSX, and OCN, were examined in osteoblasts from rats by qRT-PCR and Western blot. Additionally, we developed a lentiviral vector to overexpress miR-374-5p, which successfully infected rat osteoblast progenitors. Bone formation was subsequently assessed using Alizarin Red staining and ALP activity assays. Finally, rescue experiments were conducted to validate the involvement of the miR-374-5p/PTEN/PI3K/AKT signaling pathway.

Results Our results demonstrate that miR-374-5p significantly downregulates both the protein and mRNA levels of its target gene PTEN, as confirmed by dual luciferase assays. qRT-PCR and Western blot analyses revealed that osteoblastic markers-including Runx2, OSX, and OCN—were markedly reduced in the miR-374-5p mimic group, whereas an opposite trend was observed in the inhibitor group. In vitro, overexpression of miR-374-5p suppressed osteoblast differentiation, as evidenced by decreased calcium nodule formation and reduced ALP activity compared to controls. Furthermore, co-transfection of miR-374-5p mimics with the PI3K/AKT pathway inhibitor LY294002 in osteoblasts led to significantly lower expression of PI3K/AKT pathway-related genes, and notably, the inhibitory effect of miR-374-5p on osteoblast differentiation was reversed by LY294002 treatment.

Conclusion Our findings indicate that miR-374-5p inhibits osteogenesis in rat osteoblasts by targeting PTEN and modulating the PI3K/AKT signaling pathway.

Keywords MiRNA, PTEN, PI3K/AKT, Osteogenesis

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Introduction

Chronic periodontitis is an infectious disease characterized by progressive alveolar bone loss, posing significant challenges in maintaining the balance between osteogenesis and osteoclastogenesis, as well as in promoting oral bone repair [1]. Various chemical and physical methods have been employed to stimulate osteogenesis, which is vital for mitigating destructive alveolar bone loss, stabilizing post-orthodontic conditions, and enhancing therapeutic efficacy. Recent research has highlighted genetic therapy as a potentially more efficient treatment modality [2]. This approach modulates bone metabolism, promotes bone healing, and accelerates bone deposition by introducing exogenous genes or altering endogenous gene expression, thereby addressing specific clinical challenges in oromaxillofacial surgery, periodontology, implantology, orthodontics, and related fields.

MicroRNA (miRNA) represents a promising tool in genetic therapy by regulating the expression of specific genes, thereby influencing bone cell function and modulating bone metabolism and healing processes [3]. Recent studies increasingly emphasize the pivotal role of miRNAs in maintaining bone tissue homeostasis. With the capability to regulate up to 60% of the human genome, miRNAs exert extensive and critical effects on the proliferation and differentiation of bone cells [4–9]. For instance, miR-20a has been shown to promote the differentiation of mesenchymal stem cells into osteoblasts, concurrently upregulating osteogenic markers such as BMP-2, BMP-4, and RUNX2 [10].

Our previous study demonstrated that static magnetic fields enhance ALP production, thereby promoting osteoblast differentiation, while simultaneously altering miRNA expression in rat osteoblasts [11]. A high-throughput sequencing screen identified miR-374-5p as significantly downregulated. Subsequent bioinformatics analysis predicted PTEN—a phosphatase and tensin homolog frequently mutated in human cancers, and a critical regulator of cell growth and apoptosis—as a target of miR-374-5p [12]. Previous research has shown that PTEN is regulated by various miRNAs and that its expression in stem cells correlates with the inhibition of osteosarcoma progression, suggesting its involvement in bone tissue homeostasis [13]. As a negative regulator, PTEN suppresses the PI3K/AKT signaling pathway, which is vital for cell growth, proliferation, differentiation, migration, survival, and metabolism [14]. This pathway directly influences osteoblast function and bone development, linking it to enhanced apoptosis and osteoblast differentiation [15]. However, the specific roles of miR-374-5p and the PTEN/PI3K/AKT signaling pathway in the regulation of bone tissue homeostasis remain to be elucidated.

In this study, we aimed to investigate the effect of miR-374-5p on the PTEN/PI3K/AKT signaling pathway and its influence on bone formation in rat osteoblasts.

Table 1 The miRNA mimics and inhibitors corresponding sequences.

miRNA	Sequence
miR-374-5p mimics	5′-AUAUAAUACAACCUGCUAAGUG-3′
miR-374-5p inhibitor	5′-CACUUAGCAGGUUGUAUUAUAU-3′
Negative control (NC)	5′-UCUACUCUUUCUAGGAGGUUGUGA-3′

Materials and methods

Cell culture and treatment

Rat primary osteoblasts purchased from Shanghai Yuchi Biotechnology. All cell lines were cultivated in a complete rat osteoblast medium (PROCELL, CM-R091) under an incubator condition of 37 °C, 5% CO2.

The miRNA mimics and inhibitors were engineered and synthesized by Jiangsu Gemma Biotechnology Company, with a concentration of 100 nM for miR-374-5p mimics and inhibitors, incubated for 24 h within an incubator. The corresponding sequences are illustrated in Table 1. LY294002 (HY-10108) from MedchemExpress was used at a concentration of 50 μM.

Formation of osteogenic induction medium: Initially, a high-concentration storage solution is prepared, filtered, and aliquoted for − 20 °C freezing. Before each medium change, thaw one aliquot and incorporate it into the osteogenic culture medium. Ascorbic Acid, with an ultimate working concentration of 50 μg/mL. B-Glycerophosphate (b-GP) Disodium, with an ultimate working concentration of 10 mM. Dexamethasone, with an ultimate working concentration of 10 nM. Post-osteogenic induction for 7 days, alkaline phosphatase staining is performed, and post-osteogenic induction for 14–21 days, alizarin red staining is implemented.

