

Beneficial role of microRNA-328-3p in fracture healing by enhancing osteoblastic viability through the PTEN/PI3K/AKT pathway

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Abstract. Although fragility fracture is a global public health burden, the mechanisms underlying fracture healing remain unclear. The present study aimed to assess the dynamic expression pattern of microRNA-328-3p (miR-328-3p) during fracture healing in patients with fragility fracture and to explore the functional role and mechanisms of miR-328-3p in the regulation of osteoblastic viability. The expression levels of miR-328-3p was examined using reverse transcription-quantitative PCR (RT-qPCR). Osteoblastic proliferation and apoptosis were analyzed via MTT and flow cytometry assays. A luciferase reporter assay was adopted to confirm the interaction between miR-328-3p and its target gene PTEN, and western blotting was used to explore the activity of PI3K/AKT signaling. The results of the present study demonstrated that serum miR-328-3p expression did not significantly differ at the early stage of healing in patients with fracture, but was markedly decreased 14 and 21 days post fixation ($P < 0.01$). PTEN was demonstrated to be a target gene of miR-328-3p and was inhibited by miR-328-3p overexpression in osteoblasts ($P < 0.001$). miR-328-3p overexpression increased osteoblastic proliferation but decreased apoptotic rate, with these effects being reversed by PTEN overexpression ($P < 0.05$). The expression of phosphorylated-AKT was elevated in osteoblasts by miR-328-3p overexpression, but this effect was abolished by overexpressing PTEN. Thus, the present study revealed that miR-328-3p may accelerate fracture healing by promoting osteoblastic viability through the PTEN/PI3K/AKT pathway.

Introduction

Fragility fracture generally occurs due to an injury that would be insufficient to cause fracture in a normal bone (1). It is a

common disease in the elderly, leading to huge burdens on health and the economy, worldwide (2). Fragility fracture has become a global public health threat, and ~2,000,000 new cases occur in the USA annually (3). In general, fractures lead to acute pain, decreased mobility and loss of independence, and almost all patients require long-term fixation (4). However, fracture healing is a slow recovery process, leading to a significant reduction in patient quality of life (5). Thus, the mechanisms underlying fracture healing urgently need to be explored further, which may help to reduce the time for fracture healing. Fracture healing involves multiple growth factors and cytokines that are associated with the proliferation, apoptosis and differentiation of osteoblasts (6). To accelerate bone regeneration, strategies to facilitate osteoblastic activation are necessary.

MicroRNAs (miRNAs) are a group of small non-coding RNAs with important regulatory roles in various cellular processes, such as cell proliferation, migration, invasion, differentiation and apoptosis (7). They can directly bind the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs), leading to the inhibition in target gene expression at post-transcriptional levels (8). Emerging studies have reported the roles of miRNAs in the regulation of osteoblastic activity. For example, Zhang *et al* (9) observed that miR-146a suppressed osteoblastic proliferation and enhanced apoptosis. Li *et al* (10) revealed that miR-342-5p could inhibit osteoblastic cell proliferation, migration and differentiation by targeting Bmp7. Differentially expressed miR-125a-3p in the osteoblastic differentiation process could significantly regulate osteoblastic cell proliferation (11). The decreased expression levels of miR-491-3p in postmenopausal osteoporosis regulates osteoblastic viability and differentiation (12). miR-328-3p has also been demonstrated to regulate the proliferation of different types of cell, such as colorectal cancer (13) and osteosarcoma cells (14). Weilner *et al* (15) investigated the differentially expressed circulating miRNAs that may be associated with osteoporotic fracture, the results of which revealed that miR-328-3p was associated with osteogenic differentiation, and was downregulated in patients with osteoporosis who developed fractures. However, whether miR-328-3p is involved in fracture healing processes remains unclear.

The present study assessed the expression of miR-328-3p during healing in patients with fragility fracture and the potential regulatory effect of miR-328-3p on osteoblastic

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viability. In addition, the target gene of miR-328-3p was also predicted and explored, which may provide a novel insight into the pathogenesis of fracture healing.

Materials and methods

Patients and sample collection. The present study included 80 patients with fragility fracture (34 males, 46 females; average age, 68.94±11.30 years; age range, 48-87 years), who underwent fixation therapy at the Traditional Chinese Medicine Hospital of Weifang from January 2016 to December 2018. All patients suffered from fragility fracture without trauma or due to low-energy trauma. Before fixation therapy, blood samples were collected from the patients within 24 h of the fracture. After fixation, blood samples were obtained on day 7, 14 and 21. In addition, the blood samples of 40 healthy volunteers were also collected during the same time period. All blood samples were immediately centrifuged at 1,500 × g for 10 min at 4°C for serum extraction and stored at -80°C for subsequent use. The healthy individuals included in the current study comprised 19 males and 21 females, with an average age of 68.10 ± 12.05 years (range of 46-86 years). All the participants signed written informed consent for clinical sample collection and analysis, and the experimental procedures were approved by the Ethics Committee of Traditional Chinese Medicine Hospital of Weifang.

Cell culture and transfection. A human osteoblast cell line hFOB1.19 was purchased from the Chinese Academy of Sciences Cell Bank. The cells were cultured in RPMI-1640 (HyClone; Cytiva) medium supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.), 1,000 U/ml penicillin and 100 µg/ml streptomycin in an incubator with 5% CO₂ at 37°C.

