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Silencing of miR-330-5p stimulates osteogenesis in bone marrow mesenchymal stem cells and inhibits bone loss in osteoporosis by activating Bgn-mediated BMP/Smad pathway

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Abstract. – OBJECTIVE: To illustrate the role of micro ribonucleic acid (miR)-330-5p in regulating osteogenesis through biglycan (Bgn)-mediated bone morphogenetic protein (BMP)/Smad pathway.

MATERIALS AND METHODS: A mouse model of osteoporosis (OP) was established by ovariectomy (OVX). BMD and miR-330-5p levels in mice undergoing sham operation or OVX were determined. BMD and BV/TV in OP mice with *in vivo* knockdown of miR-330-5p were measured by Micro-CT. After silencing of miR-330-5p in mouse primary bone marrow stromal cells (BMSCs), expression changes in osteogenesis-associated genes, ALP activity, and mineralization ability were assessed. Subsequently, the interaction between miR-330-5p and Bgn was examined by Dual-Luciferase reporter gene assay and Western blotting. Then, Bgn levels in BMSCs undergoing osteogenesis at different time points were measured. At last, the regulatory effects of miR-330-5p/Bgn axis on the BMP/Smad pathway, ALP activity, and mineralization ability in BMSCs were evaluated.

RESULTS: BMD was decreased and miR-330-5p was upregulated in OP mice. OP mice with *in vivo* knockdown of miNA-330-5p presented higher BMD and BV/TV than controls. Transfection with miR-330-5p inhibitor upregulated osteogenesis-associated genes, ALP activity, and mineralization ability in BMSCs. Bgn was time-dependently upregulated in BMSCs undergoing osteogenesis, which was indicated to be the target gene of miR-330-5p. Besides, Bgn level was negatively regulated by miR-330-5p. Importantly, Bgn was able to reverse the regulatory effects of miR-330-5p on the BMP/Smad pathway, ALP activity, and mineralization ability in BMSCs.

CONCLUSIONS: Knockdown of miR-330-5p facilitates osteogenesis in BMSCs through the Bgn-induced BMP/Smad pathway, thus alleviating the progression of OP.

Key Words:
OP, MiR-330-5p, Bgn, BMP/Smad.

Introduction

Osteoporosis (OP) is an aging-associated disease that decreases bone mass and destroys bone microstructure, which is clinically manifested as skeletal pain, increased bone fragility, and high risks of fractures[1]. With the aging of population, OP has become a vial healthy issue mainly affecting middle-aged and elderly people. Postmenopausal OP is the most common type of primary OP. It occurs in women within 5-10 years after menopause, with a remarkable decrease in bone mineral density (BMD)[2]. Bilateral ovariectomy (OVX) is a classic approach for constructing the *in vivo* model of OP, which is widely applied in bone metabolism researches.

Micro ribonucleic acids (miRNAs) are endogenous, non-coding, single-stranded RNAs with 18-25 nucleotides[3,4] that exert post-transcriptional regulations on gene expressions by binding to the 3'UTR region of the target mRNAs[5]. Schmiedel et al[6] have shown the critical functions of miRNAs in a series of biological processes. Numerous researches have confirmed the involvement of certain miRNAs in maintaining the balance of bone metabolism. MiR-335 can directly inhibit the expression of Wnt inhibitor DKK1, thus triggering differentiation-dependent osteogenic formation[7]. MiR-130a and miR-27b promote osteogenesis in human marrow stem cells (MSCs) by targeting PPARγ[8]. MiR-330-5p is the mature sequence of miR-330, which is crucial in many types of tumors[9-11].

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Biglycan (Bgn) is a member of the SLRP family consisting of a 45-kDa pyrenoid and two glycosaminoglycan (GAG) chains, where chondroitin sulfate (CS) and sulphate (DS) are covalently linked to the pyrenoid[12]. Bgn is able to accelerate osteoblast differentiation. Mice with Bgn knockout present age-dependent OP-like phenotypes, including decreased growth rate, declined bone mass due to attenuated bone formation, and shortening of the femur[13-15]. Chen et al[16] first proposed that Bgn regulates osteoblast differentiation by modulating bone morphogenetic proteins (BMP) -4 pathway. Overexpression of miR-185 stimulates osteogenesis by targeting Bgn and BMP/Smad pathway[17].