Lentivirus infection

Rat primary osteoblasts were serially inoculated into a 24-well plate at 1×10^4 cells per well. Cultured in a cell incubator at 37℃ and 5% CO2 for 24 h, ensuring cellular growth density reached approximately 60%. Before infecting, the virus was retrieved from the − 80℃ freezer and placed on ice for thawing. Next, 2 mL of viral supernatant was supplemented with 100 μL Polybrene, resulting in a final concentration of 2 mg/mL. After cell replacement, diluted virus solution at a concentration of 5–8 μg/mL and rested slow virus was evenly added into each well, and gently mixed. The culture was maintained at 37℃ overnight postinfection. Following 24 h of infection, the culture medium containing the virus was aspirated, and the fresh complete culture medium continued growth at 37℃. Fluorescence observation and photo documentation commenced after 48–72 h of infection.

Western blot

Cell lysis with RIPA buffer containing 1% PMSF, centrifuge at 4 °C to transfer supernatant to a new EP tube. Mix the supernatant with buffer and boil to denature the sample, then store at -20 °C. Prepare 10% separating gel and 6% stacking gel. Post electrophoresis, set the transfer current to 200 mA, and the transfer time is dependent on protein size (60–120 min). After completion, rinse with TBST, and incubate with 5% skimmed milk for 1–2 h at room temperature for blocking. Following blocking, rinse the membrane with TBST three times, each for 10 min. Incubate the PVDF membrane with primary antibodies overnight at 4 °C (Anti-PTEN, ab32199, abcam, 1/1000; Phospho-Akt, 4060, Cell Signaling Technology, 1/2000; Osteocalcin Antibody, Affinity, DF12303, 1/500; Runx2 Antibody, Affinity, AF5186, 1/500; Anti-OSX Antibody, Santa Cruz, sc-393325, 1/500; β-Actin, 4967, Cell Signaling Technology, 1/1000). Post incubation, rinse the PVDF membrane with TBST three times, each for 10 min. Incubate the PVDF membrane with secondary antibodies for 1–2 h. Post incubation, rinse the PVDF membrane with TBST three times, each for 10 min. Then immerse the membrane in a luminescent solution for 5 min before exposure.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA is isolated using TRIzol reagent, which is converted into cDNA by reverse transcription utilizing HiFiScript cDNA First Strand Synthesis Kit instructions. The reverse transcriptase product can directly enter quantitative PCR, while miRNA reverse transcription involves miRNA polyadenylation. Quantitative PCR is performed following the standard protocol in the SYBR-Green kit. Primer information is provided in Table 2, with target gene expression levels analyzed via the $2^{-\Delta\Delta Ct}$ method.

Table 2 Primer sequences.

Gene	Primer
PTEN	F 5′- AGGCCCTGGATTTTTATGGGG-3′
	R 5′- AGCGCCTCTGACTGGGAATA-3′
OCN	F 5′- TTCTGCTCACTCTGCTGACCC – 3′
	R 5′- CTGATAGCTCGTCACAAGCAGG – 3′
RUNX2	F 5′- CGCCACCACTCACTACCACAC – 3′
	R 5′-TGGATTTAATAGCGTGCTGCC – 3′
OSX	F 5′- AGGCACAAAGAAGCCATAC – 3′
	R 5′- AATGAGTGAGGGAAGGGT – 3′
GAPDH	F 5′- CAACGGGAAACCCATCACCA-3′
	R 5′- ACGCCAGTAGACTCCACGACAT-3′
miR-374-5p	RT5′GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACCACTTA – 3′
	F 5′- GCGCGATATAATACAACCTGC-3′
	R 5′- AGTGCAGGGTCCGAGGTATT – 3′
U6	F 5′- GCTTCGGCAGCACATATACTAAAAT-3′
	R 5′- CGCTTCACGAATTTGCGTGTCAT-3′

Dual-luciferase reporter gene

PTEN wild type and 3′-UTR mutation containing the miR-374-5p binding site were developed. All transfections were conducted in 293T cells. Following Luciferase Assay System (Promega, USA) guidelines, cells were lysed and luciferase activity was measured after 48 h of incubation.

Alkaline phosphatase and Alizarin red staining

Following osteogenic induction for 7 or 21 days, the cells were fixed with 4% paraformaldehyde (Sigma) at room temp for 30 min. Subsequently, they were washed 3–5 times with 1 × PBS for 3–5 min. Next, BCIP/NBT staining solution (Cyagen) or Alizarin red (Solarbio) was added and incubated at room temp for 5–30 min in darkness. Upon achieving the desired color intensity, the dyes were decanted, washed twice with distilled water, and photographs captured.

Statistical analysis

All experimental data were collated and statistically analyzed using SPSS 25, Graphpad Prism 9.0 software was utilized for chart creation. Measurements are presented as mean ± standard deviation, and ANOVA analysis was utilized for comparison of mean values across multiple samples. All Western blot images were normalized.

Results

Overexpression of miR-374-5p reduced PTEN expression

We produced miRNA overexpressed cells via mimic transfection, utilizing RT-PCR and Western blot experiments to assess PTEN expression in 293T cells. Following transfection with miR-374-5p mimic, the RT-PCR results (Fig. 1A, B) showed a significantly elevated level of miR-374-5p, correlating with a significant reduction in PTEN mRNA expression ($P < 0.001$). Western blot analysis further examined the changes in PTEN protein levels (Fig. 1C, D), showing a significant decrease in the PTEN protein expression ($P < 0.05$), supporting that miR-374-5p negatively regulates PTEN expression.