To overexpress miR-328-3p in hFOB1.19, 50 nM miR-328-3p mimic (5'-CUGGCCUCUCUGCCCUCCG U-3') was synthesized by Shanghai GenePharma Co., Ltd. and transfected into cells using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The negative control for the mimic (mimic NC; 50 nM; Shanghai GenePharma Co., Ltd.; 5'-UUCUCCGAA CGUGUCACGU-3') was used and transfected into hFOB1.19 to serve as a negative control group. Additionally, the overexpression vector of PTEN, pcDNA3.1-PTEN (Shanghai GenePharma Co., Ltd.), was synthesized and transfected into hFOB1.19 to overexpress PTEN in osteoblasts. The cells transfected with only transfection reagent were set as the mock group. Cell transfection was performed at 37°C for 6 h, the transfection reagent was removed and fresh RPMI-1640 medium was added. After 48 h of cell transfection, cells were used for subsequent experimentation. All experiments were repeated in triplicate.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from serum and cell samples, and the obtained RNA was reversed transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. The expression of miR-328-3p and the mRNA expression of

PTEN was quantified via qPCR, which was performed using SYBR-Green I Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.) on a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were set as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 20 sec, 58°C for 10 sec and 72°C for 20 sec. The expression levels of miR-328-3p and PTEN were normalized to U6 and GAPDH, respectively, and calculated using the 2^{-ΔΔC_q} method (16). The following primer sequences were used in the reactions: miR-328-3p forward, 5'-GCCGAG CTGGCCCTCUCTGC-3' and reverse, 5'-CTCAACTGGTGT CGTGGGA-3'; PTEN forward, 5'-TCCAGACATGACAG CCATC-3' and reverse, 5'-TGCTTTGAATCCAAAAC CTTACT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH forward, 5'-CAATGACCCCTTCATTGACC-3' and reverse, 5'-TTGATTTTGGAGGGATCTCG-3'.

Cell proliferation assay. After 48 h of cell transfection, the proliferation of hFOB1.19 was estimated using an MTT assay. Cells (3×10³ cells/well) were seeded into 96-well plates and cultured at 37°C. The proliferation of cells at 0, 24, 48 and 72 h was measured by incubating cells with 5 mg/ml MTT solution (Sigma-Aldrich; Merck KGaA) for 4 h at 37°C. Subsequently, 150 µl DMSO (Sigma-Aldrich; Merck KGaA) was added into the wells to dissolve formazan for 30 min at 37°C. The optical density of samples at 490 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc.) to reflect cell proliferation.

Apoptosis assay. The apoptosis of hFOB1.19 was evaluated using an Annexin V-FITC Apoptosis Detection kit (Nanjing KeyGen Biotech Co., Ltd.). Cells (1×10⁵) were harvested at 48 h post-transcription and washed with PBS three times. After suspending with Annexin V binding buffer, cells were stained with 5 µl V-FITC and 10 µl propidium iodide for 20 min in the dark at room temperature. Apoptosis was measured using a flow cytometer (Attune NxT; BD Biosciences) and analyzed using the CellQuest Pro analysis software (version 5.1; BD Biosciences). Apoptotic rate, including early and late apoptosis, was calculated.

Bioinformatics prediction and luciferase reporter assay. The targets of miR-328-3p were predicted using TargetScan (http://www.targetscan.org/vert_72/), the results of which predicted that PTEN was a potential target gene of miR-328-3p. The wild-type (WT) and mutant type (MUT) 3'-UTR sequences of PTEN with a concentration of 100 nM were synthesized by Shanghai GenePharma Co., Ltd., and were combined into the luciferase reporter vector PMIR-RB-REPORT (Guangzhou RiboBio Co., Ltd.) to perform the luciferase reporter assay. The recombinant vectors were co-transfected with miR-328-3p mimic or mimic NC using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of transfection, the relative luciferase activity was detected using a Luciferase Reporter System (Promega Corporation) and normalized to *Renilla* luciferase activity.

Western blotting. Total protein was extracted from hFOB1.19 cells using RIPA buffer (Beyotime Institute of Biotechnology), and the concentration of proteins was determined using a

Table I. Baseline characteristics of the participants in this study.

Variables	Healthy controls (n=40)	Patients with a fracture (n=80)	P-value
Age, years	68.10±12.05	68.94±11.30	0.682
Sex			0.603
Female, n (%)	21 (52.5)	46 (57.5)	
Male, n	19 (47.5)	34 (42.5)	
BMI (kg/m ²)	23.03±2.81	22.04±2.74	0.068
25OHD (ng/ml)	30.56±7.74	25.91±6.64	0.002 ^a
Osteoporosis, n (%)	16 (40.0)	63 (78.8)	<0.001 ^a
Fracture sites, n (%)			-
Vertebrae	-	38 (47.5)	
Hip	-	22 (27.5)	
Wrist	-	20 (25.0)	

^aP<0.05. Data are presented as the mean ± SD unless otherwise stated. BMI, body mass index; 25OHD, 25-hydroxyvitamin D.

bicinchoninic acid kit (Beyotime Institute of Biotechnology). Protein were separated by 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore). After blocking with 5% skimmed milk at 37°C for 1 h, membranes were incubated with the following primary antibodies at 4°C overnight: Anti-AKT (1:1,000; cat. no. 4691), anti-phosphorylated (p)-AKT (1:2,000; cat. no. 4060), anti-PTEN (1:1,000; cat. no. 9188) and anti-GAPDH (1:1,000; cat. no. 5174; all purchased from Cell Signaling Technology, Inc.). Membranes were then incubated with horseradish peroxide-labelled anti-rabbit IgG secondary antibodies (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc.). The protein bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) and GAPDH was used as a loading control. Protein bands were quantified using ImageJ software (1.46; National Institutes of Health).

