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Original Article

MicroRNA-28-3p promotes fracture healing through inhibition of Sox6 and activation of PI3K/AKT pathway

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Abstract: Currently, microRNAs (miRNAs) participate in various biological processes. This study intends to investigate the function of miR-28-3p in fracture healing. The blood of fracture patients were obtained within 24 h after trauma as well as 7, 14, 21 days after therapy. The miR-28-3p expression in fracture and healthy human plasma was estimated by quantitative reverse transcription PCR (qRT-PCR). Then, MC3T3-E1 cells were transfected with miR-28-3p mimics, antisense oligonucleotides miR-28-3p (ASO-miR-28-3p), negative controls (mimics NC and ASO NC) or pcDNA3.1 containing Sox6 gene, respectively. Predicted by TargetScan, the target gene of miR-28-3p was verified by luciferase assay. Proliferation and apoptosis of transfected cells were respectively measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. Simultaneously, mRNA levels of osteogenesis-associated genes were calculated by qRT-PCR. Finally, the phosphorylation levels of key kinases in the phosphatidylinositol-3-kinase (PI3K)/AKT pathway were detected by Western blot analysis. The results showed the miR-28-3p expression was down-regulated at 14 d and 21 d after fracture. The cell viability and Col1a1 expression were both improved by miR-28-3p overexpression while the cell apoptosis and gene expressions (Col-II and Col-X) were suppressed. The effect of miR-28-3p mimics on cell viability, apoptosis and expressions of osteogenesis-associated genes were reversed by Sox6. Thus, Sox6 was verified as a target gene of miR-28-3p and was negatively regulated by miR-28-3p. The PI3K/AKT signaling pathway was activated by miR-28-3p mimics. In conclusion, miR-28-3p promoted fracture healing by targeting Sox6 and activation of the PI3K/AKT pathway.

Keywords: MicroRNA-28-3p, proliferation, apoptosis, Sox6, PI3K/AKT

Introduction

Fragility fractures present as one of the major public health problems worldwide, which deeply affect the societal health and economics [1, 2]. In general, factures result in acute pain and nearly all the patients suffering this disease need to be taken into hospital and receive immobilization with long-time and slow recovery [3-5]. The healing process of fracture is well-orchestrated by multiple growth factors and cytokines which consequently control the activation and cell proliferation [6]. For successful healing of fracture, there is a diamond concept consisting of four conditions illustrated as appropriate mechanical environment, osteogenic cells, osteoconductive scaffolds and growth factors which effectively induce osteogenesis [7]. Even though there are plenty of strategies to accelerate the bone-regeneration process, therapeutic limitations still exist, resulting in reduced effectiveness and availability of bone-regeneration [8]. Novel therapeutic target needs to be investigated to overcome the limitations.

As a kind of small noncoding RNAs, microRNAs (miRNA) are reported to play critical roles in multiple biological processes through mediation of mRNA cleavage, translational repression and/or degradation of mRNA at post-transcriptional level. Until 2016, there are 2588 mature human miRNAs in Release 21 of miRBase (http://www.mirbase.org) [9]. A large numbers of miRNAs are related to formation of bone. Previous study implied that intravenous administration of double-stranded miR-146a markedly suppressed the destruction of cartilage and bone [10]. Other studies claimed that miR-17-92 cluster participated in the regulation

of osteoblast proliferation and differentiation [11]. Moreover, Wang *et al.* provided evidence that reduced expressions of miR-9 and miR-181a played positive roles in promoting osteoclasts survival [12]. Murata *et al.* also demonstrated that suppression of miR-92a significantly promoted angiogenesis and thereby accelerated the fracture healing in young mice [13]. In view of these findings, miRNAs might play pivotal roles in fracture healing.

Fracture healing is a complex process which recruits an adequate number of osteoblasts [14], thus the proliferation and apoptosis of osteoblasts might greatly affect the process of fracture healing. miR-28 has been reported to negatively regulate expression of Nrf2, whose activation could inhibit cell growth and arrest cell cycle [15]. Therefore, miR-28 might possess promoting effects on proliferation of osteoblasts, which in turns accelerates fracture healing. On the basis of the evidence currently available, miR-28-5p is proved to suppress proliferation of human hepatocellular carcinoma and renal cell carcinoma [16, 17]. Considering the limited literature, the role of miR-28-3p has not been well studied. As a consequent, we focused our interest on the expression of miR-28-3p in the process of fracture healing and, for the first time, explored the function of miR-28-3p in fracture healing. The relevant signaling pathways were also investigated.

Materials and methods

Preparation of blood samples

Plasma samples of patients with fracture were obtained within 24 h after the trauma as well as 7, 14, 21 days after the standardized fixation treatment. Meantime, the blood of healthy people was also collected to act as control. After collection with EDTA-2K-containing tubes, the blood samples were immediately centrifuged at 1400 × *g* for 7 minutes in order to separate plasma. The plasma samples were stored at -80°C. All the operations were performed after receiving signatures of the informed consents and approved by our local ethical committee.

