

Expression of microRNA-27a in a rat model of osteonecrosis of the femoral head and its association with TGF- β /Smad7 signalling in osteoblasts

YUMING BAI¹, YING LIU², SHENGLI JIN¹, KE SU¹, HAISEN ZHANG¹ and SHIYUN MA¹

¹Second Department of Orthopaedics, Cangzhou Central Hospital;

²Department of Surgery, Cangzhou People's Hospital, Cangzhou, Hebei 061000, P.R. China

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Abstract. The present study assessed whether microRNA (miR)-27a is an influential factor in steroid-induced osteonecrosis of the femoral head (ONFH) and investigated the underlying mechanism of action. The results indicated that serum miR-27a was decreased in a rat model of ONFH compared with that in control rats. It was also observed that increased miR-27a expression promoted osteogenic differentiation and cell proliferation, inhibited caspase-3/9 and B-cell lymphoma-2-associated X protein expression and induced alkaline phosphatase (ALP) activity and bone morphogenetic protein (BMP)-2, runt-related transcription factor (Runx)2 and osteonectin mRNA expression in osteoblastic MC3T3-E1 cells. miR-27a mimics also induced transforming growth factor (TGF)- β and Smad7 protein expression in MC3T3-E1 cells. Furthermore, transfection with TGF- β expression plasmid was able to enhance the effects of miR-27a mimics on osteoblastic differentiation, cell proliferation, ALP activity, BMP-2, Runx2 and osteonectin mRNA expression, and Smad7 protein expression in the MC3T3-E1 cells. Transfection with a TGF- β or Smad7 expression plasmid also enhanced the effects of miR-27a mimics on osteoblastic differentiation, cell proliferation, ALP activity and osteonectin mRNA expression in the MC3T3-E1 cells. Taken together, the results of the present study suggested that the induction of TGF- β /Smad7 signaling in osteoblasts may be a potential mechanism by which miR-27a regulates steroid-induced ONFH.

Introduction

Osteonecrosis of the femoral head (ONFH) may be induced by large doses of glucocorticoids, one of its most common

causes (1). During the natural course of ONFH, ~80% of femoral heads collapse within 1-3 years, leading to the development of hip osteoarthritis, which severely affects hip joint function and eventually leads to artificial joint replacement (2). At present, the long-term therapeutic effects of artificial joint replacement are unsatisfactory; therefore, certain young patients may need to undergo 2-3 replacements, which causes substantial pain and is an economic burden for patients and their families (3,4). However, specific and effective protective drugs against ONFH for clinical use are currently lacking, as the mechanisms of hormone-induced osteonecrosis remain to be fully elucidated (5).

MicroRNAs (miRNA/miRs) are a class of single-stranded non-coding RNAs of 20-24 nucleotides in length and are produced from single-stranded RNA precursors with a hairpin loop structure that are 70-80 nucleotides in length following shearing (6). miRNAs induce the degradation or inhibit the translation of their target mRNAs through specific binding with their 3'-untranslated region (3'-UTR), thus allowing for post-transcriptional regulation of genes. Therefore, the effects of miRNAs are considered to be a common means of regulating gene expression at the post-transcriptional level in multicellular organisms (7). In particular, miRNAs serve important regulatory roles during cell proliferation, apoptosis, differentiation, physiological development and pathological processes (8).

Transforming growth factor (TGF)- β superfamily members serve important roles during bone growth, bone wound healing, skeletal muscle repair and cellular immune responses. Of note, they are considered to be important regulatory factors during wound healing, may be involved in the entire process of bone healing, and are therefore a focus of studies in this field (9,10).

The Smad signalling pathway is among those that serve key regulatory roles in osteogenic differentiation (8). Members of the Smad protein family are involved in each step of the Smad pathway, among which Smad7 functions in the medial steps of the signaling pathway. Smads belong to the universal transporter class and have important roles, which comprise binding with receptors Smad1-3, -5, -7 and -8 of the Smad protein family, assisting Smad proteins to translocate to the nucleus, and regulating the expression of downstream target genes (6).

Correspondence to: Dr Yuming Bai, Second Department of Orthopaedics, Cangzhou Central Hospital, 16 Xinhua Zhong Road, Cangzhou, Hebei 061000, P.R. China
E-mail: mmmc4009542@126.com

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The Smad7 protein is an inhibitory Smad that may block the biological effects induced by combined actions of other activated Smads, thus enabling Smad7 to antagonize bone morphogenetic protein (BMP) and TGF- β signals conferred through the Smad pathway (11). BMPs, which also belong to the TGF- β superfamily, are the only local growth factors with the ability to independently induce bone tissue formation (12). BMPs also serve a leading role in regulating bone tissue formation. It has been indicated by a previous study that a low BMP concentration may induce directional migration of mesenchymal cells, while a moderate BMP concentration promotes the chondrogenic and osteogenic differentiation of mesenchymal cells, and a high BMP concentration induces mesenchymal cell proliferation (13). As a major member of the BMP family, BMP-2 mainly serves a role in the recruitment and differentiation of mesenchymal cells and osteoblasts (14). Zeng *et al* (15) demonstrated that the miR-23a cluster miR-23a/-27a/-24-2 promoted osteocyte differentiation in osteoblasts by regulating TGF- β signalling. The present study assessed the effects of miR-27a in steroid-induced ONFH and investigated its potential underlying mechanisms of action.

Materials and methods

Animal model. A total of 20 Sprague Dawley rats (male, weight, 200–220 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and housed at 22–23°C, 55–60% humidity and 12-h light/dark cycle. The rats were randomly divided into the control (n=6) and ONFH model groups (n=6). The ONFH model rats were administered 10 mg/kg dexamethasone sodium phosphate by intramuscular injection as references (16).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from serum or cells (1x10⁶ cell/ml) using TRIzol total RNA isolation reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol and purified with the Column DNA Erase kit (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, 1 μ g total RNA was used to synthesize first-strand complementary DNA using avian myeloblastosis virus reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The thermocycling conditions were as follows: 35°C for 40 min; and 85°C for 30 sec.

The relative expression was analysed by TaqMan miRNA probes (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and a CFX96 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR was initiated by a 5-min hold at 95°C, followed by 40 cycles of denaturation at 95°C for 20 sec, annealing/extension at 60°C for 30 sec and 72°C for 30 sec. The following primer sequences were used: miRNA27a forward, 5'-ACAGGCTAGCGCCGCTAAC-3' and reverse, 5'-CCTTAAGGCCCAAGATTACG-3'; and U6 forward, 5'-TCGCTTCGGCAGCACATATAC-3' and reverse, 5'-TATGGAACGCTTCACGAATTTG-3'. The relative levels were normalized to the control using the equation $2^{-\Delta\Delta C_q}$ (17).

Cell line, culture and transfection. MC3T3-E1 cells were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. MC3T3-E1 cells were transfected with miRNA-27a mimics, miRNA-27a inhibitor (5'-GCGGAACUUAGCCACUGUGAA-3' and antisense, 5'-CAGUACUUUUGUGUAGUACAA-3'), TGF- β plasmid (5'-ACCCATGCCTCCCTCTCGGA-3' and antisense, 5'-AGTGCAGCTAAGGCTCTGGCC-3'), Smad7 plasmid (5'-TCAGCCTTTTGGAAATGTGTG-3' and antisense, 5'-CCGCGTGCGGAGGGGACAGA-3') and negative control mimic (Sangon Biotech Co., Ltd., Shanghai, China) with Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.).