Inhibition of miR-374-5p enhanced PTEN expression

The inhibitor transfection technique was used to repress the endogenous miR-374-5p mRNA level with scramble as a control. As shown in Fig. 2A, and B, the miR-374-5p levels significantly declined after transfection of the miR-374-5p inhibitor, while the PTEN mRNA expression increased significantly ($P < 0.001$). A subsequent Western blot analysis determined the alteration in PTEN protein expression levels. Results in Fig. 2C, and D reveal that the PTEN protein expression level significantly increased

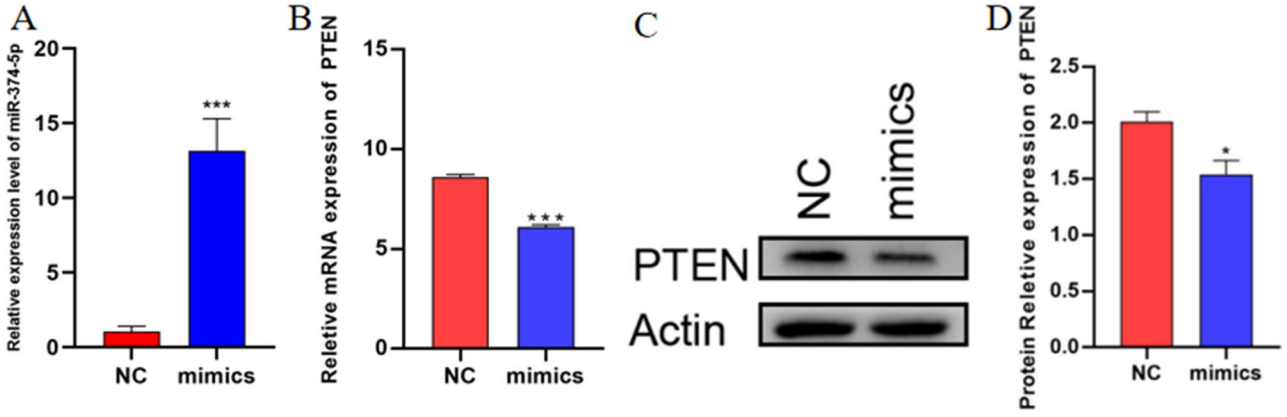

Fig. 1 Overexpression of miR-374-5p suppressed PTEN expression. (**A**). miR-374-5p expression in the NC and mimic groups; (**B**). RT-PCR quantification of PTEN mRNA relative expression levels; (**C**, **D**). Western blot analysis of PTEN protein expression level; (compared to NC group, * $P<0.05$, ***, $p<0.001$)

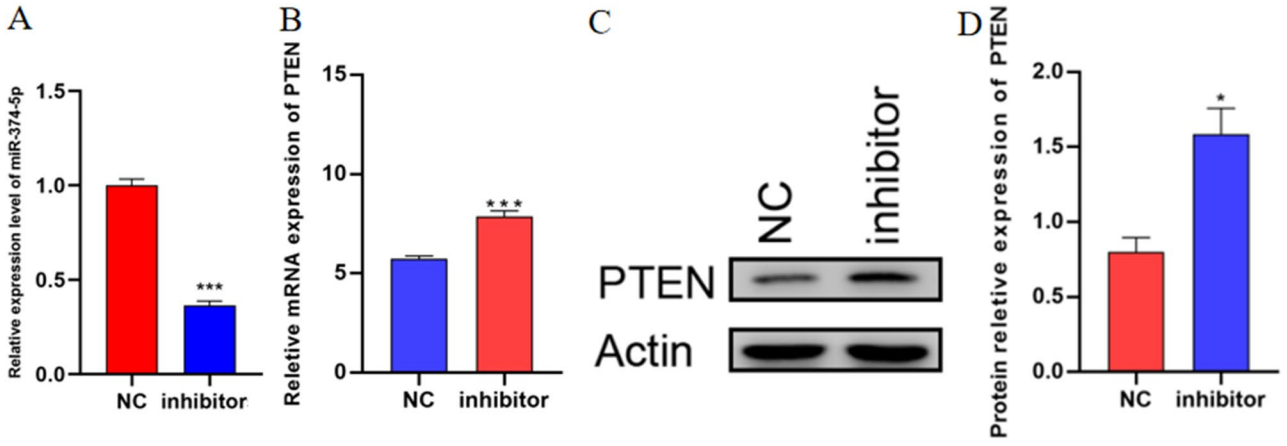

Fig. 2 Inhibition of miR-374-5p enhanced PTEN expression. (**A**). miR-374-5p expression in the NC and inhibitor groups; (**B**). RT-PCR quantification of PTEN mRNA relative expression levels; (**C**, **D**). Western blot analysis of PTEN protein expression level; (compared to NC group, * $P<0.05$, ***, $p<0.001$)

post-transfection of the miR-374-5p inhibitor ($P<0.05$). These results further confirm that miR-374-5p negatively regulates PTEN expression.

miR-374-5p targeted on PTEN

A dual-luciferase reporter assay was performed to directly verify that PTEN is a target of miR-374-5p. The binding site between miR-374-5p and the 3'UTR of PTEN is depicted in Fig. 3A, and the luciferase reporter assay results confirm that PTEN is a direct target of miR-374-5p (Fig. 3B). Furthermore, we examined the effects of miR-374-5p mimics and inhibitors on the osteogenic differentiation of rat osteoblasts. Quantitative RT-PCR and Western blot analyses were conducted to evaluate the expression of osteogenic markers, including PTEN, Runx2, OSX, and OCN. As shown in Fig. 3E, transfection with the miR-374-5p mimic led to a significant decrease in the mRNA levels of PTEN, Runx2, OSX, and OCN compared to the control group. In contrast, transfection with the miR-374-5p inhibitor resulted in a significant increase in the mRNA expression levels of these markers. These findings further substantiate the negative regulatory role of miR-374-5p on PTEN and its downstream impact on osteogenic differentiation. These results indicate that miR-374-5p may inhibit osteoblast differentiation in rat primary osteoblasts. Moreover, Western blot analyses (Fig. 3C and D) revealed that protein expression levels of PTEN and the osteogenic markers Runx2, OSX, and OCN were significantly decreased in the miR-374-5p mimic group compared to controls, while these protein levels were significantly increased in the miR-374-5p inhibitor group. Collectively, these findings suggest that miR-374-5p plays a critical regulatory role in rat osteoblast differentiation by suppressing the expression of PTEN and key osteogenic genes.