Statistical analysis. The data analyzed in the present study were presented as the mean ± SD. Each experiment was repeated at least three times. Differences between two groups were analyzed using unpaired Student's t-test or χ^2 test, and comparison between multiple groups was assessed using one-way ANOVA followed by Tukey's post hoc test. The Pearson correlation coefficient test was used to evaluate correlation. SPSS 22.0 (IBM Corp.) and GraphPad Prism 7.0 software (GraphPad Software, Inc.) were used to perform analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

Baseline characteristics of the participants. The age, sex, body mass index (BMI) and 25-hydroxyvitamin D (25OHD) of patients with a fracture and healthy controls were listed and compared in Table I. The 80 patients with a fracture included 38 (47.5%) vertebral fracture cases, 22 (27.5%) hip fracture cases and 20 (25.0%) wrist fracture cases. No significant differences were observed between the age, sex and BMI of the two groups (P>0.05). Patients with a fracture had significantly lower 25OHD levels and higher incidence rates of osteoporosis compared with healthy controls (P<0.01).

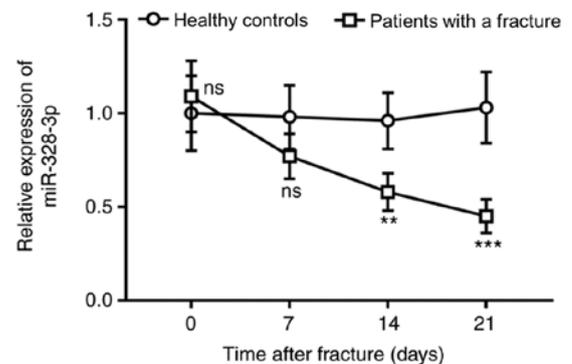


Figure 1. Dynamic serum expression of miR-328-3p in patients with fracture. **P<0.01 and ***P<0.001 vs. healthy controls. ns, not significant; miR, microRNA.

Dynamic expression patterns of miR-328-3p in patients with fracture. The serum expression levels of miR-328-3p were slightly higher in patients with fracture compared with those of healthy controls ≤24 h post fracture and before fixation therapy (P>0.05; Fig. 1). After fixation, no significant differences were observed in the serum expression levels of miR-328-3p between the patients with a fracture and healthy controls for 7 days (P>0.05). After that, miR-328-3p expression levels gradually decreased and were significantly downregulated on day 14 and 21 in patients with fracture compared with healthy individuals (P<0.01; Fig. 1). The results suggested that miR-328-3p may be involved in the progression of fracture healing.

Effects of miR-328-3p on osteoblastic proliferation and apoptosis. To understand the mechanisms underlying the aberrant expression of miR-328-3p during the healing process of patients with fracture, the effect of miR-328-3p on osteoblastic viability was investigated. The expression levels of miR-328-3p in hFOB1.19 were successfully overexpressed by the miR-328-3p mimic (P<0.001; Fig. 2A). The proliferation of osteoblasts was significantly increased by the overexpression of miR-328-3p (P<0.05; Fig. 2B). Additionally,