In this study, a mouse model of OP was constructed by OVX, and the potential functions of miR-330-5p in influencing osteogenesis and its underlying mechanism were mainly investigated.

Materials and Methods

OP Model in Mice

Female mice aged 8 weeks old were assigned into two groups and subjected to sham operation or OVX, respectively. After anesthesia, bilateral OVX was conducted from the dorsal incision. 6 weeks later, mice were sacrificed to collect bone tissues. This investigation was approved by the Animal Ethics Committee of the Animal Center of CangZhou Central Hospital.

BMSCs Isolation and Cell Culture

Mice were sacrificed and immersed in 75% ethanol for 10 min. Bilateral femora of the mice were extracted and immersed in phosphate-buffered saline (PBS). Bone marrow cavity of the femur was repeatedly washed by culture medium. The mixture containing bone marrow cavity contents was centrifuged, and the precipitant was cultured in a humidified incubator with 5% CO_2 at 37°C. Medium was replaced every three days. BMSCs were passaged for 3-4 generations, and they were cultured in osteogenic medium (DMEM-HG + 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) + 1% penicillin-streptomycin + 10^{-7} mol/L DEX + 10 mmol/L β-glycerophosphate + 50 μg/ml ascorbic acid) for inducing osteogenesis.

Cell Transfection

Cells were transfected with transfection vectors (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Micro-CT Analyses

Mouse tibia was taken and fixed in 40 g/L paraformaldehyde for 48 h. The prepared tibia sample was subjected to Micro-CT scanning using Latheta LCT-200 (source voltage: 55 kV, source current: 131 μA, exposure time: 300 ms, resolution: 10 μm). The VGStudio MAX V2.2 3D reconstruction processing software was utilized to reconstruct the 3D image of the micro-CT scan for analyzing BMD and bone mass/total volume (BV/TV).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) method was applied for isolating cellular RNAs. Then, RNAs were reversely transcribed into cDNAs, and the cDNAs were employed for PCR detection by SYBR Green method. Real-time PCR was performed with a FastStart Universal SYBR Green Master kit (Roche, Basel, Switzerland). The reaction system volume was in total 25 μl, pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 60°C for 45 sec, extension at 72°C for 3 min, with 35 cycles, and then extension at 72°C for 5 min. The primer sequences are listed as follows: miR-330-5p, F: 5'-TCTCTGGGCCTGTGTCTTAGGC-3', R: 5′-CAGTGCGTGTCGTGGAGT-3′; Bgn, F: 5'-TCCGACCTGGGTCTGAAGT-3', R: 5'-GCCTTCTCATGGATCTTGGA-3'.

Western Blot

Cellular proteins were isolated using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were immersed in PBS containing 5% skim milk for 2 h, and incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Primary antibodies, including anti-Bgn, anti-BMP2, p-Smad1/5/8, t-Smad, and GAPDH, were all purchased from Abcam (Cambridge, MA, USA). Band exposure was achieved by enhanced chemiluminescence (ECL) and processed by Image Software (NIH, Bethesda, MD, USA).

ALP Activity Determination

BMSCs were washed with pre-cold PBS for three times and lysed in pre-cold 1% Triton X-100 (Solarbio, Beijing, China) on ice for 30

min. After that, cell lysate was subjected to ALP activity determination, and the value at 405 nm was normalized to that of total protein concentration.

ALP Staining

BMSCs were washed with PBS twice, reacted in 70% ethanol for 10 min and ALP buffer (0.15 M NaCl, 0.15 M Tris-HCl, 1 mM $MgCl_2$, pH9.5) for 15 min. Subsequently, the cells were cultured in NBT-BCIP solution at 37°C in the dark for 30 min. Finally, images were captured under a microscope.

Alizarin Red S Staining (ARS)

BMSCs were induced for 7-day osteogenesis, washed, fixed in 95% ethanol for 14 min, and dyed in 2% ARS-Tris-HCL solution (pH4.3). Next, visible mineralized nodules were captured under an inverted microscope.