Overexpression of miR-374-5p lentivirus inhibits osteogenic differentiation of rat osteoblasts

Due to the lengthy osteogenic induction period, our study employed an in vitro method of constructing lentivirus overexpressing miR-374-5p and infecting it into rat osteoblasts for osteoinduction. Figure 4A demonstrates

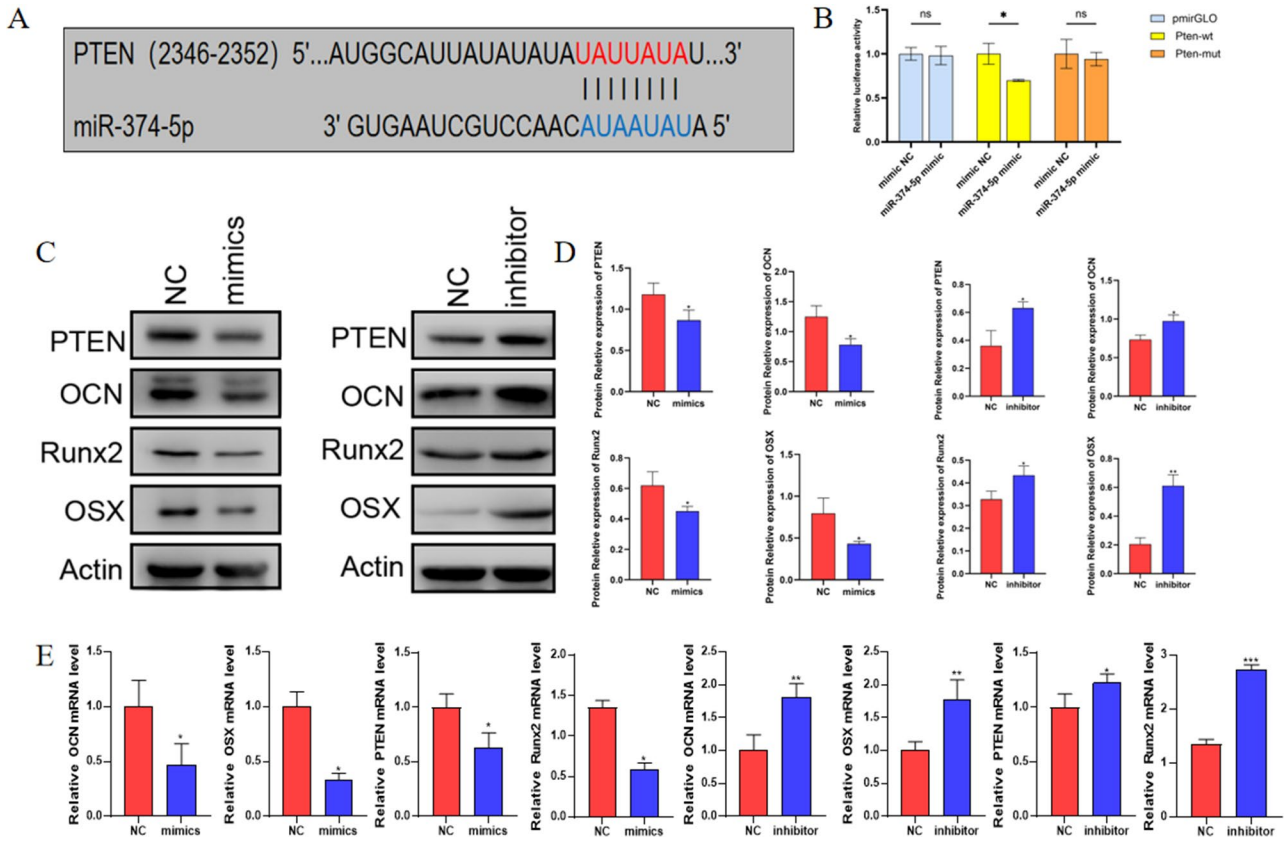

Fig. 3 miR-374-5p targeted on PTEN. (**A**) The sequences of miR-374-5p binding site within the 3'UTR of PTEN mRNA predicted by TargetScan. (**B**) Relative luminescence of NC and miR-181a-5p mimic transfected cells which are contransfected with PTEN luciferase constructs; (**C**, **D**) Upon transfection of miR-374-5p mimics or inhibitors in rat osteoblasts, Western blot analysis measured the protein expression level of PTEN, OCN, Runx2, and OSX; (**E**) PTEN, Runx2, OSX, OCN mRNA expression in rat osteoblasts after transfection with miR-374-5p mimics and inhibitor; (compared to NC group, * $P<0.05$, **, $p<0.01$, ***, $p<0.001$)

the infection status of rat osteoblasts via microscopy, indicating that both the empty vector group and overexpression group could adhere to the wall, and maintain ideal cell morphologies post-infection, with efficacies exceeding 80%. This validated the successful lentiviral infection.

Following a 7-day osteogenic induction, the differentiation status was evaluated by assessing ALP activity. As illustrated in Fig. 4C, rat osteoblasts overexpressing miR-374-5p exhibited a significant reduction in ALP activity compared to the control group ($P<0.01$), underscoring the potential suppressive role of miR-374-5p in osteoblast differentiation.