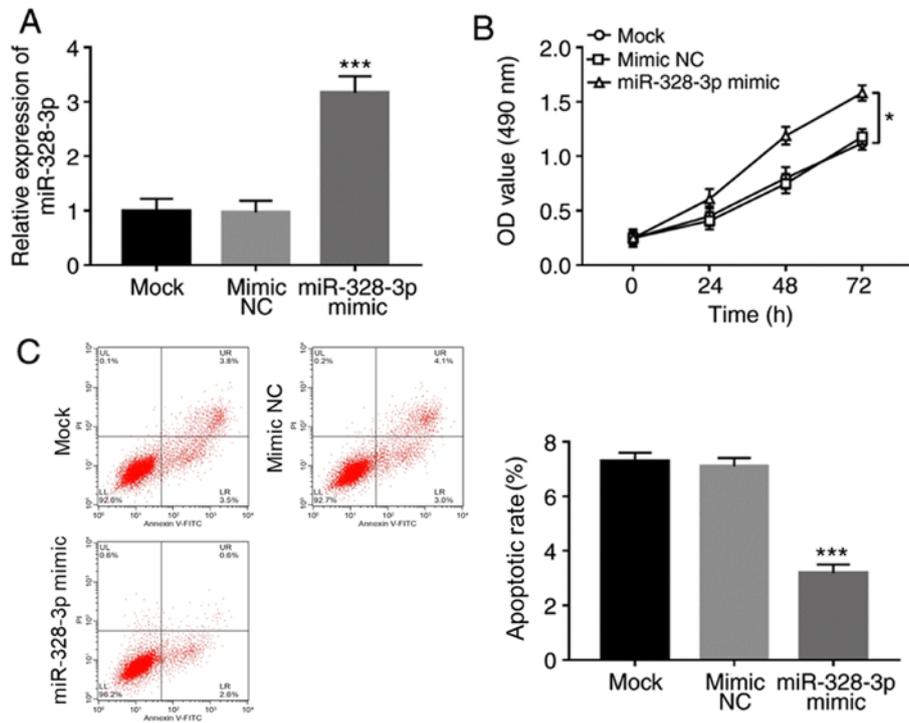


Figure 2. Effect of miR-328-3p on osteoblastic proliferation and apoptosis of hFOB1.19. (A) Expression of miR-328-3p was overexpressed by miR-328-3p mimic. (B) Overexpression of miR-328-3p led to enhanced osteoblastic proliferation. (C) The apoptosis of hFOB1.19 cells was inhibited by the overexpression of miR-328-3p. *P<0.05 and ***P<0.001 vs. mimic NC. miR, microRNA; OD, optical density; NC, negative control.

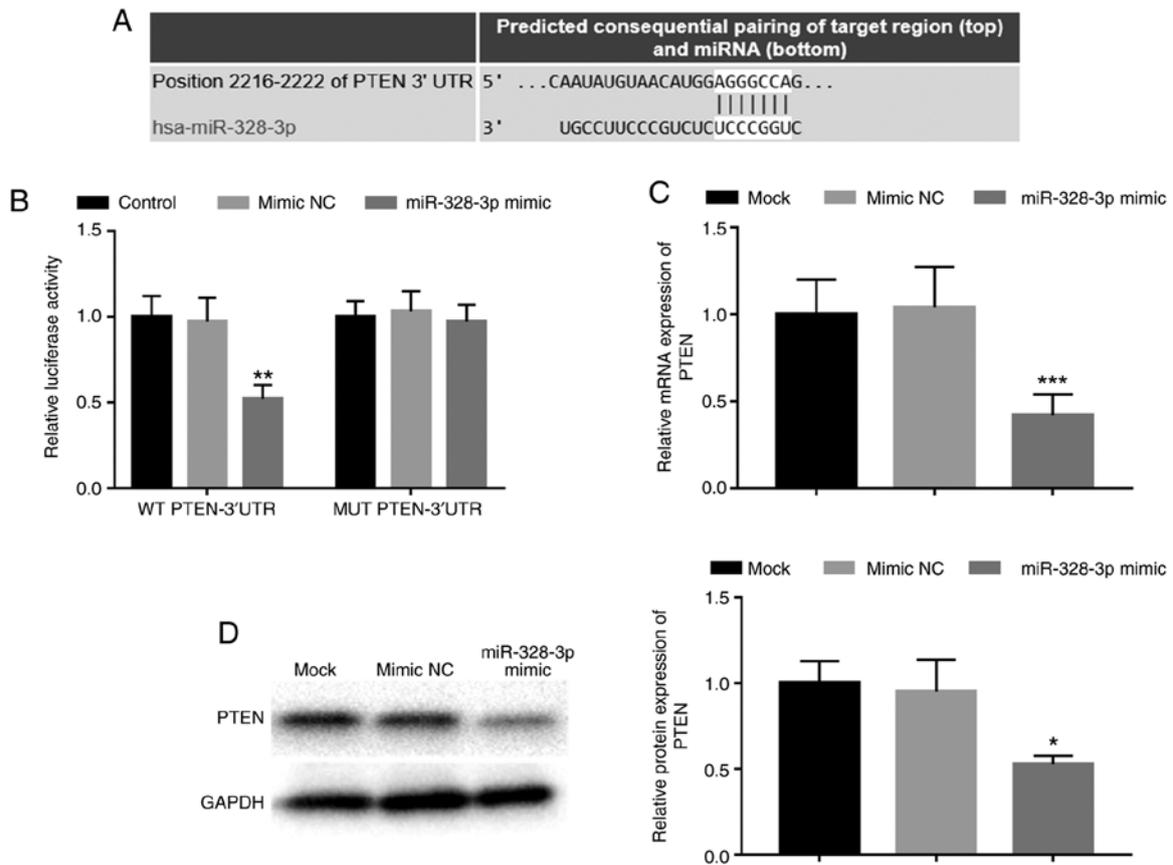


Figure 3. miR-328-3p directly inhibits the expression of PTEN in osteoblasts. (A) The putative binding site of miR-328-3p at the 3'-UTR of PTEN. (B) The relative luciferase activity was inhibited by the overexpression of miR-328-3p in WT PTEN-3'UTR group. (C) The mRNA expression of PTEN was significantly reduced in hFOB1.19 cells with miR-328-3p overexpression. (D) The protein expression of PTEN was also decreased by miR-328-3p overexpression in hFOB1.19 cells. *P<0.05, **P<0.01 and ***P<0.001 vs. mimic NC. miR, microRNA; UTR, untranslated region; WT, wild-type; MUT, mutated; NC, negative control.