Dual-Luciferase Reporter Gene Assay

The cells were co-transfected with miR-NC/miR-330-5p inhibitor and Bgn-WT/Bgn-MUT, respectively for 48 h. Subsequently, the cells were lysed and centrifuged, and the supernatant was collected. Besides, the luciferase activity was measured using the Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 7 (La Jolla, CA, USA) was adopted for all statistical analyses. Data were expressed as mean ± SD. The *t*-test was used for analyzing differences between two groups. $p<0.05$ indicated that the difference was statistically significant.

Results

Knockdown of MiR-330-5p Protected Bone Loss in Mice Undergoing OVX

A mouse model of OP was established *via* OVX. Compared with mice in sham group, those undergoing OVX presented lower BMD and higher level of miR-330-5p (Figure 1A, 1B). *In vivo* knockdown of miR-330-5p in OP mice greatly enhanced BMD and BV/TV than controls (Figure 1C). In addition, knockdown of miR-330-5p upregulated Osterix (Osx), Col1a1, and OCN in OP mice (Figure 1D).

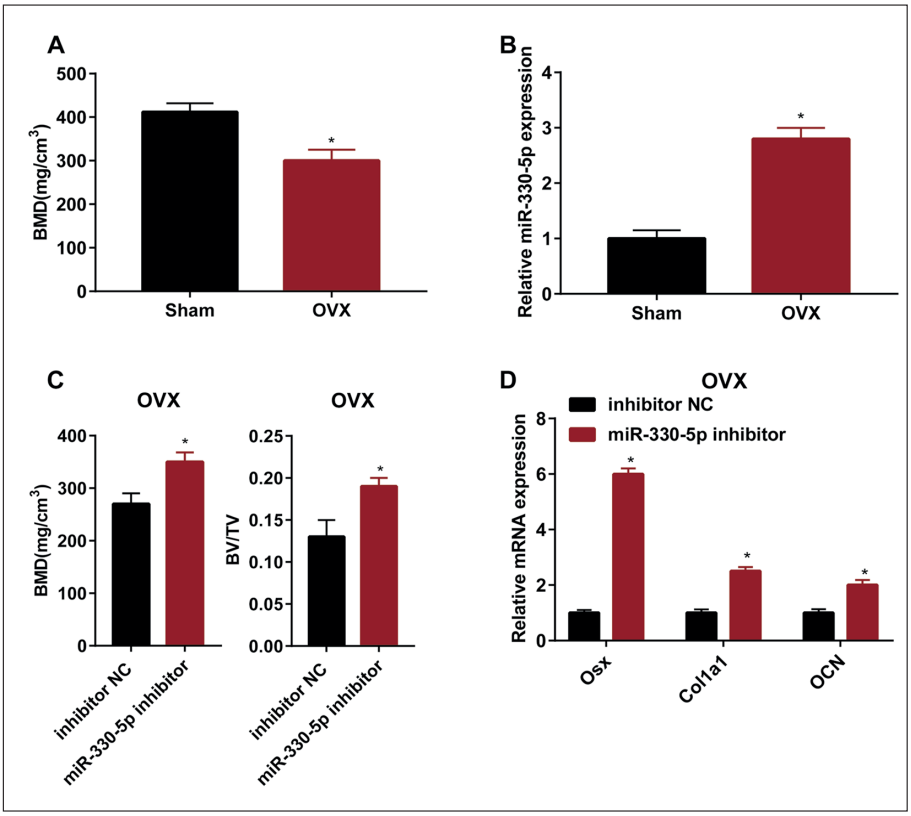

Figure 1. Knockdown of miR-330-5p protects bone loss in mice undergoing OVX. **A,** BMD in mice undergoing sham operation of OVX. **B,** MiR-330-5p level in mice undergoing sham operation of OVX. **C,** BMD in OP mice administrated with inhibitor NC or miR-330-5p inhibitor. **D,** Relative levels of Osx, Col1a1, and OCN in OP mice administrated with inhibitor NC or miR-330-5p inhibitor.