After 21 days of osteogenesis induction, the effect of overexpression of miR-374-5p on calcium nodule generation was evaluated using alizarin red staining (Fig. 4B). The results demonstrated a significant reduction in calcium nodule generation in rat osteoblasts overexpressing miR-374-5p compared to the empty vector group. This finding suggests that miR-374-5p negatively affects bone matrix deposition and mineralization, further supporting its role in inhibiting osteoblast differentiation.

Rescue experiments: interplay between miR-374-5p overexpression and PI3K Inhibition

The PI3K/AKT signaling pathway is a key downstream regulator of PTEN, yet its interplay with miR-374-5p during osteogenic differentiation in rat osteoblasts remains underexplored. To investigate this, we examined the effects of miR-374-5p modulation on the pathway. Western blot analysis revealed that transfection with the miR-374-5p mimic significantly enhanced AKT phosphorylation (pAKT) levels (Fig. 5A), indicating that miR-374-5p targets PTEN to promote activation of the PI3K/AKT pathway. Conversely, treatment with the miR-374-5p inhibitor resulted in a marked reduction in pAKT expression (Fig. 5B). Furthermore, rescue experiments were conducted by co-culturing osteoblasts overexpressing miR-374-5p with the PI3K inhibitor LY294002. This co-treatment effectively counteracted the effects of miR-374-5p overexpression on osteogenesis, suggesting that the enhanced activation of the PI3K/AKT pathway by miR-374-5p is a critical mediator of its inhibitory effects on osteoblast differentiation. Collectively, these data indicate that miR-374-5p promotes activation of the PI3K/

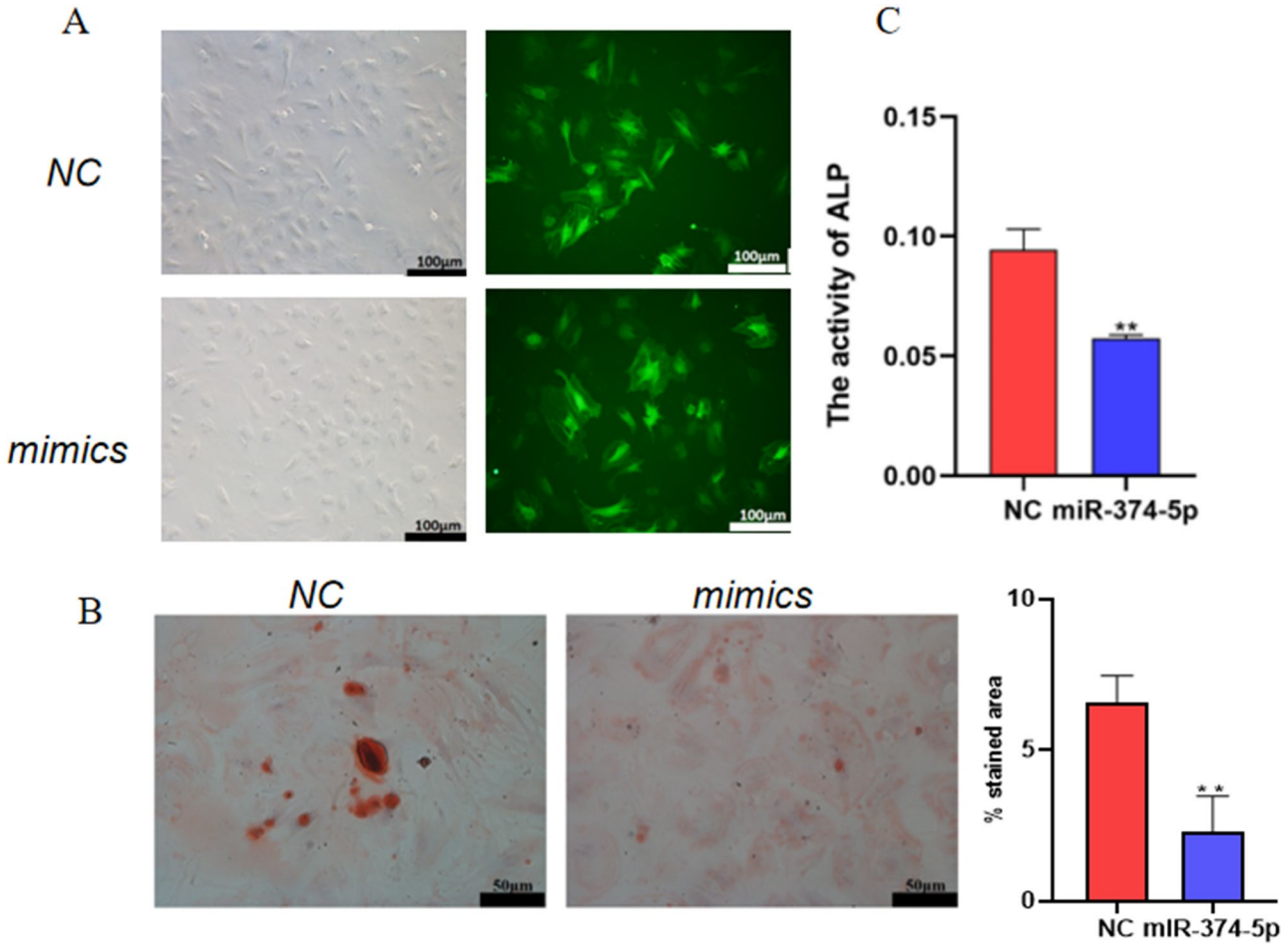

Fig. 4 Overexpression of miR-374-5p lentivirus inhibits osteogenic differentiation of rat osteoblasts. (**A**). Microscopy of overexpression of miR-374-5p lentivirus infection into rat osteoblasts; (**B**, **C**) ALP and ARS staining of the NC and mimic groups treated with osteogenesis induction medium, (compared to NC group, **, $p < 0.01$)

AKT pathway by targeting PTEN, thereby modulating osteogenic differentiation in rat osteoblasts.