Cell culture

Mouse osteoblastic cell line MC3T3-E1 was purchased from the Centre for Cell Culture (Chinese Academy of Science, Shanghai, China). With a density of 1×10^5 cells/cm^2, the cells were incubated in α-modified minimal essential medium (α-MEM, Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), and 100 U/mL penicillin/streptomycin (Sigma-Aldrich, Gillingham, UK). The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C (medium was changed every 2-3 days).

Complete cDNAs of the mouse Sox6 gene was cloned into pcDNA3.1/Zeo vector (Invitrogen) to generated pcDNA3.1/Sox6 (pc-Sox6) for overexpression of Sox6. Cells for further experiments were divided into three or four groups by transfected with different miRNA mimics, antisense oligonucleotides (ASO) or pc-Sox6. All the nucleotides were purchased from Genepharma (Shanghai, China) and transfected into cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the instructions provided by the manufacturer. In addition, PI3K inhibitor, LY294002 (20 μM, Selleck, Shanghai, China), was added to the cells.

Cell viability assay

The cell viability was performed using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) in line with the method described previously [18]. In brief, cells were seeded in 96-well plates with a density of 1.5×10^3 cells/well. After 1 d, 2 d and 3 d of incubation, 10 μL of MTT solution (5 mg/mL) was added into each well and then the mixture was maintained at 37°C for 4 h. After that, 150 μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to dissolve formazan. The absorbance at 570 nm was measured by a microplate reader (Bio-rad, Hercules, CA, USA).

Cell apoptosis assay

Annexin V-FITC/PI apoptosis detection kit (Biosea Biotechnology, Beijing, China) was employed to measure the cell apoptosis. According to the manufacture's instruction, the harvested MC3T3-E1 cells were firstly rinsed by cold phosphate-buffered saline (PBS) and resuspended in binding buffer with a density of 1×10^6 cells/mL. Subsequently, the cells were stained in order with 10 μL of Annexin V-FITC and 5 μL propidium iodide (PI). Finally, the stained cells were analyzed by flow cytometer

Table 1. Primers used in this study

Gene	Primer sequence
Col-II	Forward 5'-CTCCCAGAACATCACCTACC-3'
	Reverse 5'-TCGTGCAGCCATCCTTCAGG-3'
Col-X	Forward 5'-AGGCTACCTGGATCAGGCTTC-3'
	Reverse 5'-ACATTACTTTTAGCCTACCTCC-3'
Col1a1	Forward 5'-GCTCCTCTTAGGGGCCACT-3'
	Reverse 5'-CCACGTCTCACCATTGGGG-3'
Sox6	Forward 5'-TTGGGGAGTACAAGCAACTGATGC-3'
	Reverse 5'-ATCTGAGGTGATGGTGTGGTCGTT-3'
GAPDH	Forward 5'-AGGTCGGTGTGAACGGATTTG-3'
	Reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3'

(Beckman Coulter, Miami, FL, USA) to estimate the percentage of apoptotic cells.

Luciferase assay

TargetScan algorithm was employed to predict the target gene for miR-28-3p. Wild 3'-untranslated region (3'UTR) fragment containing putative binding site for miR-28-3p and mutant 3'UTR with site-mutagenesis at binding site were respectively inserted into pmirGLO vector (Promega, Fitchburg, WI, USA) between Nhe I and Sal I restriction sites. Directed Mutagenesis System (Invitrogen) was employed to construct mutant, resulting in three point mutations: ACC CTA A (TtoA) C (TtoA) A (GtoC) TTG TCA T. After sequencing, the vectors containing luciferase reporter gene and 3'UTR fragment or mutant were transfected into MC3T3-E1 cells along with miR-28-3p mimics, negative control of miR-28-3p (mimics NC), ASO-miR-28-3p or negative control of ASO-miR-28-3p (ASO NC) (Genepharma, Shanghai, China) utilizing X-tremeGENE siRNA Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA). Together, the plasmid pRL-TK (Promega) containing renilla gene was also transfected into the MC3T3-E1 cells acting as control. Forty-eight hours after transfection, the luciferase activity was assayed using Dual-Luciferase Reporter Assay System (Promega) in line with the manufacturer's protocol.

Quantitative reverse transcription PCR (qRT-PCR)

The mRNA expression levels were detected by qRT-PCR. Total RNA was isolated by Trizol (Invitrogen) in line with the manufacture's protocol. The cDNA was obtained by reverse transcription utilizing GoScript™ Reverse Transcription System (Promega). The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, West Sussex, UK) was employed to conduct quantitative PCR. Primers were synthetized by Sangon Biotech Co., Ltd. (Shanghai, China). Primers for type I procollagen (Col1a1), collagen II (Col-II), collagen X (Col-X), Sex-determining region Y-box 6 (Sox6) and GAPDH were shown in **Table 1**. The expression levels of mRNAs were normalized to levels of GAPDH who acted as an housekeeping gene.