Cell proliferation assay and alkaline phosphatase (ALP) activity. The proliferation was assessed using an MTT assay. A total of 150 μ l MTT solution (5 mg/ml in PBS; Invitrogen; Thermo Fisher Scientific, Inc.) was added to the cells (1x10³ cell/well) in a 96-well cell culture plate following transfection at 48 h, followed by incubation at 37°C for 4 h. Following removal of the supernatant, 200 μ l dimethyl sulfoxide was added, followed by incubation at 37°C for 20 min. The optical density (OD) was measured at 490 nm on a microplate reader (Bio-Rad 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

ALP activity was measured with an ALP activity assay (A059-2; Nanjing Jiancheng Biology Engineering Institute) following transfection for 48 h according to the manufacturer's protocol. The OD was measured at 405 nm on a microplate reader (Bio-Rad 550; Bio-Rad Laboratories, Inc.).

Oil red O and ALP staining. For Oil red O staining, MC3T3-E1 cells (1x10⁵ cell/ml) were gently washed twice with PBS and fixed with 15% neutral formalin for 1 h at room temperature. Subsequently, the MC3T3-E1 cells were stained with oil red O solution at 37°C for 30 min.

For ALP staining, the MC3T3-E1 cells (1x10⁵ cell/ml) were gently washed twice with PBS and fixed with 4% neutral formalin for 5 min at room temperature. MC3T3-E1 cells were gently washed twice with Tris-buffered saline/Tween-20 (0.5% TBST) and stained with ALP (cat. no. D001-2; Nanjing Jiancheng Biology Engineering Institute) at 37°C for 30 min in the dark. Cell morphology and staining patterns were then examined using a microscope.

Protein extraction and western blot analysis. Cells (1x10⁶ cell) were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) following transfection at 48 h and the protein concentration in the supernatant was quantified with a bicinchoninic acid assay (Beyotime Institute of Biotechnology). Total cell lysates were separated by 8–12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked in 5% non-fat milk in TBST for 1 h at 37°C and hybridized with antibodies for B-cell lymphoma-2-associated X protein

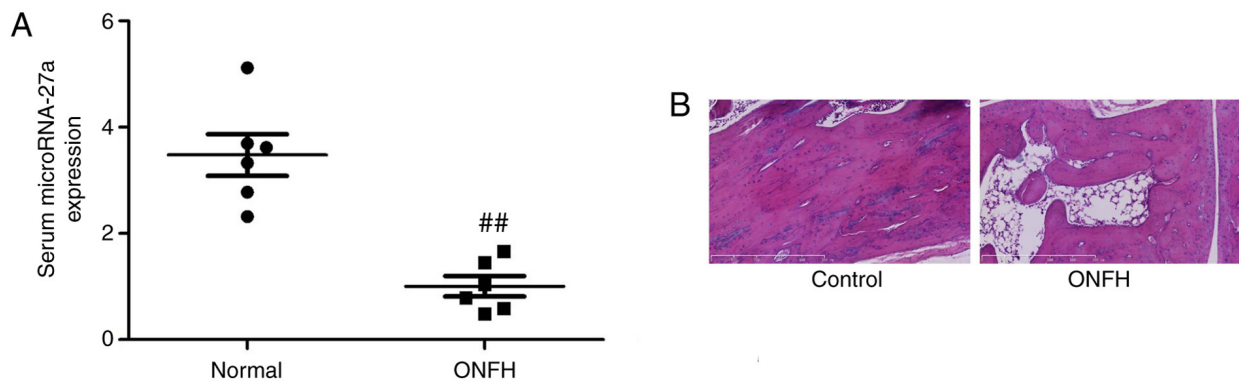


Figure 1. Serum miR-27a in ONFH rats. (A) Serum miR-27a levels in ONFH rats and (B) expression of miR-27a in ONFH tissue detected by hematoxylin and eosin staining (magnification, x100). $^{##}P < 0.01$ vs. control group. miR, microRNA; ONFH, osteonecrosis of the femoral head.

(Bax; cat. no. sc-6236; 1:1,000; Santa Cruz Biotechnology, Inc.), TGF- β (cat. no. sc-31609; 1:1,000; Santa Cruz Biotechnology, Inc.), Smad7 (cat. no. sc-9183; 1:1,000; Santa Cruz Biotechnology, Inc.) and GAPDH (cat. no. sc-51631; 1:5,000; Santa Cruz Biotechnology, Inc.) at 4°C overnight. Subsequently, the membranes were incubated with anti-rabbit immunoglobulin G secondary antibody (cat. no. sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.) and bands were visualized with an enhanced chemiluminescence system kit (EMD Millipore). Membranes were analyzed using Image_Lab_3.0 (Bio-Rad Laboratories, Inc.).

Assessment of caspase activity. Total cell lysates prepared as in the western blot protocol were used to measure caspase-3/9 activity with caspase-3 or caspase-9 activity kits (C1116 or C1158; Beyotime Institute of Biotechnology). The OD was measured at 405 nm on a microplate reader (Bio-Rad 550; Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol.

Statistical analysis. Values are expressed as the mean \pm standard deviation using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical analysis of differences between multiple groups was performed using Student's t-test or one-way analysis of variance with Tukey's post-hoc test. Experiments were repeated three times. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Serum miR-27a in ONFH rats. The levels of miR-27a in the serum of ONFH rats were significantly decreased compared with those in normal controls (Fig. 1A). HE staining demonstrated that bone cell appeared mass deaths in ONFH group, compared with control group (Fig. 1B).

miR-27a regulates the proliferation and osteogenic differentiation of MC3T3-E1 cells. miR-27a mimics were used to increase the miR-27a expression in MC3T3-E1 cells. As demonstrated in Fig. 2A, transfection with miR-27a mimics significantly increased miR-27a expression of the MC3T3-E1 cells compared with that in the negative control transfection group. Over-expression of miR-27a promoted

osteogenic differentiation and increased the proliferation of the MC3T3-E1 cells compared with that in the negative control transfection group (Fig. 2B and C). The inhibitor of miR-27a was used to decrease the levels of miR-27a in MC3T3-E1 cells. Transfection with miR-27a inhibitor decreased the expression of miR-27a, inhibited osteogenic differentiation and reduced cell proliferation in the MC3T3-E1 cells compared with those in the negative control transfection group (Fig. 3).

miR-27a mimic and inhibitor affect caspase-3/9 activity and Bax protein expression in MC3T3-E1 cells. Subsequently, it was identified that miR-27a mimics inhibited caspase-3/9 activity and Bax protein expression in MC3T3-E1 cells (Fig. 4), while the miR-27a inhibitor increased caspase-3/9 activity and Bax protein expression in the MC3T3-E1 cells (Fig. 5), relative to the respective control groups. These results indicate that miR-27a has an anti-apoptotic function in osteoblasts.

miR-27a mimic and inhibitor affect ALP activity, and BMP-2, runt-related transcription factor (Runx)2 and osteonectin mRNA expression in MC3T3-E1 cells. As demonstrated in Fig. 3A, miR-27a mimics effectively increased ALP activity, as well as BMP-2, Runx2 and osteonectin mRNA expression (Fig. 6), while miR-27a inhibitor suppressed ALP activity, as well as BMP-2, Runx2 and osteonectin mRNA expression in MC3T3-E1 cells, compared with those in the respective control groups (Fig. 7). Therefore it was demonstrated that miR-27a regulates osteogenic differentiation.

miR-27a mimic and inhibitor affect TGF- β and Smad7 protein expression in MC3T3-E1 cells. Western blot analysis was used to examine TGF- β and Smad7 protein expression in MC3T3-E1 cells. It was observed that miR-27a overexpression significantly promoted TGF- β and Smad7 protein expression (Fig. 8), while the miR-27a inhibitor significantly suppressed TGF- β and Smad7 protein expression in the MC3T3-E1 cells (Fig. 9), compared with those in the respective control groups.