Knockdown of MiR-330-5p Promoted Osteogenesis in Primary BMSCs

To validate the influence of miR-330-5p on osteogenesis, primary BMSCs undergoing 7-day osteogenesis were transfected with miR-330-5p inhibitor. Knockdown of miR-330-5p markedly elevated ALP activity in BMSCs (Figure 2A, 2C). Moreover, the relative levels of Osx, Col1a1, and OCN were upregulated in BMSCs transfected with miR-330-5p inhibitor (Figure 2B). Mineralization ability was accelerated after the knockdown of miR-330-5p (Figure 2D).

Bgn Was the Direct Target of MiR-330-5p

During the osteogenesis process, Bgn was time-dependently upregulated in BMSCs at day 0, 3, and 7 (Figure 3A). Potential binding sequences in the promoter regions of miR-330-5p and Bgn were predicted (Figure 3B). Subsequently, the decreased luciferase activity after co-transfection of miR-330-5p mimics and Bgn-WT verified the binding between Bgn and miR-330-5p (Figure 3C). Transfection efficacies of miR-330-5p mimics and inhibitor were verified (Figure 3D). Moreover, both mRNA and protein levels of Bgn were negatively regulated by miR-330-5p (Figure 3E, 3F).

Knockdown of MiR-330-5p Activated the BMP/Smad Pathway by Targeting Bgn

The BMP/Smad pathway is of significance during osteogenesis. In this study, the protein levels of Bgn, BMP2, p-Smad1/5/8 were upregulated after transfection with miR-330-5p inhibitor, which was partially reversed by the knockdown of Bgn (Figure 4A). Expression level of t-Smad1 was not influenced by miR-330-5p or Bgn. In addition, ALP activity (Figure 4B, 4C) and mineralization ability (Figure 4D) in BMSCs transfected with miR-330-5p inhibitor were enhanced, and they were further inhibited by co-transfection with si-Bgn.

Discussion

OP is a systematic disease that mainly affects the middle-aged and elderly people. It is characterized by high incidence, high mortality, high disability, high medical cost, and low life quality[18]. Many miRNAs have been identified to serve as therapeutic targets in bone diseases[19-21]. In this pa-