For the detection of human miRNAs, reverse transcription was performed using miScript II RT kit which was followed by quantitative RT-PCR utilizing miScript SYBR® Green PCR kit and miR specific (has-miR-28-3p) primers (all purchased from SABiosciences, Frederick, MD, USA). The expression levels of miRNAs were normalized to levels of U6 snRNA.

Western blot analysis

After being rinsed with pre-cold PBS, the cells were resuspended with RIPA lysis buffer (Beyotime, Shanghai, China). Then, the lysate was centrifuged at 13000 × *g* for 20 min at 4°C followed by measurement of protein concentration using bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking by Tris-buffered saline with Tween 20 (TBST) containing 5% skim milk (Nestlé, Shuangcheng, China), the membranes were incubated at 4°C with primary antibodies: anti-mammalian B cell lymphoma-2 (Bcl-2, ab32124), anti-Sox6 (ab30455), anti-Col-II (ab34712), anti-Col-X (ab58632) (all from Abcam, Cambridge, MA, UK); anti-Bcl-2-associated X protein (Bax, 147-96), anti-β-actin (8457), anti-phosphoinositide 3-kinase (PI3K, 4249), anti-phosphorylated PI3K (p-PI3K, 4228), anti-AKT (4691), anti-phosphorylated AKT (p-AKT, 4060) (all from Cell Signaling Technology, Danvers, MA, USA); anti-Col1a1 (PAB17205, Abnova, Taiwan, China). Then, the rinsed membranes were incubated in horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody at room temperature for 2 h. Finally, the specific bands in membranes were visualized utilizing EasyBlot enhanced chemiluminescence (ECL) kit (Sangon,

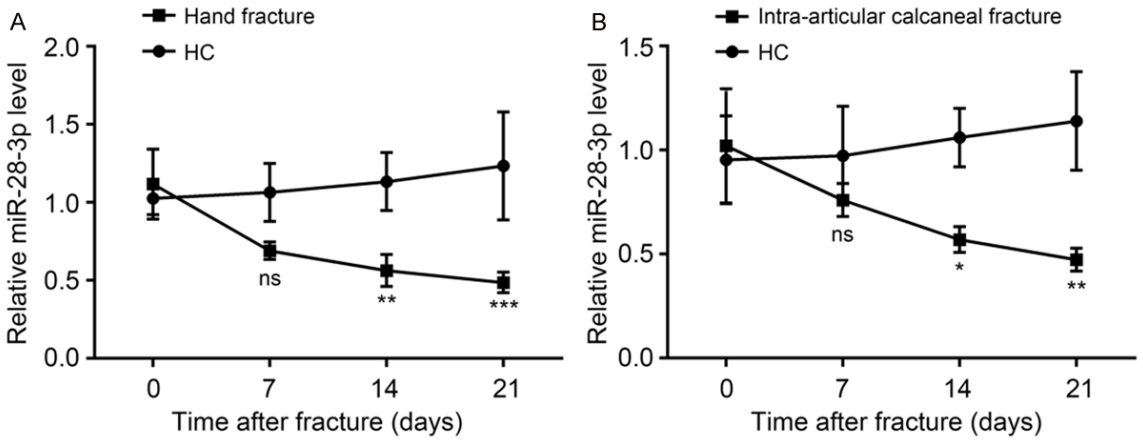

Figure 1. The relative miR-28-3p level in human plasma during healing process of hand fracture (A) and intra-articular calcaneal fracture (B) both accompanied by relative level in human plasma of HC. Data presented were the mean of ten independent experiments. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. ns, no significance; HC, healthy control.

Shanghai, China). The intensity analysis of bands was operated with Image Lab™ software (Bio-Rad). The internal control was β-actin.

Statistical analysis

All experiments were performed in triplicate. The results were presented as the mean ± standard deviation (SD). Statistical analysis was performed using Graphpad Prism 5 software (GraphPad, San Diego, CA, USA). The *P*-values were calculated using the two-way analysis of variance (ANOVA) or unpaired two-tailed *t* test. Multiple comparisons of two-way ANOVA were performed with Bonferroni correction. *P* values of < 0.05 were considered significant.

Results

The expression of miR-28-3p was down-regulated in serum after fracture

In order to explore the changes of miR-28-3p expression level in fracture-healing process, qRT-PCR was employed to estimate the relative levels in patients who suffered from fracture and in healthy controls (HCs). A total of 20 patients, 10 of which suffered a hand fracture (half males and half females, average age 24.9 ± 2.08 years), and the other 10 of which suffered intra-articular calcaneal fractures (half males and half females, average age 24.1 ± 2.69 years). Besides, blood was also obtained from 10 HCs (age-matched and sex-matched) who had never been treated for heart failure, renal failure, arthralgia or autoimmune disease. In comparison with miRNA levels in HC, the difference on expression levels at 7 d after trauma was not significant. With time went by, however, the expression levels in patients were significantly suppressed at 14 d and 21 d after hand fracture or intra-articular calcaneal fracture ($P < 0.05$, $P < 0.01$ or $P < 0.001$) (**Figure 1A** and **1B**). The results indicated that the expression of miR-28-3p was down-regulated in fracture-healing process.