Ectopic overexpression of TGF- β enhances miR-27a-mediated expression of TGF- β and Smad7 in MC3T3-E1 cells. MC3T3-E1 cells were transfected with miR-27a mimics and simultaneously with TGF- β to evaluate the potential role of TGF- β in the effect of miR-27a on osteogenic differentiation.

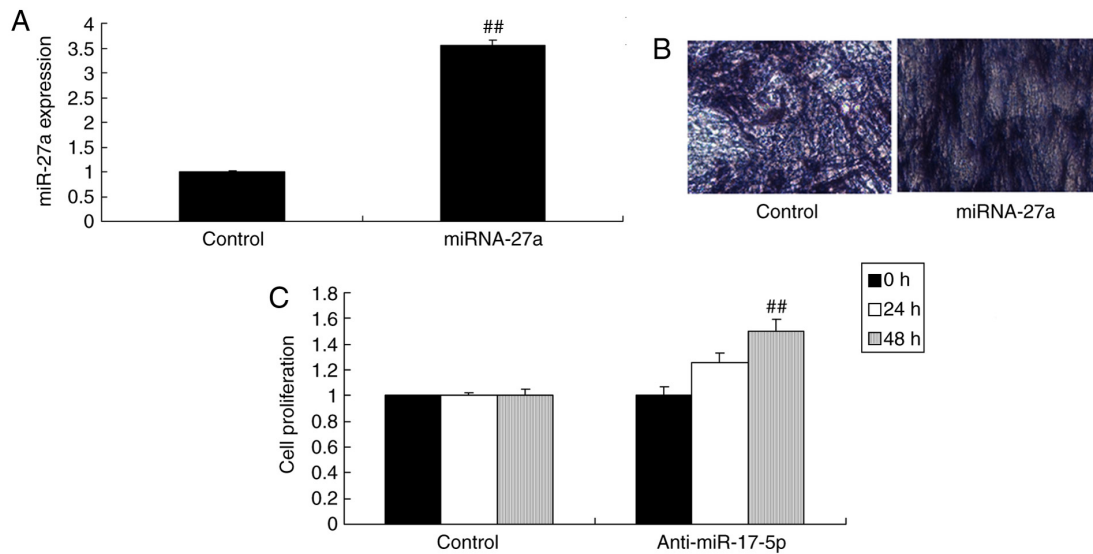


Figure 2. miR-27a affects the proliferation and osteogenic differentiation of MC3T3-E1 cells. (A) miR-27a expression, (B) osteogenic differentiation by ALP staining (magnification, x100) and (C) proliferation of MC3T3-E1 cells (magnification, x100). ^{##} $P < 0.01$ vs. control group. Groups: Control, negative control group; miR-27a, miR-27a mimic group. miR, microRNA.

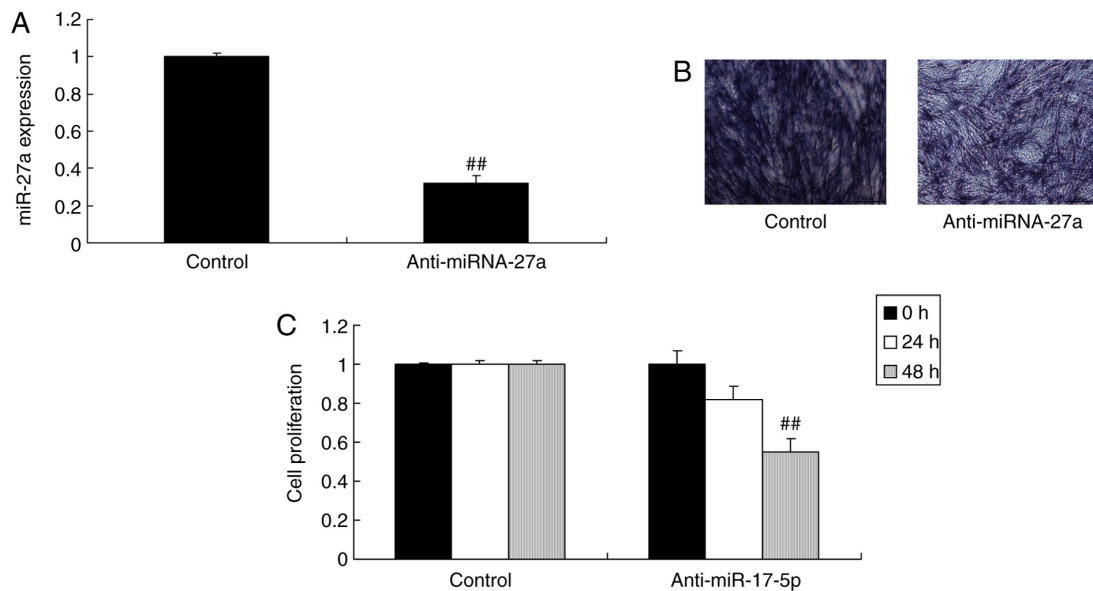


Figure 3. Inhibition of miR-27a affects the proliferation and osteogenic differentiation of MC3T3-E1 cells. (A) miR-27a expression, (B) osteogenic differentiation by ALP staining (magnification, x100) and (C) proliferation of MC3T3-E1 cells. ^{##} $P < 0.01$ vs. control group. Groups: Control, negative control group; anti-miR-27a, miR-27a inhibitor group. miR, microRNA.

As demonstrated in Fig. 10, TGF- β -expressing plasmid significantly increased TGF- β and Smad7 protein expression in MC3T3-E1 cells transfected with miR-27a mimics, compared with that in cells transfected with miR-27a mimics alone. In this study, the results demonstrated that TGF- β is an important for the effects of miR-27a on osteogenic differentiation of MC3T3-E1 cells.

TGF- β contributes to the function of miR-27a in osteogenic differentiation of MC3T3-E1 cells. Next, the role of TGF- β in the function of miR-27a in osteogenic differentiation was investigated. Ectopic overexpression of TGF- β significantly increased the effect of miR-27a mimics on the proliferation

and osteogenic differentiation of MC3T3-E1 cells when compared with that of cells transfected with miR-27a mimics alone (Fig. 11).

TGF- β enhances the effects of miR-27a on caspase-3/9 activity and Bax protein expression in MC3T3-E1 cells. Ectopic overexpression of TGF- β increased the inhibitory effect of miR-27a on caspase-3/9 activity and Bax protein expression in MC3T3-E1 cells when compared with those in cells transfected with miR-27a mimics alone (Fig. 12).

TGF- β enhances the effects of miR-27a on ALP activity, as well as BMP-2, Runx2 and osteonectin mRNA expression in