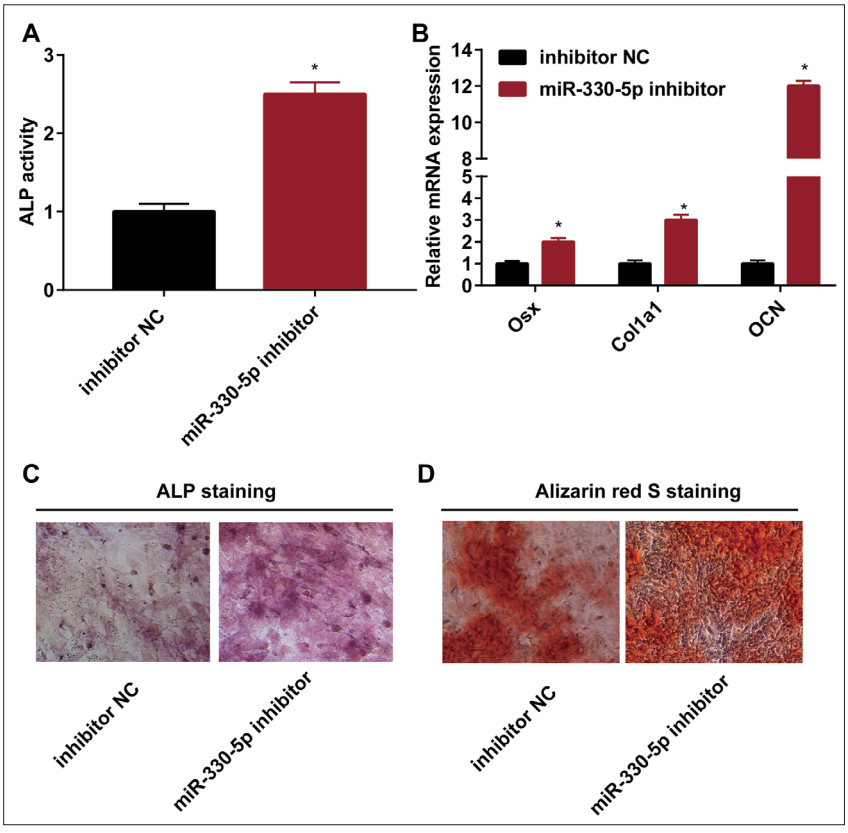

Figure 2. Knockdown of miR-330-5p promotes osteogenesis in primary BMSCs. Primary BMSCs are transfected with inhibitor NC or miR-330-5p inhibitor on the 7th day after osteogenesis. **A,** ALP activity. **B,** Relative levels of Osx, Col1a1, and OCN. **C,** ALP staining (magnification: 40×). **D,** Alizarin red S staining (magnification: 40×).