The cell viability and Col1a1 expression were both enhanced while the cell apoptosis and gene expressions (Col-II and Col-X) were inhibited by miR-28-3p mimics

After transfection with mimics NC, miR-28-3p mimics, ASO NC or ASO-miR-28-3p, the cell viability, cell apoptosis and mRNA levels of osteogenesis-associated genes were analyzed. When compared to cells transfected with mimics NC, viabilities of cells transfected with miR-28-3p mimics were significantly enhanced at 1 d, 2 d and 3 d (**Figure 2A**, $P < 0.05$ or $P < 0.01$) while apoptosis of cells transfected with miR-28-3p mimics was markedly suppressed (**Figure 2B**, $P < 0.01$). As well, the pro-apoptotic Bax was down-regulated and the anti-apoptotic Bcl-2 was up-regulated in cells transfected with miR-28-3p mimics when compared with transfection of mimics NC (**Figure 2C**). As shown in **Figure 2D**, the Col1a1 expression was

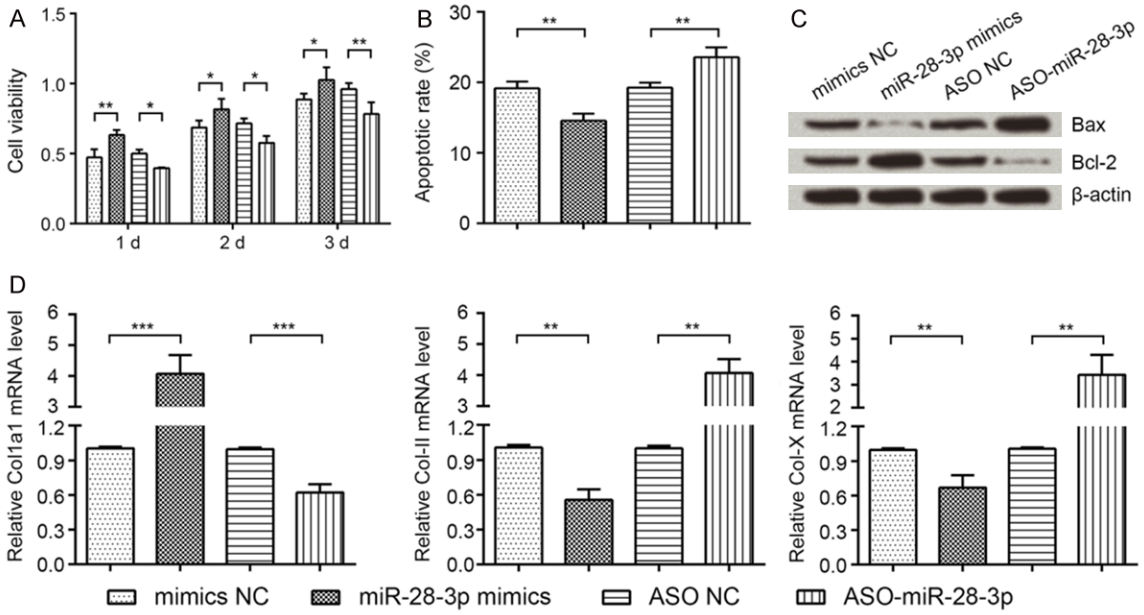

Figure 2. miR-28-3p improved proliferation and type I procollagen (Col1a1) expression while reduced apoptosis and expressions of collagen II (Col-II) and collagen X (Col-X). MC3T3-E1 cells were respectively transfected with miR-28-3p mimics, miR-28-3p mimics negative control (mimics NC), antisense oligonucleotides miR-28-3p (ASO-miR-28-3p) or ASO-miR-28-3p negative control (ASO NC). A. Cell viability after transfection; B. Apoptotic rate after transfection; C. Protein expression levels of apoptosis-associated proteins; D. Relative mRNA levels of osteogenesis-associated genes. Data presented were the mean of three independent experiments. Error bars indicated SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

remarkably increased while Col-II and Col-X expressions were observably decreased by transfection of miR-28-3p mimics when compared with transfection of mimics NC ($P < 0.01$ or $P < 0.001$). Furthermore, the effects of ASO-miR-28-3p on cell viability, cell apoptosis and expression levels of genes (Col1a1, Col-II and Col-X) exhibited just the opposite ($P < 0.05$, $P < 0.01$ or $P < 0.001$). All the results described above suggested that miR-28-3p enhanced cell viability and Col1a1 expression while inhibited cell apoptosis and gene expressions (Col-II and Col-X).