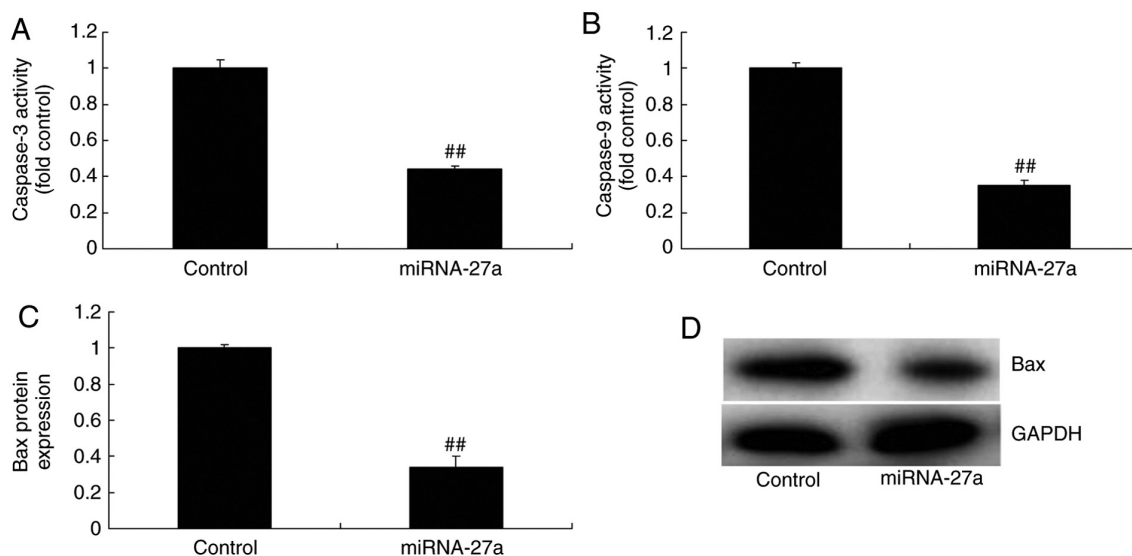


Figure 4. miR-27a affects caspase-3/9 activity and Bax protein expression in MC3T3-E1 cells. Activity of (A) caspase-3 and (B) caspase-9. (C) Quantified protein expression levels of Bax detected by western blot analysis and (D) representative western blot bands. ^{##}P<0.01 vs. control group. Groups: Control, negative control group; miR-27a, miR-27a mimics group. miR, microRNA; Bax, B-cell lymphoma-2-associated X protein.

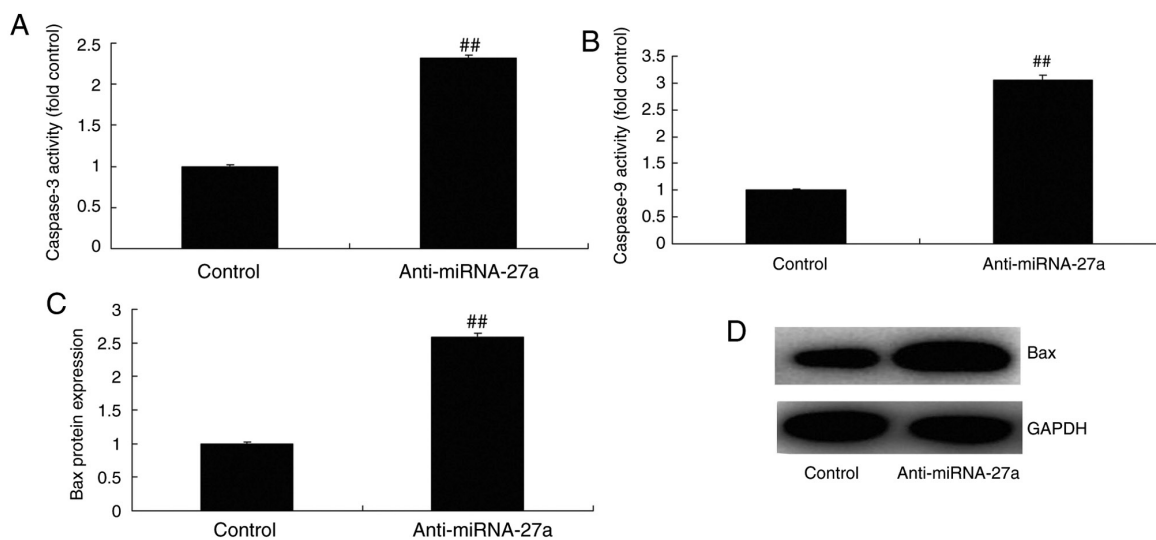


Figure 5. Inhibition of miR-27a affects caspase-3/9 activity and Bax protein expression in MC3T3-E1 cells. Activity of (A) caspase-3 and (B) caspase-9. (C) Quantified protein expression levels of Bax detected by western blot analysis and (D) representative western blot bands. [#]P<0.01 vs. control group. Groups: Control, negative control group; anti-miR-27a, miR-27a inhibitor group. miR, microRNA; Bax, B-cell lymphoma-2-associated X protein.

MC3T3-E1 cells. Ectopic overexpression of TGF- β promoted the effects of miR-27a on ALP activity, as well as on BMP-2, Runx2 and osteonectin mRNA expression in MC3T3-E1 cells when compared with those in cells transfected with miR-27a only, suggesting that miR-27a/TGF- β expression to activate osteogenic differentiation of MC3T3-E1 cells (Fig. 13).

Ectopic overexpression of Smad7 enhances miR-27a-mediated expression of Smad7 in MC3T3-E1 cells. To determine the potential role of Smad7 in the function of miR-27a on the osteogenic differentiation of MC3T3-E1 cells, transfection with Smad7 mimics was performed. As demonstrated in Fig. 14, Smad7-expressing plasmid enhanced Smad7 protein expression in MC3T3-E1 cells following miR-27a transfection, compared with that in cells transfected with miR-27a alone.

These results demonstrated that miR-27a regulates Smad7 to activate osteogenic differentiation of MC3T3-E1.

Smad7 enhances the stimulatory effect of miR-27a on the osteogenic differentiation of MC3T3-E1 cells via. Vector-mediated upregulation of Smad7 promoted the effect of miR-27a mimics on the proliferation and osteogenic differentiation of MC3T3-E1 cells compared with that in the group transfected with miR-27a mimics alone (Fig. 15).

Smad7 enhances the anti-apoptotic effects of miR-27a on MC3T3-E1 cells. Vector-mediated upregulation of Smad7 also enhanced the inhibitory effect of miR-27a on caspase-3/9 activity and Bax protein expression in MC3T3-E1 cells compared with that in cells transfected with miR-27a alone (Fig. 16).

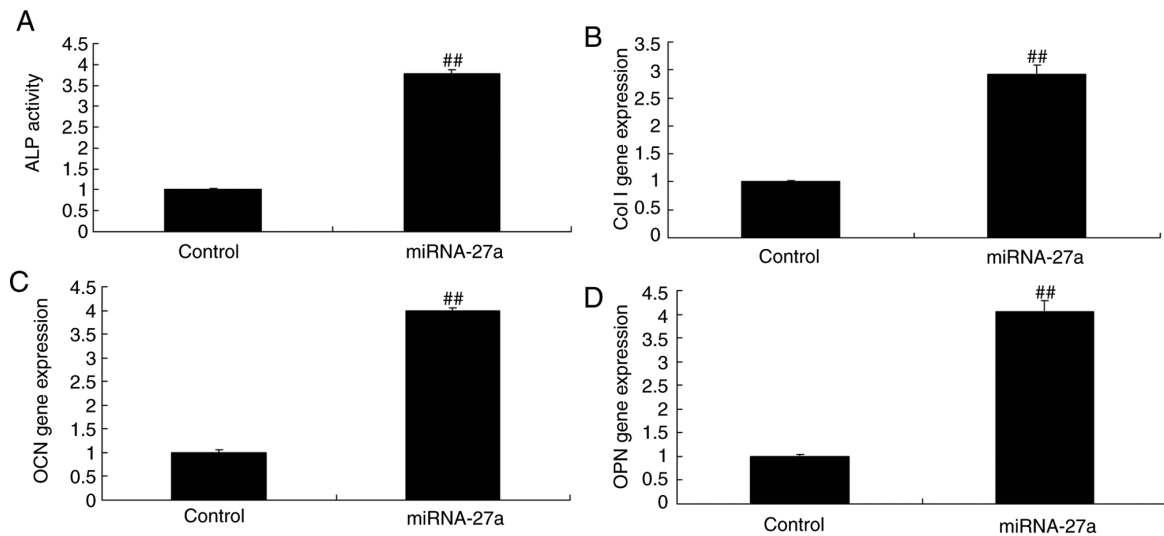


Figure 6. miR-27a affects ALP activity, and Col I, OCN and OPN mRNA expression in MC3T3-E1 cells. (A) ALP activity. mRNA expression levels of (B) Col I, (C) OCN and (D) OPN in MC3T3-E1 cells. ^{##}P<0.01 vs. control group. Control, negative control group; miR-27a, miR-27a mimics group. miR, microRNA; ALP, alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin; Col I, collagen I.