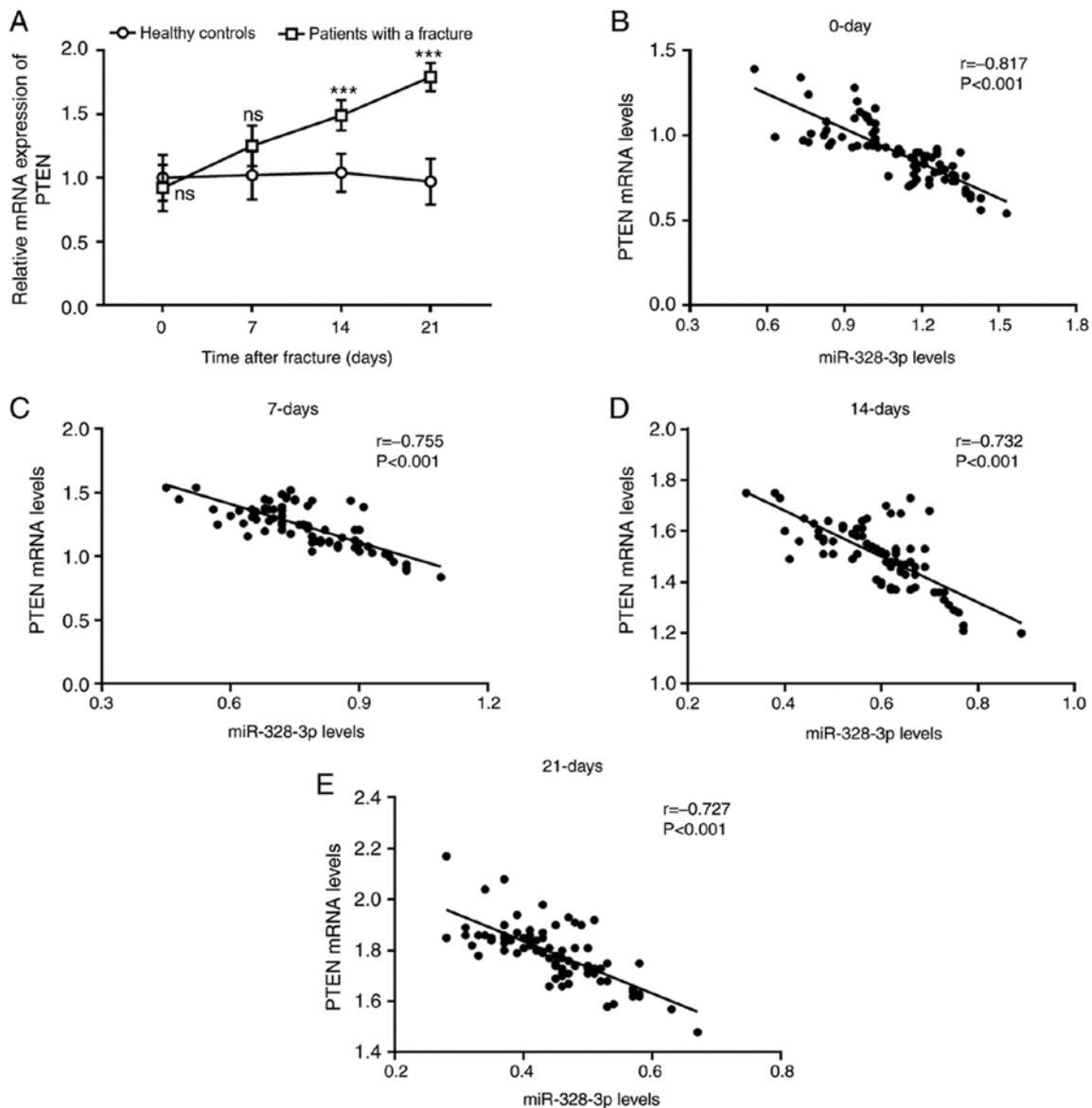


Figure 4. Correlation of miR-328-3p serum levels and PTEN in patients with fracture. (A) Dynamic expression of PTEN in patients with fracture. Serum miR-328-3p expression levels were negatively correlated with PTEN expression in patients (B) before fixation (0) and (C) 7, (D) 14 and (E) 21 days post fixation therapy. *** $P < 0.001$ vs. healthy controls. miR, microRNA; ns, not significant.

apoptotic rate was significantly reduced in cells transfected with miR-328-3p mimic compared with cells transfected with mimic NC ($P < 0.001$; Fig. 2C).

miR-328-3p directly regulates PTEN in osteoblasts. Bioinformatics prediction revealed a putative binding site of miR-328-3p at the 3'-UTR of PTEN (Fig. 3A). Thus, a luciferase reporter assay was performed to confirm the relationship between miR-328-3p and PTEN. The results demonstrated that the relative luciferase activity in the WT PTEN-3'UTR group was significantly inhibited by the overexpression of miR-328-3p when compared with cells co-transfected with mimic NC ($P < 0.01$; Fig. 3B), whereas no significant differences were observed in the MUT PTEN-3'UTR group ($P > 0.05$; Fig. 3B). Additionally, the regulatory effect of miR-328-3p on PTEN in osteoblasts was examined. The results demonstrated that overexpression of miR-328-3p expression significantly decreased the mRNA and protein expression levels of PTEN

compared with those of the mimic NC group in hFOB1.19 cells ($P < 0.05$; Fig. 3B and C).