Sox6 was one of target genes of miR-28-3p

As predicted by TargetScan algorithm, miR-28-3p was partially complemented with Sox6 3'UTR as shown in **Figure 3A**. To test the hypothesis, luciferase reporter assay was employed to evaluated the ability of miR-28-3p in regulation of Sox6 3'UTR. In **Figure 3B**, miR-28-3p mimics markedly decreased the luciferase activity of wild type (WT) 3'UTR reporter but not 3'UTR mutant (Mut) reporter ($P < 0.01$). Meanwhile, ASO-miR-28-3p significantly increased the luciferase activity of WT 3'UTR reporter but not 3'UTR Mut reporter ($P < 0.01$) in **Figure 3C**. In **Figure 3D**, **3E**, the mRNA and protein expression levels of Sox6 were both reduced in cells with miR-28-3p mimics compared with cells with mimics NC ($P < 0.01$). While, the expression levels were both elevated in cells with ASO-miR-28-3p compared with cells with ASO NC ($P < 0.05$). All the results described above indicated that Sox6 was one of target genes of miR-28-3p and its expression was negatively regulated by miR-28-3p.

The effects of miR-28-3p on cell viability, apoptosis and expressions of osteogenesis-associated genes were inhibited by Sox6

miR-28-3p mimics was transfected into cells alone or accompanied by pc-Sox6 which was followed up with transfection of mimics NC acting as control. The cell viability, cell apoptosis and expression levels of osteogenesis-associated genes (Col1a1, Col-II and Col-X) were all determined in transfected cells. In **Figure 4A**, cell viabilities were remarkably increased in miR-28-3p mimics transfected cells at 1 d, 2 d and 3 d after transfection when compared with cells transfected with mimics NC ($P < 0.05$ or P