To further elucidate the role of miR-374-5p and the PTEN/PI3K/AKT pathway in rat osteoblast differentiation, we co-treated cells with miR-374-5p mimic and LY294002, a PI3K/AKT pathway inhibitor. Western blot analysis (Fig. 5C) revealed that the group receiving both the mimic and LY294002 exhibited the lowest pAKT protein expression, confirming that LY294002 effectively suppresses the AKT pathway. Additionally, in the absence of LY294002, osteogenesis-related genes Runx2, OSX, and OCN were upregulated compared to the group treated with the mimic alone, suggesting that the bone inhibitory effect of miR-374-5p can be reversed by PI3K/AKT pathway inhibition. These findings indicate that the PI3K/AKT pathway plays a crucial role in rat osteoblast differentiation and that miR-374-5p inhibits osteoblast differentiation by activating the PTEN/PI3K/AKT pathway.

To further explore the effects of miR-374-5p and LY294002 on rat osteoblast differentiation, we constructed a lentivirus overexpressing miR-374-5p and infected rat osteoblasts. We then compared the outcomes of ALP activity and Alizarin Red S staining between cells overexpressing miR-374-5p alone and those co-treated with LY294002. As shown in Fig. 5D and E, the ALP activity in the co-treated group was significantly higher than in the miR-374-5p overexpression-only group, suggesting that suppression of the PI3K/AKT pathway can reverse the inhibitory effect of miR-374-5p on osteoblast differentiation. Similarly, Alizarin Red S staining revealed an increased number of calcium nodules in the co-treated cells compared to those treated solely with miR-374-5p overexpression. These findings support the conclusion that miR-374-5p suppresses osteoblast differentiation by activating the PTEN/PI3K/AKT pathway.

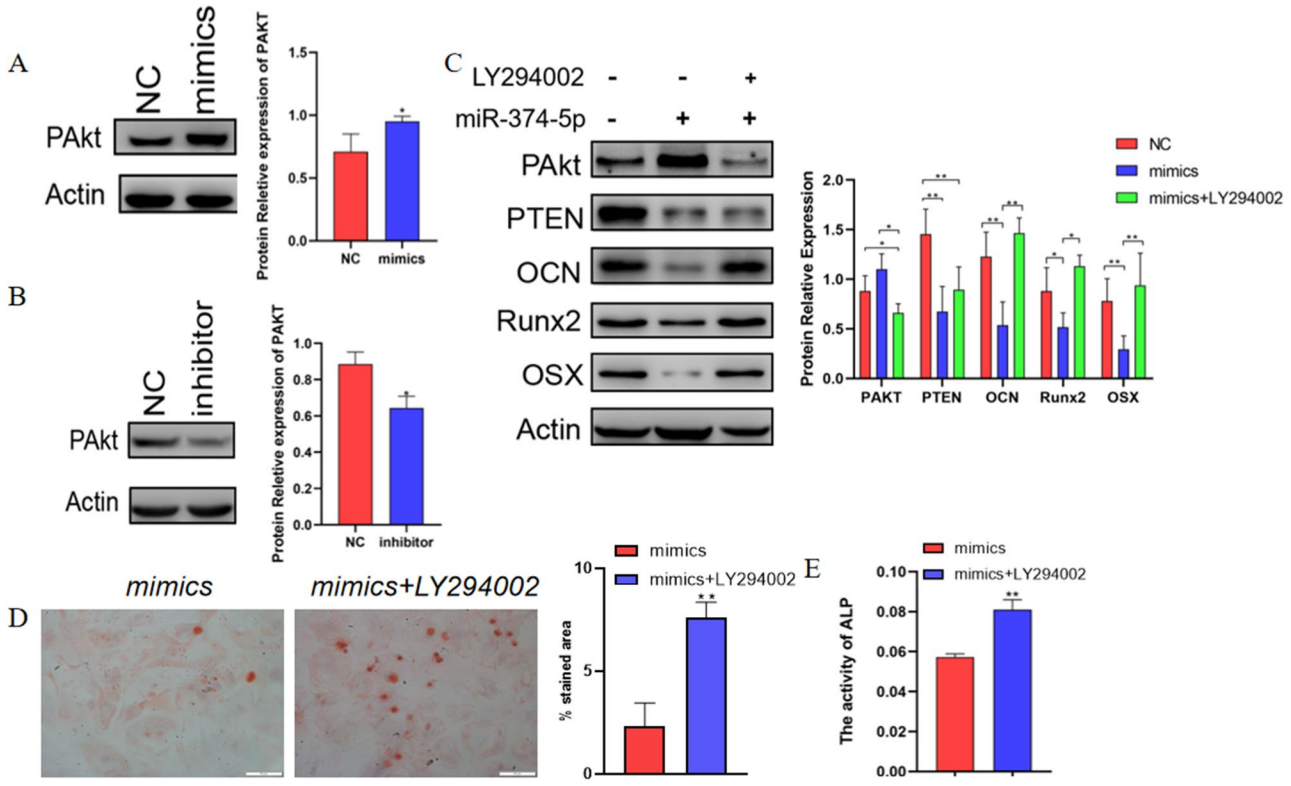

Fig. 5 LY294002 plus miR-374-5p mimics enhanced the efficiency of osteogenesis. (**A**, **B**). Effect of miR-374-5p mimics or inhibitor in rat osteoblasts on the protein expression of pAKT; (**C**). Western blot assay to detect PTEN, pAKT, Runx2, OSX, and OCN protein expression levels after transfection of miR-374-5p mimic plus LY294002 in rat osteoblasts; (**D**, **E**). ALP activity and alizarin red staining in the miR-374-5p overexpression group and the miR-374-5p overexpression combined with LY294002 group; (compared to NC group, * $P < 0.05$, **, $p < 0.01$)

Discussion

Skeletal formation is a complex process that necessitates cellular coordination and multilevel regulation systems [16]. Proliferation and differentiation signals are usually provided by extracellular signal molecules that subsequently regulate gene expression. As a crucial, noncoding RNA, miRNA plays an integral role in gene expression regulation in various organisms.