Correlation between miR-328-3p and PTEN in patients with fracture. The mRNA expression levels of PTEN in patients with fracture were evaluated. No significant differences were observed in the expression levels of PTEN between patients with fracture and healthy controls before fixation therapy and within 7 days post therapy ($P > 0.05$). However, the expression levels of PTEN were significantly increased on days 14 and 21 day in patients with fracture compared with healthy controls ($P < 0.001$; Fig. 4A). Additionally, the correlation of serum miR-328-3p and PTEN levels was analyzed; at each time point, a negative correlation was observed between serum levels of miR-328-3p and PTEN ($P < 0.001$; Fig. 4B-E).

PTEN mediates the regulatory effects of miR-328-3p on osteoblastic viability. PTEN is known to serve pivotal roles in the

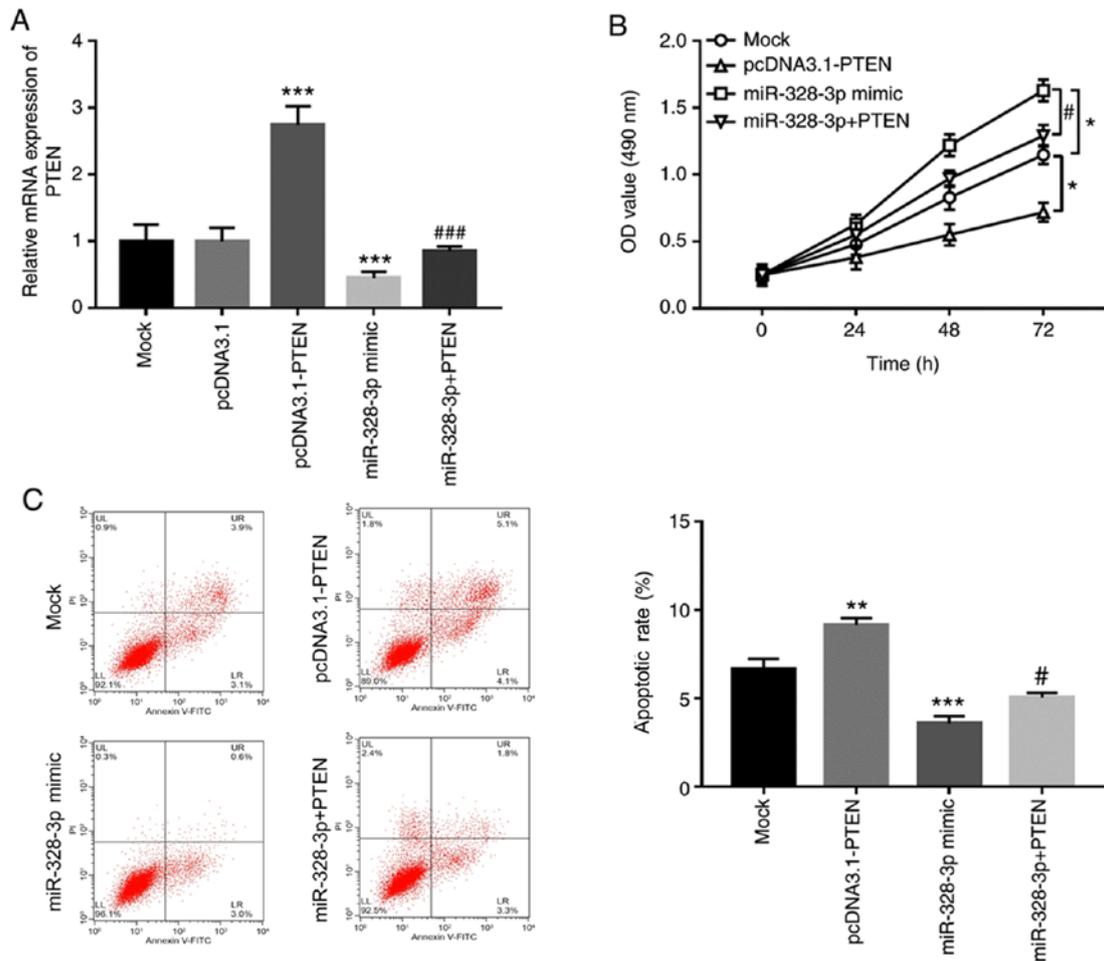


Figure 5. miR-328-3p regulates osteoblastic viability by targeting PTEN. (A) pcDNA3.1-PTEN-induced overexpression of PTEN in hFOB1.19 cells and reversed the effect of miR-328-3p overexpression on PTEN expression. (B) PTEN overexpression inhibited osteoblastic proliferation as well as the miR-328-3p mimic-induced increased cell proliferation. (C) Osteoblastic apoptosis was increased in cells with PTEN reduction and the inhibited cell apoptosis caused by miR-328-3p overexpression was abolished by the overexpression of PTEN. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. mock; # $P < 0.05$ and ### $P < 0.05$ vs. miR-328-3p mimic. miR, microRNA.

regulation of a cell's biological function (17), and the inhibition of PTEN has been reported to promote long non-coding RNA GHET1 on osteoblast proliferation and differentiation (18). Thus, the effect of miR-328-3p on osteoblastic viability may be mediated by PTEN. pcDNA3.1-PTEN was used to overexpress PTEN in hFOB1.19 cells (Fig. 5A). Overexpression of PTEN significantly inhibited osteoblastic proliferation, but significantly increased osteoblastic apoptosis ($P < 0.05$; Fig. 5B and C). Additionally, in cells co-transfected with miR-328-3p mimic and pcDNA3.1-PTEN, the expression levels of PTEN were significantly reduced compared with those of cells transfected with pcDNA3.1-PTEN alone ($P < 0.001$; Fig. 5A). Notably, overexpression of PTEN by pcDNA3.1-PTEN significantly reversed the effect of miR-328-3p on osteoblastic viability, which was demonstrated by the inhibited cell proliferation and enhanced apoptosis in hFOB1.19 ($P < 0.05$; Fig. 5B and C).