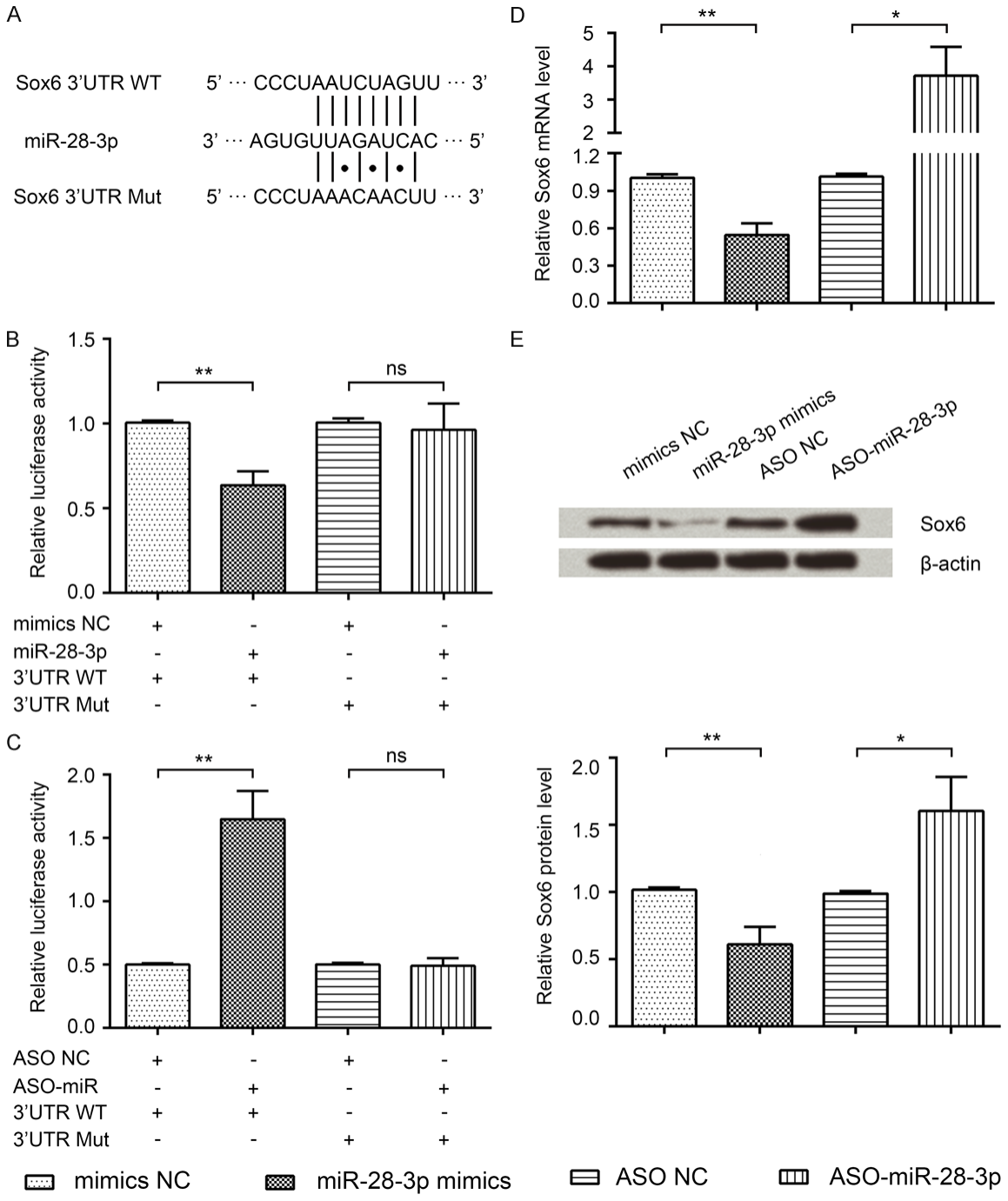

Figure 3. miR-28-3p post transcriptionally regulated Sox6 expression by targeting its 3'-untranslated region (3'UTR). (A) Sequence complementarity between wild miR-28-3p or mutant miR-28-3p and the seed region in the Sox6 3'UTR. Short vertical lines indicated complementary nucleotides while dark spot indicated uncomplimentary nucleotides; (B) Relative luciferase activity in MC3T3-E1 cells after co-transfection of miR-28-3p mimics or its negative control (mimics NC) with wild-type (WT) or mutant (Mut) Sox6 3'UTR-luciferase reporter vector; (C) Relative luciferase activity in MC3T3-E1 cells after co-transfection of antisense oligonucleotides miR-28-3p (ASO-miR-28-3p) or its negative control (ASO NC) with WT or Mut Sox6 3'UTR-luciferase reporter vector; Relative Sox6 mRNA (D) and protein (E) expression levels after transfection of mimics NC, miR-28-3p mimics, ASO NC or ASO-miR-28-3p. Data presented were the mean of three independent experiments. Error bars indicated SD. *, $P < 0.05$; **, $P < 0.01$; ns, $P > 0.05$; miR-28-3p, miR-28-3p mimics; ASO-miR; ASO-miR-28-3p.