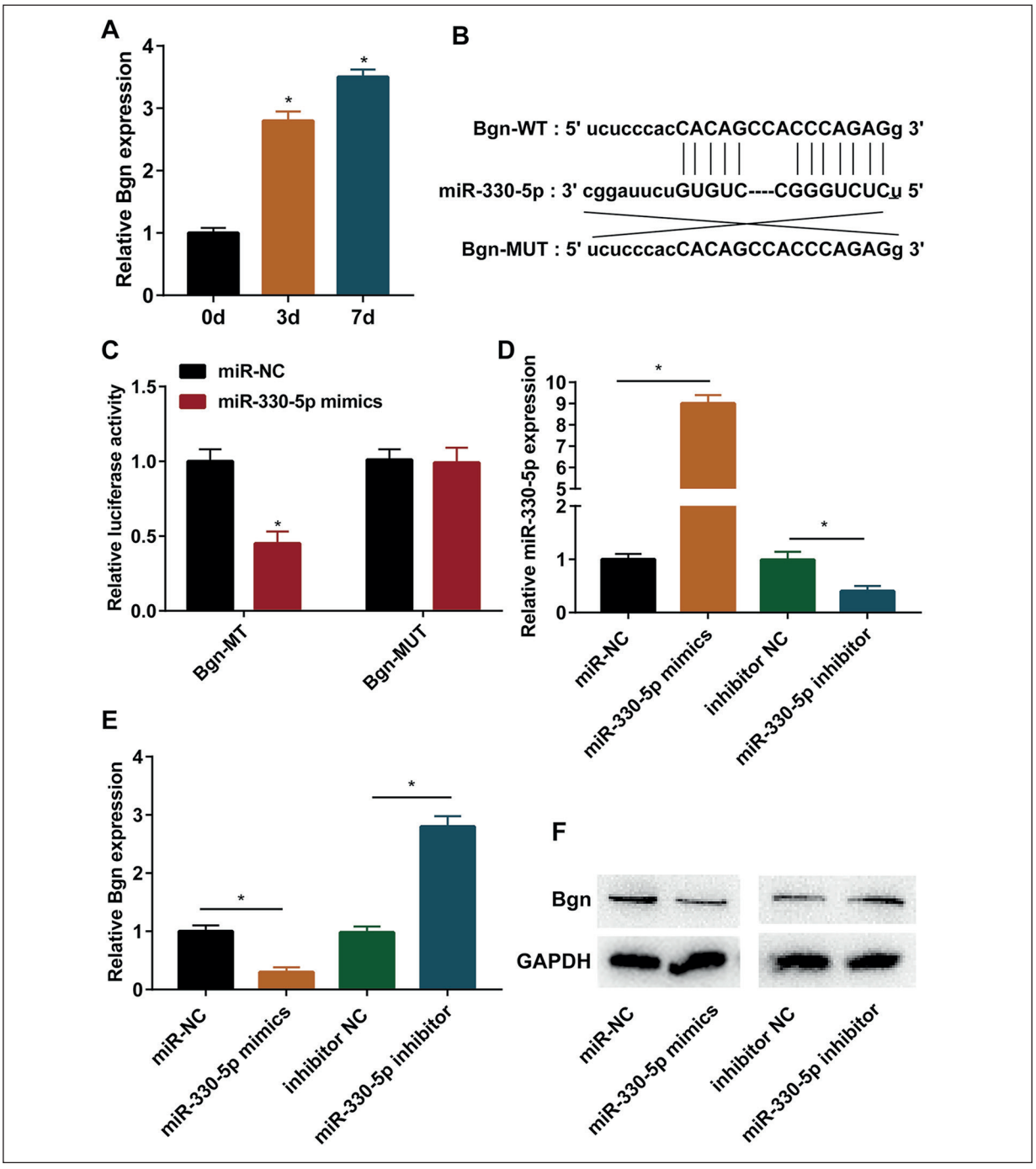

Figure 3. Bgn is the direct target of miR-330-5p. **A,** Bgn levels in BMSCs undergoing osteogenesis at 0, 3 and 7 day. **B,** Potential binding sequences in the promoter regions of miR-330-5p and Bgn. **C,** Luciferase activity in cells co-transfected with miR-NC/miR-330-5p inhibitor and Bgn-WT/Bgn-MUT. **D,** Transfection efficacies of miR-330-5p mimics and miR-330-5p inhibitor. **E, F,** The mRNA (**E**) and protein (**F**) levels of Bgn in BMSCs transfected with miR-NC, miR-330-5p mimics, inhibitor NC or miR-330-5p inhibitor.

per, *in vivo* knockdown of miR-330-5p in OP mice decreased BMD, and upregulated osteogenesis-associated genes. Similarly, the knockdown of miR-330-5p in primary BMSCs enhanced the osteogenesis, ALP activity, and mineralization ability.

MiRNAs effectively regulate the expressions of transcription factors by mediating mRNA activities, thus influencing physiological processes. Runx2, Osx, and other homologous domain proteins are the major transcription factors influenc-