In previous research, our team discovered that miR-374-5p expression was significantly altered when rat osteoblasts were exposed to static magnetic fields. Based on this finding, we hypothesized that miR-374-5p may play a significant role in osteogenesis. Therefore, the objective of this study was to investigate the potential mechanism of miR-374-5p regulating osteoblast differentiation. According to our experimental findings, miR-374-5p can target and suppress PTEN, thereby inhibiting osteoblast differentiation using the PTEN/PI3K/AKT signaling pathway. Notably, little research had been previously conducted on the correlation between miR-374-5p and bone tissue formation, it had been reported that miR-374-5p could regulate proliferation and hypertrophic differentiation of growth plate chondrocytes [17]. Peng's study found [18] that the potential mechanism of action of schisandrin therapy for breast cancer may be through regulation of the miR-374a/ PTEN/ Akt signaling pathway. Moreover, Hu's research showed that miR-374b can prevent cell proliferation and dysregulated glycosylation by targeting PTEN, providing a new potential treatment for IgA nephritis [19]. Thus, this study has filled the experimental void in the novel field of miR-374-5p maintenance of bone tissue homeostasis.

Mounting evidence indicates that miRNAs play a crucial role in regulating bone formation through their targeting of PTEN. For example, miR-136-3p has been shown to modulate angiogenesis and bone formation by targeting PTEN, thereby mitigating alcohol-induced bone mass reduction [20]. Similarly, miR-374b enhances MSC osteogenic differentiation and fracture healing by degrading PTEN [21], while miR-29b promotes the osteogenic differentiation of human adipose mesenchymal stem cells via PTEN targeting [22]. Through bioinformatics analysis and Target Scan website prediction, we postulated that the target gene of miR-374-5p is PTEN. To verify the direct targeting relationship between miR-374-5p and PTEN, we conducted a dual luciferase assay, which revealed that miR-374-5p significantly suppressed the luciferase activity of PTEN compared to the control group, indicating a direct targeting relationship between miR-374-5p and PTEN. To further validate the regulatory

effect of miR-374-5p on PTEN, we performed Western blot and qRT-PCR experiments to detect the expression of PTEN at the protein and mRNA levels after transfection with miR-374-5p mimics and inhibitors. The results indicated that transfection with miR-374-5p mimics significantly suppressed the mRNA and protein expression levels of PTEN, while transfection with miR-374-5p inhibitors resulted in the opposite effect, further confirming the results of the dual luciferase assay. Therefore, these experimental results suggest that miR-374-5p can directly target and suppress the expression of PTEN.

According to a previously obtained high-throughput sequencing result, miR-374-5p expression was significantly downregulated in rat primary osteoblasts after static magnetic field induction. This suggests a suppressive role for miR-374-5p during osteogenesis. Runx2, OSX, and OCN are critical bone formation markers involved in this process [23]. Runx2, a potent osteogenic transcription factor, interacts with osteoblast-specific cis-acting elements (OSEs) on osteogenic gene promoters, controlling osteoblast proliferation and differentiation. OSX also plays an essential role in osteoblast proliferation, differentiation, and bone formation by regulating several specific osteoblastic differentiation markers. OCN, expressed at the post-osteoblastic stage, coordinates calcium homeostasis and bone mineralization. Currently, serum OCN expression levels are thought to reflect osteoblastic function [24]. Therefore, we used Western blot and qRT-PCR experiments to examine the expression of osteogenic markers Runx2, OSX, and OCN in osteogenic rat primary osteoblasts to determine the mechanism of miR-374-5p's role in osteogenic differentiation. The results indicated that PTEN and the protein and mRNA expression levels of osteogenic markers Runx2, OSX, and OCN were suppressed after transfection with miR-374-5p mimic; conversely, transfection with miR-374-5p inhibitor resulted in a significant increase in these markers. These findings further support the hypothesis that miR-374-5p negatively regulates osteogenic differentiation in rat primary osteoblasts.

To explore miR-374-5p's regulatory role more comprehensively, we constructed a lentiviral vector overexpressing miR-374-5p in vitro and generated stable osteogenic cell lines for long-term induction. Alkaline phosphatase (ALP) is a key enzyme synthesized by osteoblasts to hydrolyze phosphates for hydroxyapatite formation, and its activity serves as an indicator of osteoblast biosynthetic function. In our study, osteoblasts overexpressing miR-374-5p exhibited significantly reduced ALP activity compared to control cells, indicating that miR-374-5p exerts a strong inhibitory effect on early osteoblast differentiation. We also employed alizarin red staining to detect the degree of calcification in osteoblasts, where the osteogenic induction procedure makes calcium ions precipitate as calcium salts and form calcium nodules that produce deep red compounds under miR-374-5p overexpression conditions. This indicates that miR-374-5p can negatively regulate rat osteoblast calcification, thereby reducing the number of deposited nodules. The results from the ALP activity detection experiment and alizarin red staining experiment were consistent with those from the Western blot and qRT-PCR experiments, all suggesting that miR-374-5p negatively regulates osteogenic differentiation in rat primary osteoblasts by targeting PTEN.