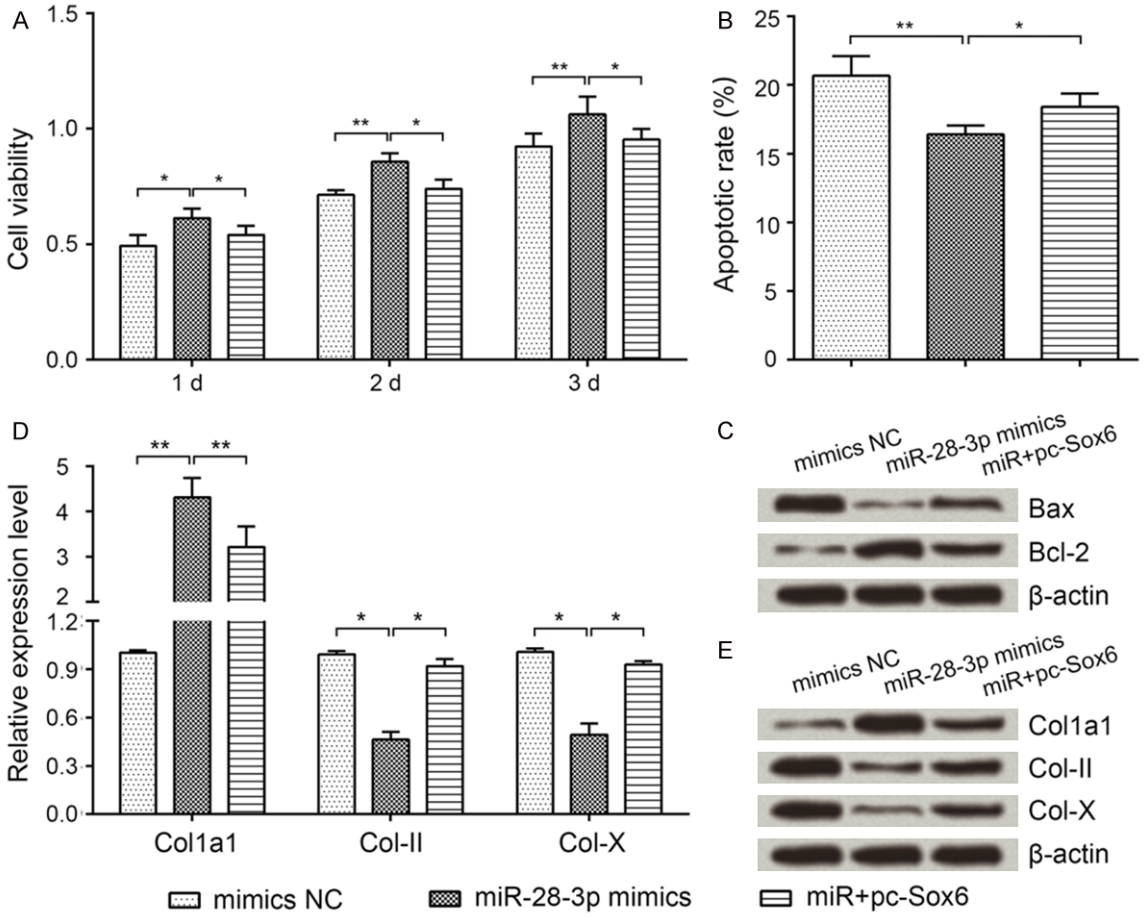

Figure 4. The effects of miR-28-3p on cell proliferation, apoptosis and expressions of osteogenesis-associated genes were all reversed by Sox6. MC3T3-E1 cells were respectively transfected with miR-28-3p mimics, miR-28-3p mimics negative control (mimics NC) or miR-28-3p mimics and pcDNA3.1/Sox6 (pc-Sox6). (A) Cell viability after transfection; (B) Apoptotic rate after transfection; (C) Protein expression levels of apoptosis-associated proteins; Relative mRNA (D) and protein (E) levels of osteogenesis-associated genes. Data presented were the mean of three independent experiments. Error bars indicated SD. *, $P < 0.05$; **, $P < 0.01$; miR+pc-Sox6, miR-28-3p mimics + pc-Sox6.

< 0.01). In **Figure 4B**, miR-28-3p mimics suppressed the cell apoptosis in comparison with mimics NC ($P < 0.01$). The protein expression level of pro-apoptotic Bax was decreased by transfection of miR-28-3p mimics while the anti-apoptotic Bcl-2 was enhanced in **Figure 4C**. In comparison with cells transfected with mimics NC, the expression of Col1a1 was significantly elevated ($P < 0.01$) followed with notable decrease of Col-II and Col-X ($P < 0.05$) as shown in **Figure 4D**, **4E**. Interestingly, all the effects of miR-28-3p mimics were declined by co-transfection of pc-Sox6 ($P < 0.05$ or $P < 0.01$). Thus, we drew a conclusion that miR-28-3p might modulate cell viability, apoptosis and expressions of osteogenesis-associated genes by targeting Sox6.

The PI3K/AKT signaling pathway was activated by miR-28-3p mimics

Cells were transfected with mimics NC, miR-28-3p mimics, ASO NC, ASO-miR-28-3p or ASO-miR-28-3p accompanied with the inhibitor of PI3K (LY294002). After that, the expression levels of proteins involved in PI3K/AKT signaling pathway were evaluated by Western blot analysis. As shown in **Figure 5A**, the miR-28-3p mimics enhanced both the phosphorylation levels of PI3K and AKT when compared with mimics NC while the ASO-miR-28-3p exhibited just the opposite regulation. Simultaneously, the up-regulation induced by miR-28-3p was ameliorated by LY294002 in **Figure 5B**. As a conse-

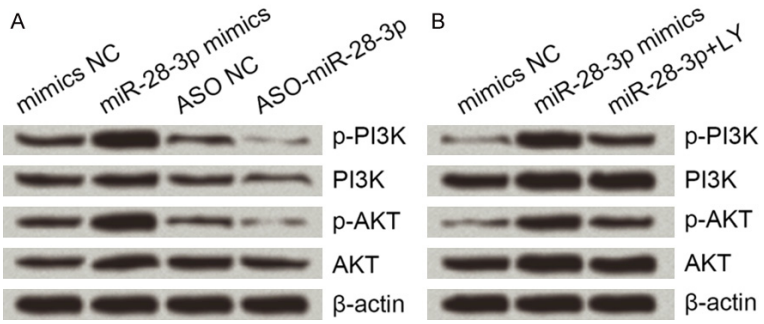

Figure 5. miR-28-3p mimics activated PI3K/AKT signaling pathway. A. Protein expression levels of different kinases in transfected cells, MC3T3-E1 cells were respectively transfected with miR-28-3p mimics, miR-28-3p mimics negative control (mimics NC), antisense oligonucleotides miR-28-3p (ASO-miR-28-3p) or ASO-miR-28-3p negative control (ASO NC); B. Protein expression levels of different kinases in transfected cells, MC3T3-E1 cells were respectively transfected with miR-28-3p mimics, miR-28-3p mimics negative control (mimics NC) or miR-28-3p mimics and LY294002 (LY) acting as PI3K inhibitor.

quence, miR-28-3p might activate the PI3K/AKT pathway.

Discussion

With the urgent need for breaking the limitations in the clinical therapies for fracture, we focused our interest on the burgeoning researches of miRNAs. Assays on human plasma suggested that the expression levels of miR-28-3p were both markedly down-regulated during hand and intra-articular calcaneal fracture healing. Subsequent experiments showed that miR-28-3p mimics significantly promoted cell proliferation and inhibited cell apoptosis. Sox6 was verified to act as one target gene of miR-28-3p and was negatively regulated by miR-28-3p mimics. Meanwhile, the effects of miR-28-3p mimics on cell viability, cell apoptosis and expressions of osteogenesis-associated genes were all reversed by Sox6 overexpression. Finally, the phosphorylation levels of PI3K and AKT were both up-regulated by transfection of miR-28-3p mimics, while the up-regulations were both weakened by inhibitor of PI3K.