Regulatory effect of miR-328-3p on the PI3K/AKT signaling pathway. PTEN is a negative regulator of the PI3K/AKT signaling pathway, which is one of the most important pathways during the processes of cell proliferation and apoptosis (19). Thus, the present study further explored the

effect of miR-328-3p on the activity of PI3K/AKT signaling. The results demonstrated that the ratio of p-AKT/AKT was significantly increased in hFOB1.19 cells with overexpression of miR-328-3p when compared with the mock group ($P < 0.001$; Fig. 6), which suggested that the activity of the AKT signaling pathway was enhanced by the overexpression of miR-328-3p. Additionally, in the cells overexpressing PTEN, the increase in p-AKT/AKT ratio induced by miR-328-3p overexpression was significantly attenuated ($P < 0.01$; Fig. 6), suggesting that miR-328-3p may activate the PI3K/AKT signaling by inhibiting PTEN.

Discussion

Bone regeneration is the key to fracture healing, which involves the proliferation, apoptosis and differentiation of osteoblasts (20), implying that methods that regulate osteoblastic activity may assist fracture healing. miRNAs have important regulatory effects on various cellular processes, such as cell proliferation, migration, invasion, differentiation and apoptosis (21). Emerging studies have focused on deregulated miRNAs during fracture healing, which are expected

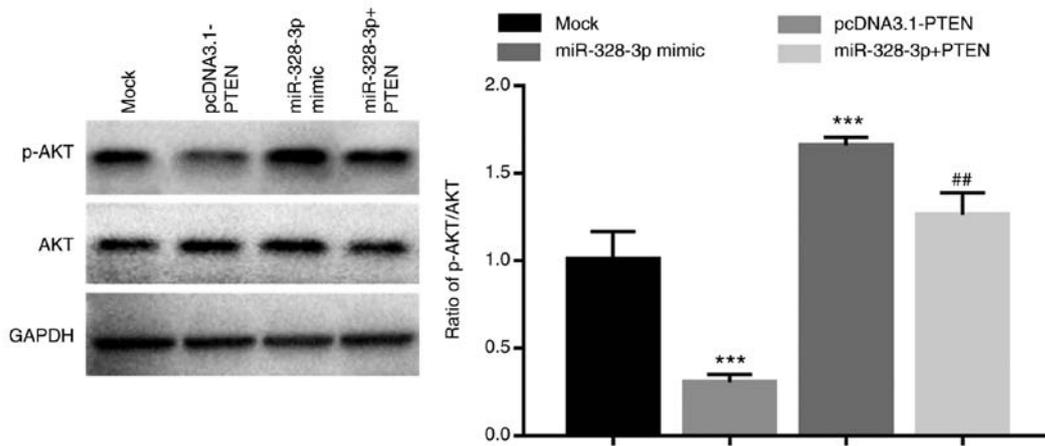


Figure 6. Effect of miR-328-3p and PTEN on the activation of PI3K/AKT signaling pathway. The ratio of p-AKT/AKT was enhanced by the overexpression of miR-328-3p. ***P<0.001 vs. mock; ##P<0.01 vs. miR-328-3p mimic. miR, microRNA; p, phosphorylated.

to promote the development of novel strategies to accelerate healing by improving osteoblastic viability. For example, Lang *et al* (22) provided evidence for the use of miR-25 to promote fracture healing by enhancing osteoblastic proliferation and differentiation through activation of the Wnt signaling pathway. Mi *et al* (23) demonstrated that decreased miR-7223-5p during fracture healing regulated osteoblastic proliferation and apoptosis by targeting PI3KR1, mediating the promoting effect of circRNA AFF4 on fracture healing. The expression of miR-203 in patients with fractures was also aberrant during healing and could inhibit the proliferation and promote the apoptosis of osteoblast cells by targeting prostate and breast cancer overexpressed 1 (24). These data indicate the important roles of miRNAs, which have regulatory effects on osteoblastic viability in fracture healing.