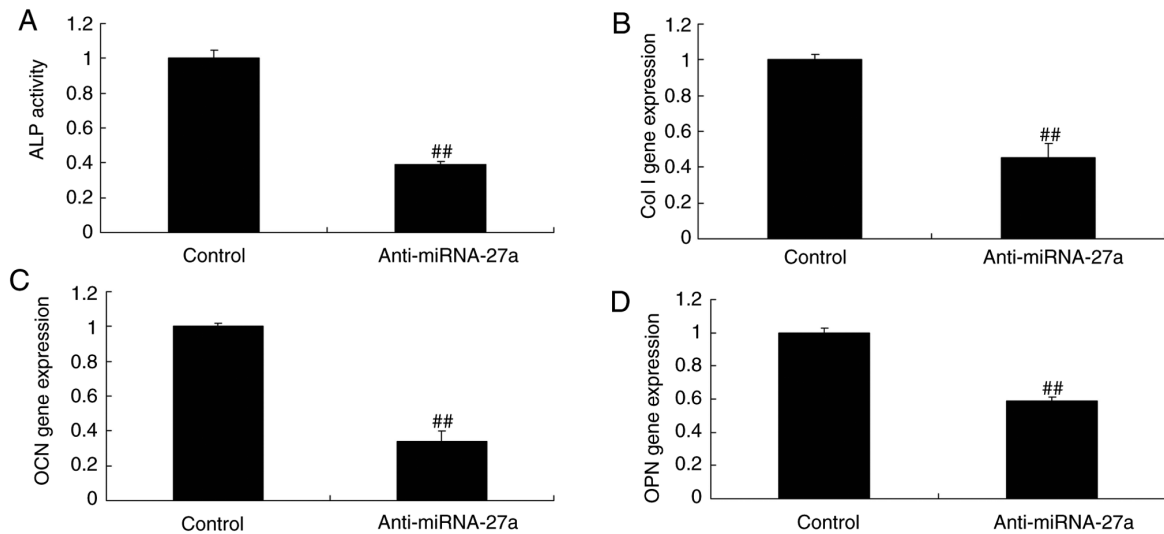


Figure 7. Inhibition of miR-27a affects ALP activity, and Col I, OCN and OPN mRNA expression in MC3T3-E1 cells. (A) ALP activity. mRNA expression levels of (B) Col I, (C) OCN and (D) OPN in MC3T3-E1 cells. ^{##}P<0.01 vs. control group. Groups: Control, negative control group; anti-miR-27a, miR-27a inhibitor group. miR, microRNA; ALP, alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin; Col I, collagen I.

Smad7 amplifies the effects of miR-27a on ALP activity, as well as *BMP-2*, *Runx2* and *osteonectin* mRNA expression in MC3T3-E1 cells. To evaluate the potential role of *Smad7* in the effect of miR-27a on the osteogenic differentiation of MC3T3-E1 cells, ALP activity and *BMP-2*, *Runx2* and *osteonectin* mRNA expression were measured following transfection with *Smad7* and/or miR-27a mimics. As demonstrated in Fig. 17, upregulation of *Smad7* increased the effects of miR-27a on ALP activity and *BMP-2*, *Runx2* and *osteonectin* mRNA expression in the MC3T3-E1 cells, compared with that in the group transfected with miR-27a mimics alone.

Discussion

With the extensive application of corticosteroids in clinical practice, it has gradually become apparent that they are key

inducing factors of osteonecrosis. Corticosteroid-induced ONFH is the most common type of ONFH in Chinese patients (3) and numerous research groups have focused on investigating and exploring its pathogenesis for a number of years. However, specific and effective therapeutic methods for the treatment of hormone-induced ONFH are still lacking in the clinic at present, due to an incomplete understanding of its precise pathogenesis (18). In the present study, the serum levels of miR-27a were decreased in a rat model of ONFH when compared with those in normal controls.

miRNAs are a class of small non-coding RNAs that collectively regulate thousands of genes; it is estimated that miRNAs regulate the expression of 30% of human genes, generally by blocking the expression or promoting the degradation of their target mRNAs through binding with their 3'-UTR (8). Although the roles of miRNAs have been

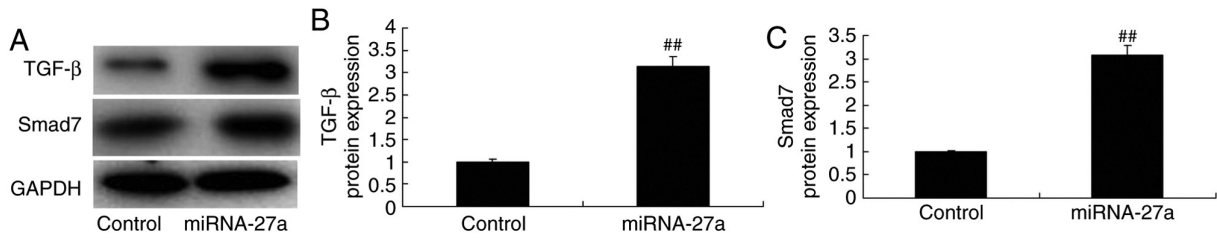


Figure 8. miR-27a affects TGF-β and Smad7 protein expression in MC3T3-E1 cells. (A) Representative western blot image and (B and C) quantified protein levels of TGF-β and Smad7 in MC3T3-E1 cells. ##P<0.01 vs. control group. Groups: Control, negative control group; miR-27a, miR-27a mimics group. miR, microRNA; TGF, transforming growth factor.

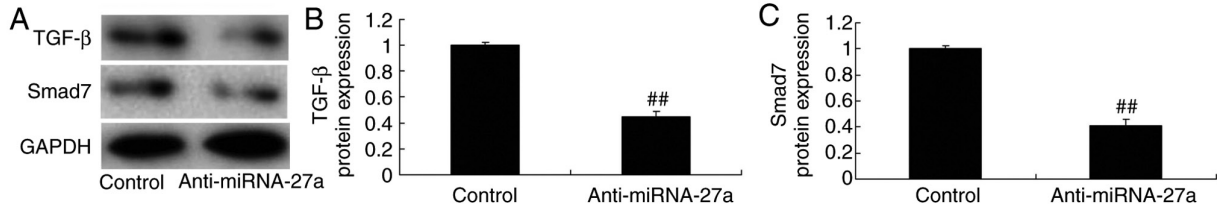


Figure 9. Inhibition of miR-27a affects TGF-β and Smad7 protein expression in MC3T3-E1 cells. (A) Representative western blot image and quantified expression levels of (B) TGF-β and (C) Smad7 protein in MC3T3-E1 cells. ##P<0.01 vs. control group. Groups: Control, negative control group; anti-miR-27a, miR-27a inhibitor group. miR, microRNA; TGF, transforming growth factor.