Accumulating evidence has demonstrated that miRNA expression is remarkably associated with cell proliferation and apoptosis [19, 20]. In terms of miR-28-3p, related researches are focused on the function as potential bio-marker for specific disease [21, 22]. Recent study declared that the effects of miR-28-5p were significantly distinguished with miR-28-3p on colorectal cancer cells [23]. Considering the suppression of miR-28-5p on cell proliferation as well as the promotion on cell apoptosis in hepatocellular carcinoma (HCC) cells [24], and the promoted effects of miR-28 on cell proliferation, we hypothesized miR-28-3p might induce proliferation but repress apoptosis. A proposed "rheostat" model provides an explanation for the pro-survival and pro-death switch with relative expression of Bcl-2 family proteins [25]. The Bcl-2 family proteins are consisted of two subfamilies, one is anti-apoptotic effectors such as Bcl-2, and another one is pro-apoptotic effectors such as Bax [26]. The ratio of Bax/Bcl-2 strongly influences cytochrome c release, which further activates caspase-9 and caspase-3 in turn [27]. The activation of caspase-3 thereby fragments nucleosome and executes apoptotic program [28]. In the present study, miR-28-3p mimics down-regulated Bax while up-regulated Bcl-2, leading to pro-survival and anti-apoptosis. In common, fracture healing is a complex process and an adequate number of osteoblasts were required [14]. Experiments operated with osteoblasts in our study suggested remarkable promotion on cell proliferation and inhibition on apoptosis of miR-28-3p mimics, implying potential promotion of miR-28-3p for fracture healing.

Previous studies declared that cartilages played as obligatory templates for the endochondral bone formations [29]. After a consequence of differentiation and hypertrophy, chondrocytes are dead accompanied with calcification, resulting in formations of bone. In the conversion process, Col-II is encoded firstly and is followed by further differentiation, resulting in production of Col-X which is followed by calcification. Lefebvre *et al.* stated that only traces of α-1 type II collagen (Col2a1) and Sox6 RNA were found after cartilage was replaced by bone and the expression of Col1a1 was activated in osteoblasts [30]. In our study, the expression level of Col1a1 was up-regulated by miR-28-3p while levels of Col-II and Col-X were both down-regulated, implying potential promotion of miR-28-3p for fracture healing.

The influence of miRNAs varies depending on the complemented target genes of miRNAs. In our study, luciferase assay illustrated that Sox6 was one of the target genes for miR-28-3p. Plenty of investigations reported that Sox6 was a member of the Sox transcription-factor family which involved in cell proliferation and apoptosis. A study once provided evidence of the suppression generated by Sox6 on cell proliferation and apoptosis via up-regulation of p53 and p21 expressions [31]. More recently, Li *et al.* demonstrated the same effects of Sox6 in human colorectal cancer cells [32]. Therefore, the promotion on proliferation as well as the inhibition on apoptosis of miR-28-3p might partially come into play through down-regulation of Sox6. In consideration of the effect of osteoblasts on fracture healing, we concluded that miR-28-3p might promote fracture healing by targeting Sox6.

The PI3K/AKT pathway is reported to participate in the regulation of Bcl-2 family proteins [33]. Considering that AKT acts as one of the upstream signal proteins of the Bcl-2 family and its phosphorylation appears to promote the pro-survival pathway [34], the activation of PI3K/AKT pathway tremendous influences the cell proliferation and apoptosis. In the present study, miR-28-3p mimics remarkably activated the PI3K/AKT signaling pathway, resulting in down-regulation of pro-apoptotic Bax and up-regulation of anti-apoptotic Bcl-2, which might lead to down-regulation of the ratio of Bax/Bcl-2 and inhibition of apoptosis. As a result, the fracture healing was promoted by increasing number of osteoblast. On the other hand, a previous study proposed that TGF-β2 induced expression of Cal1a1 by activation of PI3K/AKT pathway [35]. Moreover, activation of PI3K is required in the stimulation of bone growth induced by C-type natriuretic peptide (CNP) [36]. Additionally, a possible link between up-regulation of TWIST and activation of AKT pathway was suggested to be existed [37]. Activating of PI3K/AKT might play roles in fracture healing, because TWIST suppresses Runx2 and thereby regulates bone formation [38]. In a word, we drew a conclusion that miR-28-3p accelerated process of fracture healing by activation of PI3K/AKT pathway. The experiments operated on cells transfected with ASO-miR-28-3p or ASO NC as well as induction of PI3K inhibitor verified the conclusion.

To summarize, we explored a novel function of miR-28-3p in acceleration of fracture healing. The possible mechanism might be the effects of Sox6 which is one of the target genes of miR-28-3p, and PI3K/AKT pathway. The detailed interactions of Sox6 suppression and PI3K/AKT activation need to be deep studied.

Disclosure of conflict of interest

None.

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