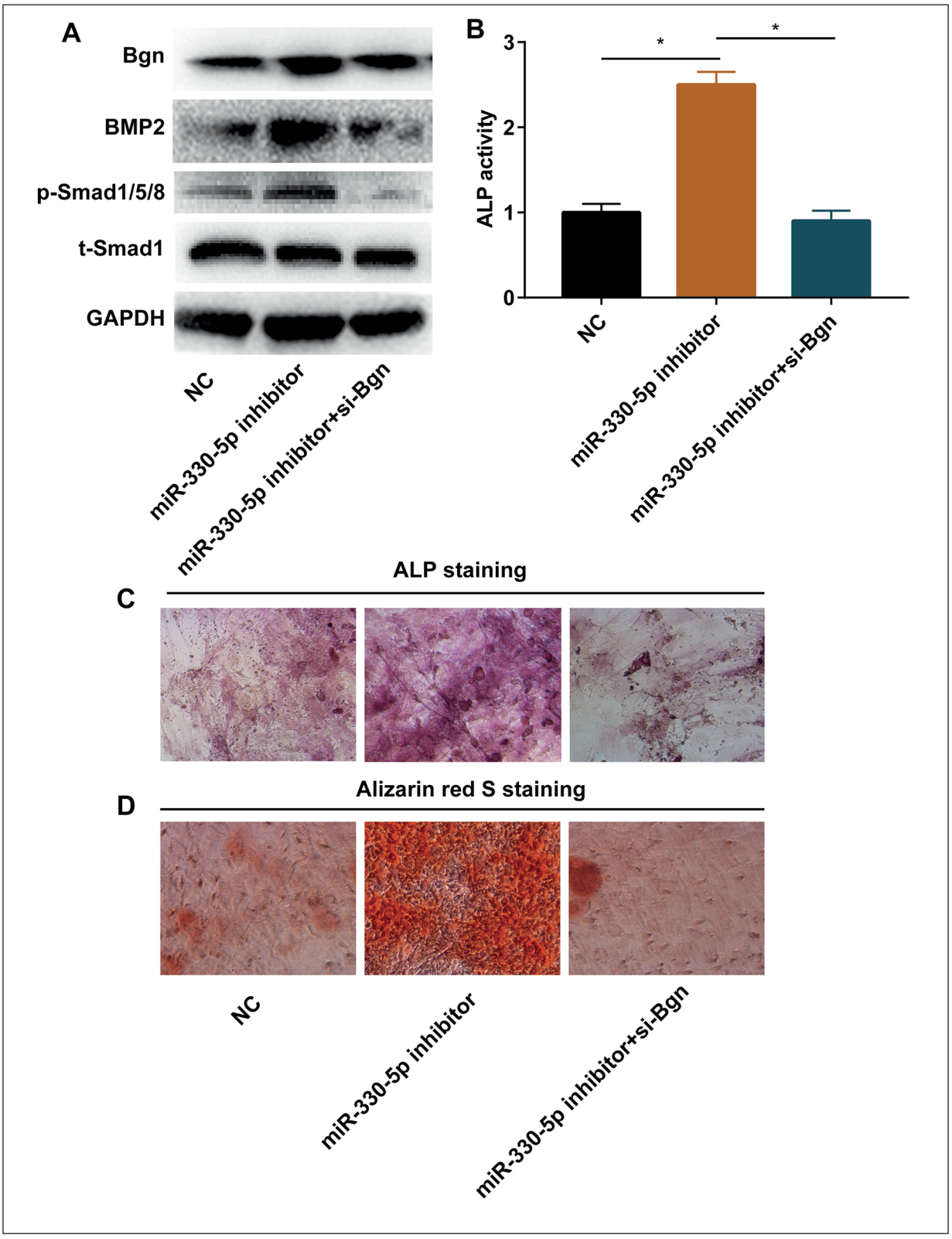

Figure 4. Knockdown of miR-330-5p activates the BMP/Smad pathway by targeting Bgn. Primary BMSCs are transfected with NC, miR-330-5p inhibitor or miR-330-5p inhibitor+si-Bgn on the 7th day after osteogenesis. **A,** Protein levels of Bgn, BMP2, p-Smad1/5/8, and t-Smad1. **B,** ALP activity. **C,** ALP staining (magnification: 40×). **D,** Alizarin red S staining (magnification: 40×).

ing osteogenesis in MSCs and differentiation of osteogenic precursor cells[22]. They interact with miRNAs to form a complex regulatory network, which further coordinates bone formation process[22]. The findings in this study indicated that Bgn was the direct target of miR-330-5p. Bgn was time-dependently upregulated during osteogenesis. Bgn is an extracellular matrix proteoglycan in the SLRP family. It is highly expressed in bones and bone connective tissues[23]. Previous studies[24-26] have shown that Bgn interacts with TGF-β, BMP2/4, and Wnt signaling pathways to promote osteogenesis.

It is reported that Bgn can directly bind to BMP2 and its receptors by actively regulating the activity of BMP2/4, thus accelerating osteoblast differentiation[27]. BMPs are currently recognized as the strongest and the only cytokines to induce ectopic osteogenesis. They can be applied clinically for bone tissue regeneration and reconstruction, and promote fracture healing[28,29]. BMP2 contributes to skeletal development and MSC differentiation[30,31]. BMP/Smad pathway is generally considered as a vital pathway involved in osteogenesis. BMP2 specifically activates Smad1/5/8 after binding to BMP receptors on the cell membrane. Phosphorylated Smad1/5/8 binds to Smad-4 and thus translocates into the nucleus, where the transcription of downstream factors is activated[32]. Xie et al[33] suggested that the overexpression of miR-146a suppresses BMP2-induced osteogenesis of adipose-derived MSCs. Administration of miR-146a inhibitor markedly upregulated Runx2 and Osx. In the experiments of this study, the knockdown of miR-330-5p upregulated the protein levels of Bgn, BMP2, and p-Smad1/5/8, which were reversed by the knockdown of Bgn. As a result, Bgn was responsible for the activation of BMP/Smad pathway induced by miR-330-5p.

Conclusions

In summary, the knockdown of miR-330-5p facilitates osteogenesis in BMSCs through the Bgn-induced BMP/Smad pathway, thus alleviating the progression of OP.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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