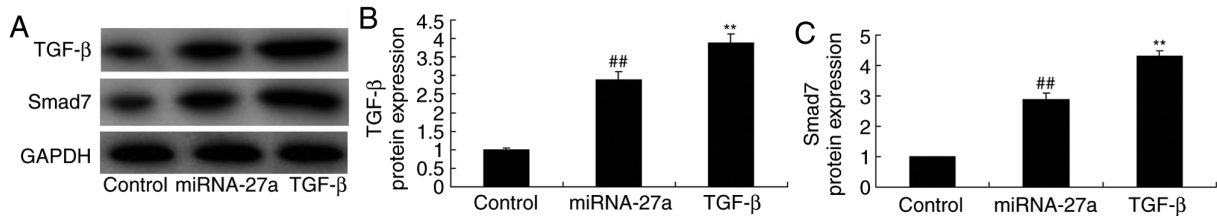


Figure 10. Vector-mediated upregulation of TGF-β enhances TGF-β and Smad7 protein expression in MC3T3-E1 cells transfected with miR-27a mimics. (A) Representative western blot image and quantified expression levels of (B) TGF-β and (C) Smad7 protein in MC3T3-E1 cells. ##P<0.01 vs. control group, **P<0.01 vs. miR-27a mimics group. Groups: Control, negative control group; miR-27a, miR-27a mimics group; TGF-β, miR-27a mimics + TGF-β group. miR, microRNA; TGF, transforming growth factor.

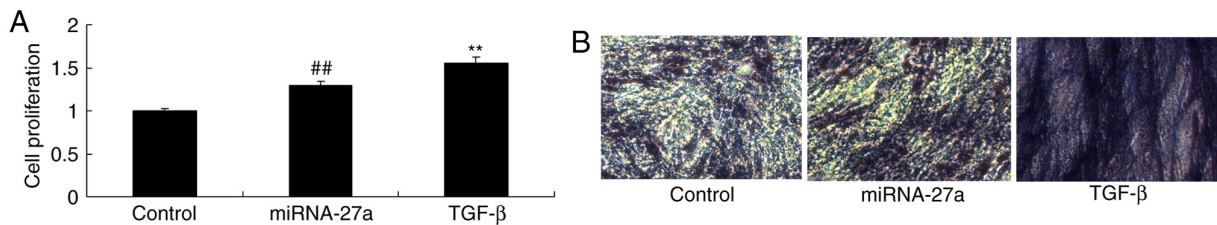


Figure 11. TGF-β enhances to the effect of miR-27a in the osteogenic differentiation of MC3T3-E1 cells. (A) Cell proliferation and (B) osteogenic differentiation of MC3T3-E1 cells (magnification, x100). ##P<0.01 vs. control group, **P<0.01 vs. miR-27a mimics group. Groups: Control, negative control group; miR-27a, miR-27a mimics group; TGF-β, miR-27a mimics + TGF-β group. TGF, transforming growth factor; miR, microRNA.

investigated for numerous diseases, few studies have reported on their regulatory role in the differentiation of stem cells and osteoblasts, or their differential expression profiles in bone diseases including osteoporosis and ONFH (19). The present study identified that miR-27a expression promoted osteogenic differentiation, and reduced caspase-3/9 activity and Bax protein expression in MC3T3-E1 cells, while the miR-27a inhibitor inhibited osteogenic differentiation and increased caspase-3/9 activity and Bax protein expression in MC3T3-E1

cells, compared with that in the respective control groups. In contrast to these results, Wang *et al* (20) reported that miR-27a promoted the apoptosis of cochlear sensory epithelium and may thus have opposing functions in the survival of different cell types.

TGF-β is an important cytokine involved in the function and metabolism of bone cells, and according to a previous study, it not only exerts a mitogenic effect on bone cells, but also reduces ossein loss, increases the bone deposition rate

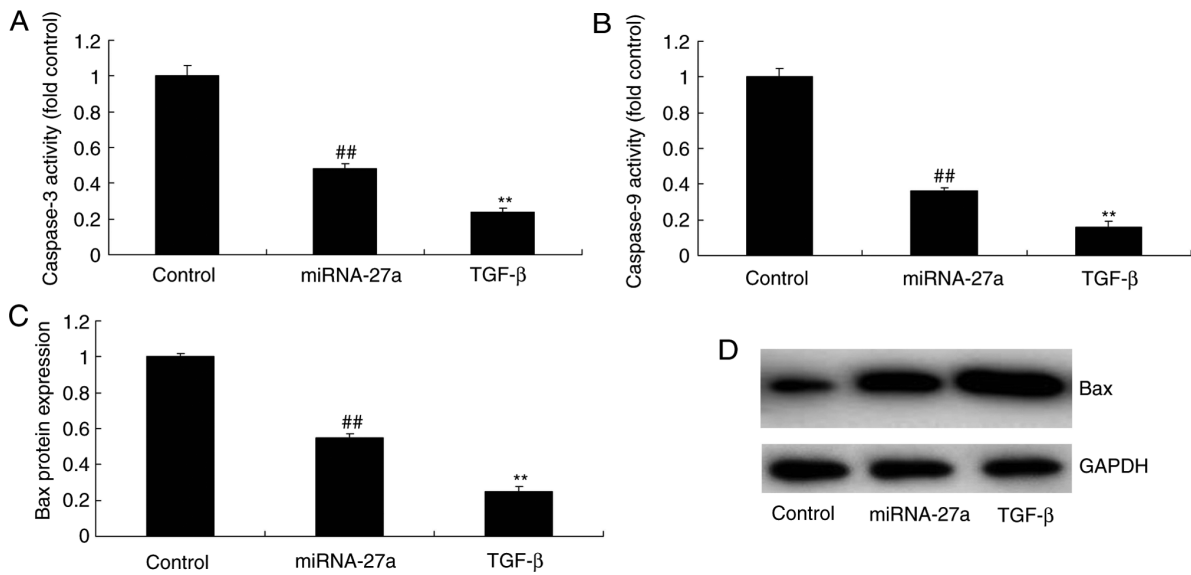


Figure 12. TGF-β enhances the effects of miR-27a on caspase-3/9 activity and Bax protein expression of MC3T3-E1 cells. Activity of (A) caspase-3 and (B) caspase-9. (C) Quantified protein expression levels of Bax detected by western blot analysis and (D) representative western blot bands. ##P<0.01 vs. control group, **P<0.01 vs. miR-27a mimics group. Groups: Control, negative control group; miR-27a, miR-27a mimics group; TGF-β, miR-27a mimics + TGF-β group. TGF, transforming growth factor; miR, microRNA; B-cell lymphoma-2-associated X protein.