As a negative regulatory factor, PTEN modulates the PI3K/AKT pathway by inhibiting AKT phosphorylation [25]. Specifically, PI3K phosphorylates PIP2 (phosphatidylinositol-4,5-bisphosphate), converting it to PIP3. PTEN dephosphorylates PIP3 back to PIP2, thereby modulating PI3K signaling. PIP3 recruits AKT to the plasma membrane and induces its phosphorylation, leading to downstream activation. If PI3K is likened to the accelerator of signal transmission, PTEN is considered the brake, limiting or halting further activation of the PI3K/AKT pathway. Growing evidence suggests that the PTEN/PI3K/AKT pathway plays a crucial role in maintaining bone tissue homeostasis. Overexpression of miR-181a/b-1 intensifies osteogenic function by regulating PTEN/PI3K/AKT signals and mitochondrial metabolism [26]. miR-26a-5p boosts osteoblast differentiation in MC3T3E1 cells through the PTEN/PI3K/AKT pathway [26]. During RANKL-induced osteoclastogenesis, miR-21 targets PTEN to activate the PI3K/AKT pathway, increasing osteoclast formation and bone resorption [27]. Additionally, as a key regulator of bone metabolism, miR-214 enhances osteoclast activity by targeting PTEN to stimulate the PI3K/AKT pathway [28].

To investigate the potential downstream signaling pathways of miR-374-5p inhibiting osteoblast differentiation in rat primary osteoblasts, we used Western blot analysis to examine the effects of miR-374-5p mimics, inhibitors, and the PI3K/AKT pathway inhibitor LY294002 on the expression levels of osteoblast-specific markers Runx2, OSX, OCN, and pAKT proteins. The results indicated that miR-374-5p mimics significantly increased pAKT protein expression and suppressed the expression of osteoblast-specific markers PTEN, OCN, Runx2, and OSX. Conversely, miR-374-5p inhibitors exerted an opposite effect. Interestingly, LY294002 reversed the inhibitory effect of miR-374-5p mimics on osteoblast markers, suggesting that miR-374-5p suppresses osteoblast differentiation in rat primary osteoblasts by potentially activating the PTEN/PI3k/AKT pathway. To gain a more comprehensive understanding of the role of miR-374-5p and the PTEN/PI3K/AKT pathway in regulating rat osteoblast differentiation, we employed Western blot analysis, alizarin red staining, and ALP activity detection.

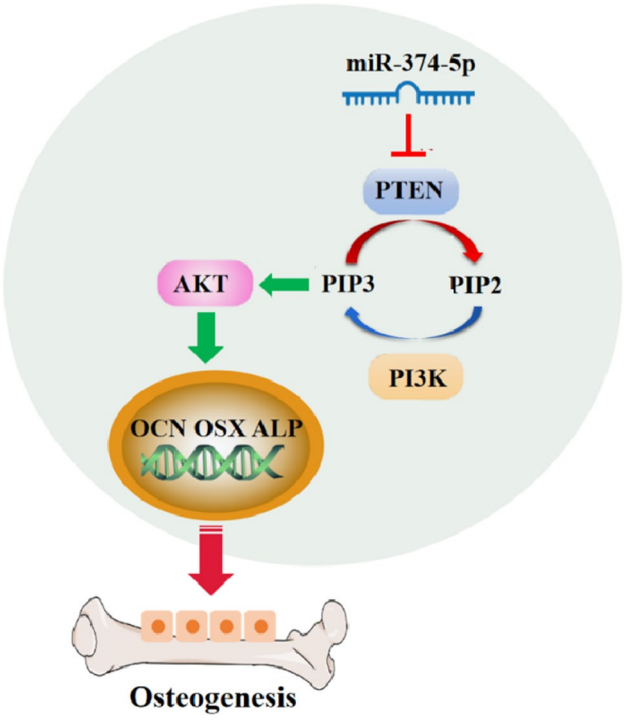

Fig. 6 Diagram of the mechanism of miR-374-5p regulating osteogenic differentiation

The results revealed a significant reduction in calcium nodule formation and ALP activity levels in the miR-374-5p overexpression group compared to the group co-treated with miR-374-5p and LY294002. These findings suggest that miR-374-5p may inhibit osteoblast differentiation in rat primary osteoblasts, potentially through the regulation of the PTEN/PI3K/AKT signaling pathway.

As population aging and lifestyle modifications elevate the prevalence of bone-related diseases, which influence various aspects of bone growth, development, repair, and metabolism, these disorders have become a significant health issue [29]. Hence, it is crucial to discover novel strategies for treating these diseases. Recent studies indicate that miRNAs play a pivotal role in bone tissue development, growth, and repair, becoming a novel target for treating bone-related diseases and maintaining bone tissue reconstruction. Notably, this study unveiled that miR-374-5p regulates osteoblast differentiation by targeting PTEN and activating the PTEN/PI3K/AKT signaling pathway in rat primary osteoblasts. This discovery provides robust support for developing new therapeutic strategies using miR-374-5p and its target gene PTEN for treating bone-related diseases, as well as new avenues for exploring bone tissue treatment and maintenance. Figure 6 presents a schematic diagram based on experimental findings. In conclusion, miR-374-5p suppresses PTEN and activates the PI3K/AKT cascade, preventing osteogenic differentiation of rat osteoblasts.

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Author contributions
ZHL and FT is responsible for conceptualization and funding acquisition. GNL and FL is responsible for methodology, formal analysis and writing original draft. HMC, JG, PW and YFL is responsible for validation and writing review and editing. All authors have approved the final article.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Conflict of interest
No competing financial interests exist.

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