A previous study on differentially expressed miRNAs in patients with osteoporosis reported that patients with osteoporosis who develop fractures had significantly decreased miR-328-3p levels (15), suggesting that miR-328-3p may be associated with bone regeneration and progression of osteoporosis. There is some evidence that miR-328-3p regulates cell proliferation and apoptosis; the decreased expression of miR-328-3p in patients with osteosarcoma has been reported to be involved in tumor progression by regulating osteosarcoma cell proliferation, migration and apoptosis (25). The expression of miR-328-3p in hepatocellular carcinoma cells was also demonstrated to be reduced and could decrease tumor cell proliferation and increase apoptosis (26). However, the relationship between miR-328-3p and osteoblastic viability has rarely been reported. In the present study, the serum expression levels of miR-328-3p was demonstrated to be slightly higher in patients with fracture before fixation treatment and exhibited no significant changes 7 days post fixation. However, its expression was markedly decreased 14 and 21 days after fixation. The abnormal expression patterns of miR-328-3p suggested its role during fracture healing. Osteoporosis is the most frequent underlying cause of fragility fracture (27). A total of 78.8% of the patients with fracture enrolled in the current study had osteoporosis, and the majority of female patients were postmenopausal women, who have high risk of osteoporosis (28). Circulating miR-328-3p has been

identified as a miRNA associated with osteoblast differentiation in osteoporosis (29), which also indicated the crucial role of miR-328-3p in osteoporosis pathogenesis and progression. The present study demonstrated that the overexpression of miR-328-3p markedly increased osteoblastic proliferation but decreased cell apoptosis, indicating the important regulatory effect of miR-328-3p on osteoblastic viability. Considering the strong demand for enough osteoblasts for fracture healing, the present study hypothesized that the slight increase in miR-328-3p at an early time of fracture is required to obtain a sufficient number of osteoblasts. After the accumulation of osteoblasts, the expression of miR-328-3p was downregulated, the proliferation of osteoblasts was decreased and the inhibition of apoptosis was attenuated. At the later stage of fracture healing, miR-328-3p expression was significantly reduced, leading to the osteoblasts proliferating slowly but further differentiating into mature bone cells and chondrocytes, thereby achieving bone regeneration. Therefore, the methods to increase miR-328-3p may reduce the time spent in the early stage of fracture healing by promoting osteoblastic viability.

According to bioinformatics prediction, PTEN was identified as a target gene of miR-328-3p, and its interaction with miR-328-3p was demonstrated using a luciferase reporter assay. PTEN is a key molecule involved in various cellular processes (30,31). For example, PTEN acted as a target gene of miR-29a-3p and could mediate the regulatory effects of miR-29a-3p on vascular smooth muscle cell proliferation and apoptosis (30). Another example demonstrated that the regulatory role of methyltransferase-like protein 3 (METTL3) regulated retinal pigment epithelium cell proliferation, apoptosis and pyroptosis by inhibiting PTEN (31). In addition, Ge *et al* (32) reported that PTEN inhibited fracture healing by suppressing osteogenic differentiation of mesenchymal stem cells. Mice with PTEN knockdown in osteoblasts demonstrated significantly improved fracture healing (33). The present study demonstrated lower PTEN expression levels at the early stage of fracture, but its expression was significantly increased 14 days post fixation, which was negatively correlated with serum miR-328-3p levels in patients with fracture. The results suggested that the inhibition of PTEN was required for the rapid proliferation of osteoblasts for fracture healing.

Furthermore, the overexpression of miR-328-3p inhibited PTEN expression in osteoblasts, and PTEN overexpression reversed the effect of miR-328-3p on osteoblastic proliferation and apoptosis, which suggested that miR-328-3p may promote osteoblastic viability by targeting PTEN.

The PI3K/AKT pathway is a well-known signaling pathway that is important in regulating cell proliferation, cell cycle progression and apoptosis, and its beneficial role in fracture healing has been well reported (34,35). PTEN acts as a major inhibitor of this signaling pathway, thereby participating in various cellular processes (36). In the osteoblasts overexpressing miR-328-3p, the expression of p-AKT was elevated, suggesting that the overexpression of miR-328-3p promoted the activation of the PI3K/AKT signaling pathway. Furthermore, elevated p-AKT induced by miR-328-3p was abolished by the overexpression of PTEN, which implied that the regulatory effect of miR-328-3p on the PI3K/AKT signaling may be mediated by PTEN. Collectively, miR-328-3p may facilitate osteoblastic viability by activating the PI3K/AKT signaling pathway via PTEN.

The present study may provide novel insights into the pathogenesis of fracture healing, and the methods to increase miR-328-3p expression may be used to shorten healing time in patients with fracture. However, the present study lacks animal experiments, and further *in vivo* studies are required. Another limitation of the present study is that the osteoblastic viability under the inhibition of miR-328-3p was not assessed. Thus, future studies should include *in vitro* functional experiments using both miR-328-3p mimics and inhibitors. In addition, fracture healing involves complex biological processes and although the present study demonstrated the regulatory effect of miR-328-3p on osteoblastic viability, further studies are necessary to confirm the role of miR-328-3p in fracture healing and the underlying mechanisms by considering other aspects that are associated with bone repair, such as osteoblast differentiation, bone matrix protein production and mineralization. In conclusion, the present study suggested that the aberrant expression of miR-328-3p in patients with a fracture may promote fracture healing by accelerating osteoblastic viability through the PTEN/PI3K/AKT pathway.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WX and ZZ contributed to the study design, the data collection, data interpretation and manuscript preparation. ZW and YZ contributed to data collection, data analysis and statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Traditional Chinese Medicine Hospital of Weifang and written informed consent was obtained from each patient.

Patient consent for publication

Written informed consent for publication was obtained from each participant.

Competing interests

The authors declare that they have no competing interests.

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