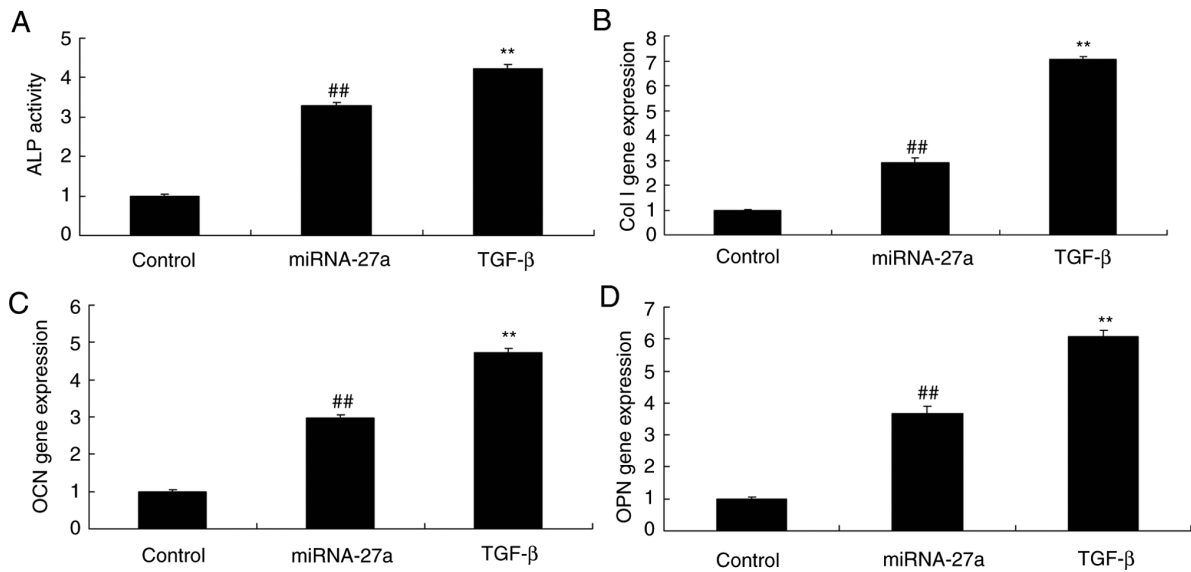


Figure 13. TGF-β enhances the effects of miR-27a on ALP activity, and Col I, OCN and OPN mRNA expression in MC3T3-E1 cells. (A) ALP activity. mRNA expression levels of (B) Col I, (C) OCN and (D) OPN in MC3T3-E1 cells. ##P<0.01 vs. control group, **P<0.01 vs. miR-27a mimics group. Groups: Control, negative control group; miR-27a, miR-27a mimics group; TGF-β, miR-27a mimics + TGF-β group. TGF, transforming growth factor; miR, microRNA; ALP, alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin; Col I, collagen I.

and promotes osteoblast differentiation (13). Suppression of programmed cell death is also among the mechanisms by which TGF-β prolongs cell survival (21). TGF-β has also been reported to promote metaplasia of the periosteal and aponeurotic layers on the greater trochanter surface of the femoral head of articular cartilage (10). The present study demonstrated that miR-27a significantly promoted TGF-β and Smad7 protein expression, while the miR-27a inhibitor significantly suppressed TGF-β and Smad7 protein expression in MC3T3-E1 cells when compared with that in the control groups. Similarly, Zeng *et al* (15) demonstrated that the miR-23a cluster miR-23a/-27a/-24-2 promoted

osteocyte differentiation in osteoblasts by regulating TGF-β signaling.

The Smad pathway is responsible for transducing BMP and TGF-β signals during osteogenic and chondrogenic differentiation (13). BMPs and TGF-β belong to the TGF-β superfamily, with BMPs, as the largest family in the TGF-β superfamily, classified as acid glycoproteins that are extensively distributed over the extracellular matrix (21). In particular, BMP-2 is an important extracellular signaling molecule that promotes osteogenic differentiation and bone formation (22). Regulation via the BMP-2/Smad/Runx2 pathway leads to increases and decreases in bone mass during the growth, metabolism and

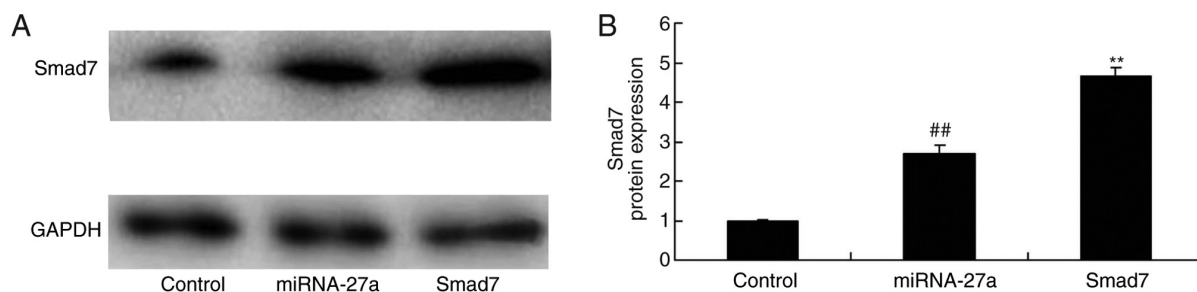


Figure 14. Upregulation of Smad7 protein expression in MC3T3-E1 cells following transfection with miR-27a mimics. (A) Representative western blot image and (B) quantified expression levels of Smad7 protein in MC3T3-E1 cells. ^{##}P<0.01 vs. control group, ^{**}P<0.01 vs. miR-27a mimics group. Groups: Control, negative control group; miR-27a, miR-27a mimics group; Smad7, miR-27a mimics + Smad7 group. miR, microRNA.

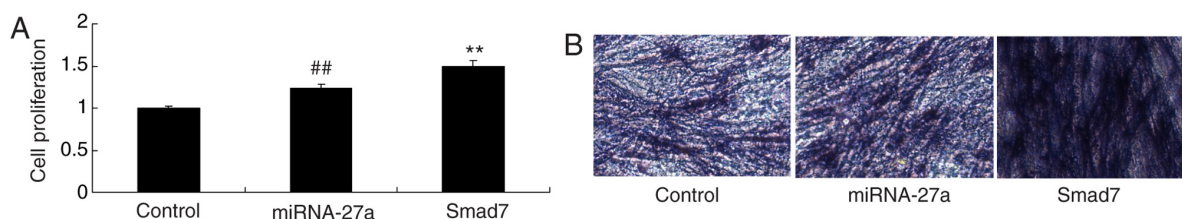


Figure 15. Smad7 enhances the effect of miR-27a on osteogenic differentiation of MC3T3-E1 cells. (A) Cell proliferation and (B) osteogenic differentiation of MC3T3-E1 cells (magnification, x100). ^{##}P<0.01 vs. control group, ^{**}P<0.01 vs. miR-27a mimics group. Groups: Control, negative control group; miR-27a, miR-27a mimics group; Smad7, miR-27a mimics + Smad7 group. miR, microRNA.

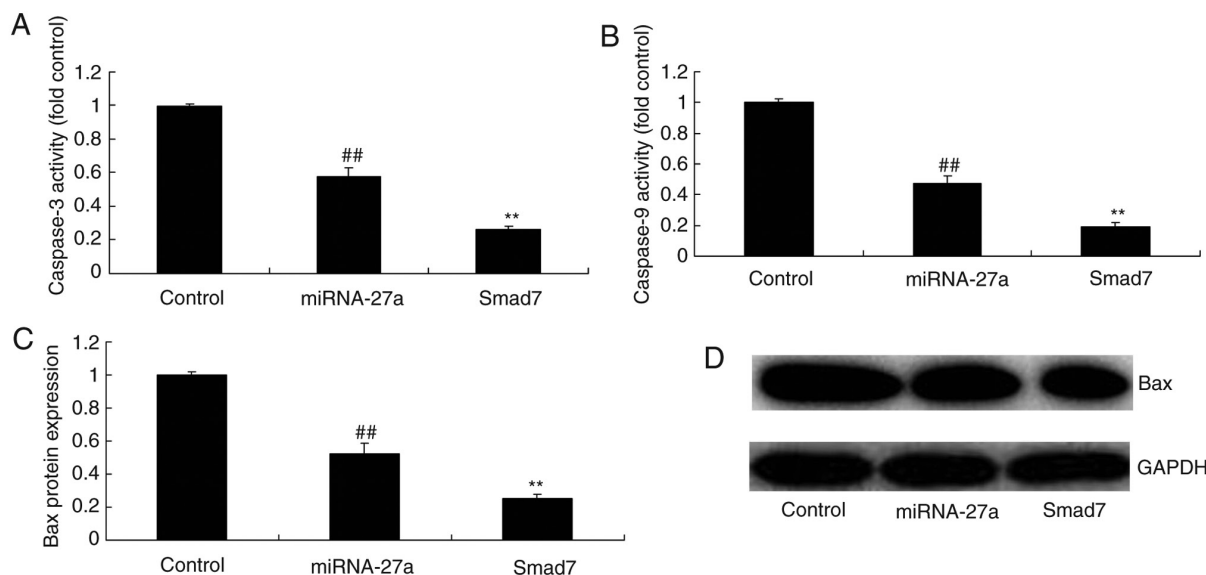


Figure 16. Smad7 enhances the effect of miR-27a on caspase-3/9 activity and Bax protein expression in MC3T3-E1 cells. Activity of (A) caspase-3 and (B) caspase-9. (C) Quantified protein expression levels of Bax detected by western blot analysis and (D) representative western blot bands. ^{##}P<0.01 vs. control group, ^{**}P<0.01 vs. miR-27a mimics group. Groups: Control, negative control group; miR-27a, miR-27a mimics group; Smad7, miR-27a mimics + Smad7 group. miR, microRNA; Bax, B-cell lymphoma-2-associated X protein.

development of bone tissues, in addition to bone formation and reconstruction, the osteogenic differentiation of stem cells, the maturation of osteoblasts and the secretion and mineralization of extracellular matrix (23). BMP-2 regulates the transcription of genes involved in osteogenesis by activating the Smad pathway, which thus enhances its osteogenic effects (24). Type I and II serine/threonine kinase receptors are receptors of the TGF- β receptor family; type I receptors are also known as activin-receptor-like kinases. The two receptor types may be

activated by TGF- β signaling to form tetramers, in which type II receptors phosphorylate type I receptors. In the present study, upregulation of Smad7 protein expression enhanced the effects of miR-27a overexpression on osteoblastic differentiation, cell proliferation, ALP activity, osteonectin mRNA expression and Smad7 protein expression in MC3T3-E1 cells. Wang *et al* (25) demonstrated that miR-27a ameliorates chronic kidney disease-induced muscle atrophy. Smad7 enhanced osteogenic differentiation via a different/additional mechanism, and an

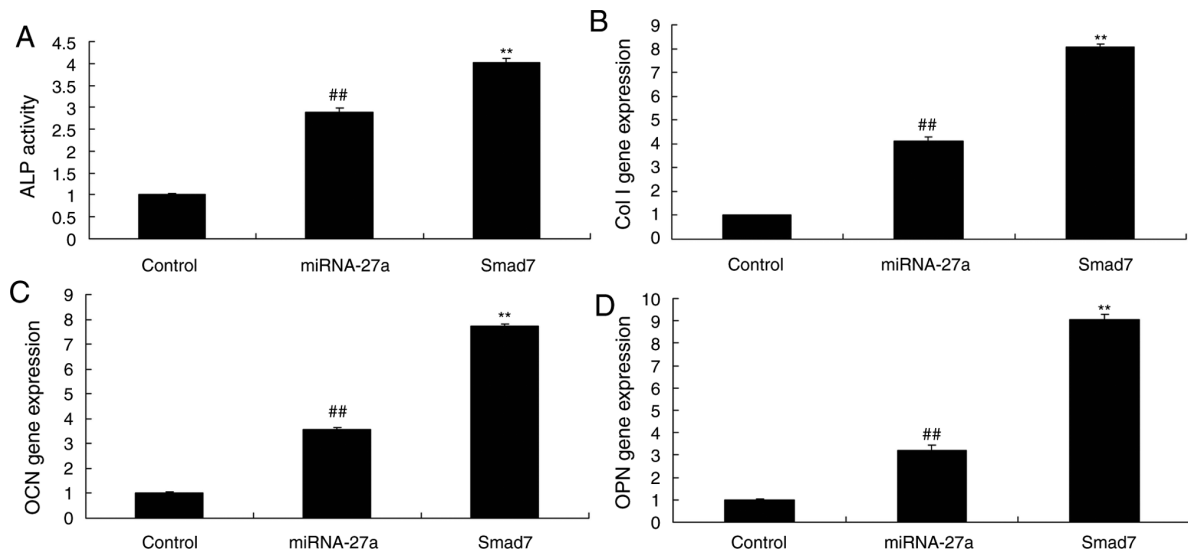


Figure 17. Smad7 enhances the effects of miR-27a on ALP activity, and Col I, OCN and OPN mRNA expression in MC3T3-E1 cells. (A) ALP activity. mRNA expression levels of (B) Col I, (C) OCN and (D) OPN in MC3T3-E1 cells. ^{##}P<0.01 vs. control group, ^{**}P<0.01 vs. miR-27a mimics group. Groups: Control, negative control group; miR-27a, miR-27a mimics group; Smad7, miR-27a mimics + Smad7 group. miR, microRNA; ALP, alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin; Col I, collagen I.

experiment with Smad7 knockdown to inhibit the effect of miR-27a on osteogenic differentiation would have provided more information. The present study did not investigate the direct or indirect binding of miR-27a and TGF or Smad7 by a luciferase assay, which is one of its limitations, however the regulation is probably an indirect one and that the steps in between are likely to be those reported by Chae *et al* (26).

The accumulation of activated Smad compounds in the nucleus serves a crucial role in the transmission of TGF- β signals from transmembrane receptors to the cell nucleus (27). It is currently thought that the distribution of Smads is in a dynamic balanced state between the cell nucleus and cytoplasm, which means that Smads undergo constant trafficking between the cytoplasm and nucleus in the presence or absence of signal stimulation to reach a certain equilibrium (13). Part of the mechanism regulating the nuclear-cytoplasmic trafficking of Smad has previously been documented (27). The nuclear-cytoplasmic trafficking of Smad allows cells to sense changes in TGF- β signaling in a continuous manner, which enables rapid cellular responses to changes in signalling (13). Smad7, along with Smad6, is classified as an inhibitory Smad, although it differs from Smad6, which specifically inhibits BMP signalling (21), as Smad7 inhibits BMP and TGF- β signals to exert marked negative regulatory effects (21). Furthermore, overexpression of Smad7 has been demonstrated to inhibit BMP-induced osteogenesis (21). The results of the present study demonstrated that vector-mediated upregulation of TGF- β enhanced the effects of miR-27a mimics on osteoblastic differentiation, cell proliferation, ALP activity, as well as the expression of BMP-2, Runx2 and osteonectin mRNA and Smad7 protein in MC3T3-E1 cells. TGF- β -expressing plasmid increased TGF- β and Smad7 protein expression in MC3T3-E1 cells transfected with miR-27a mimics, miR-27a induced TGF- β expression to activate osteogenic differentiation of MC3T3-E1 cells. Smad7-expressing plasmid enhanced Smad7 protein expression in MC3T3-E1 cells following miR-27a transfection. Chae *et al* (26) demonstrated that miR-27a induced the TGF- β

signaling pathway by targeting Smad2 and -4 in lung cancer. The present study only analyzed the extent to which the expression of miR-27a regulated TGF- β /Smad7 signaling in osteoblasts, which is a limitation of the present study. An analysis of the expression profile of downstream genes following transfection with miR-27a mimics in MC3T3-E1 cells should be pursued in a future study. In addition, these experiments with additional overexpression of TGF- β may not provide sufficient mechanistic evidence. It may have been more appropriate to inhibit TGF- β to then demonstrate that the effect of miR-27a is abrogated

In conclusion, the present study demonstrated that the effects of miR-27a on TGF- β /Smad7 signaling in osteoblasts may be a potential mechanism by which miR-27a regulates steroid-induced ONFH. miR-27a was indicated to have a role in regulating osteoblast differentiation and cell proliferation, although its exact role in the pathogenesis of ONFH remains to be elucidated.

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Availability of data and materials

The analysed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YB designed the experiment; YL, SJ, KS, HZ and SM performed the experiment; YB and YL analysed the data; YB wrote the manuscript.

Ethics approval and consent to participate

Full ethical approval was granted by the Medical Ethics Committee of Cangzhou City Central Hospital (Cangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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