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LncRNA Bmncr alleviates the progression of osteoporosis by inhibiting RANML-induced osteoclast differentiation

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Abstract. – OBJECTIVE: To elucidate whether long non-coding RNA (lncRNA) Bmncr could inhibit RANML-induced osteoclast differentiation, thus alleviating the progression of osteoporosis.

MATERIALS AND METHODS: Expression level of lncRNA Bmncr at different stages of osteoclast differentiation was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). After Bmncr overexpression or knockdown in RAW 264.7 cells, expression levels of osteoclast-related genes were detected. Bone marrow mesenchymal stem cells (BMMs) isolated from rats undergoing ovariectomy (OVX) were induced with RANKL (50 ng/mL) and M-CSF (50 ng/mL) for 120 h. TRAP staining was conducted to count the number of TRAP-positive osteoclasts containing more than three nuclei. Bone resorption area of bone fragments was quantitatively analyzed. Osteoporosis model in mice was established. Mice were subjected to MicroCT analyses for recording BMD and BV/TV. The expression level of lncRNA Bmncr in the marrow and spleen of osteoporosis mice was examined.

RESULTS: LncRNA Bmncr was lowly expressed in the marrow and spleen of osteoporosis mice. Besides, Bmncr expression gradually downregulated during RANKL-induced *in vitro* osteoclast differentiation, reaching the lowest level at 72 h. The overexpression of Bmncr reduced the amount of osteoclasts, inhibited bone resorption capacity, and downregulated expression levels of Atp6v0d2, Acp5, Ctr, and Mmp9. Conversely, Bmncr knockdown obtained the opposite trends.

CONCLUSIONS: LncRNA Bmncr inhibits RANKL-induced osteoclast differentiation, thus alleviating the progression of osteoporosis.

Key Words:
LncRNA Bmncr, RANKL, Osteoclasts, Osteoporosis.

Introduction

Osteoclasts and osteoblasts maintain the balance of bone resorption and formation. Dysregulated osteoclast or osteoblast differentiation directly leads to diseases. Previous studies[1-3] have shown that over-activation of osteoclast differentiation and osteoclast dysfunction are the direct pathological factors for osteoporosis and rheumatoid arthritis. Therefore, clarification of osteoclast formation and function contributes to reveal the etiology of bone diseases and develop novel effective drugs.

Osteoclast differentiation and maturation depend on the activation of the RANK/RANKL/OPG signaling pathway[4,5]. During the process of osteoclast differentiation, stimulation of nuclear factor-κB receptor activator (RANKL) and macrophage colony-stimulating factor (M-CSF) is required[6]. After binding to its receptor RANK, RANKL can recruit adapter molecules, such as the TNF receptor-associated factor (TRAF), ultimately promoting osteoclast formation[7]. RANKL is a well-defined regulator of osteoclast differentiation and a key cytokine that activates the regulation of bone remodeling[8-10]. In the destructive bone metabolic disorders, the high expression of RANKL is considered to be a detrimental factor. Targeted inhibition of osteoclast-inducing activity helps to enhance the therapeutic efficacy of osteoporosis[11,12]. Therefore, inhibition of RANKL-induced osteoclasts is beneficial to prevent and treat osteoporosis.

Long non-coding RNA (LncRNA) is an important member of the transcript family with over 200 nucleotides in length and does not encode proteins. LncRNA exerts a complex and precise regulatory role in biological development and gene expression. It participates in the regulation of many cellular processes, including X chromosome inactivation, genomic imprinting, transcription, and post-transcriptional regulation[13-19]. Orthopedic diseases and osteoclast differentiation also require the involvement of lncRNAs. Liu et al[20] found that lncRNA AK077216 accel-

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erates RANKL-induced osteoclast differentiation and enhances osteoclast bone resorption. A recently discovered lncRNA Bmncr acts as a scaffold to promote the interaction between TAZ and ABL, and it promotes the assembly of TAZ and RUNX2/PPARG transcriptional complexes. Functionally, Bmncr promotes osteogenesis and inhibits lipogenesis, showing a vital role in osteoporosis[21]. However, the role of Bmncr in osteoclast differentiation is rarely reported.

This investigation mainly explored the role of lncRNA Bmncr in osteoclast differentiation and function. Through establishing osteoporosis model in mice, our work elucidated the regulatory effect of Bmncr on the progression of osteoporosis, thus providing a theoretical basis for clinical prevention and therapy.

Materials and Methods

Establishment of Osteoporosis Model in Mice

Female C57BL/6J mice with eight-week-old were randomly assigned into OVX group and sham group. Mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium. Dorsal skin and muscle were cut open alongside the mouse spine. Both ovaries and surrounding adipose tissues were resected in mice of OVX group. Mice in sham group were only resected with adipose tissues surrounded both the ovaries. BMMs were isolated from mice of sham group and OVX group. The study was approved by the Animal Ethics Committee of Fujian Medical University Animal Center.

BMMs Extraction and Osteoclast Differentiation

Mice were sacrificed by cervical vertebra dislocation and soaked in alcohol for 15 min, followed by the collection of mouse femur and tibia. The bone marrows of the collected femur and tibia were placed in a 10-cm dish with α-Minimum Eagle's Medium (α-MEM; HyClone, South Logan, UT, USA) containing 1% penicillin/streptomycin, 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 30 ng/mL M-CSF. The medium was replaced every two days for removing the unattached cells. At 6 day, cell morphology was similar with an oval shape, indicating the purified BMMs. Until 90% of confluence, purified BMMs were induced with M-CSF (30 ng/mL) and RANKL (50 ng/mL) in a 96-well plate with 8×10^3 cells per well. The medium was replaced every other day. Larger multinucleated cells were observed, which were osteoclasts.

Lentivirus Transfection

Overexpression (LV-Bmncr), interference (sh-Bmncr) lentiviral vectors and negative control were provided by Geno-meditech Co. Ltd, Shanghai. RAW 264.7 cells were infected in α-MEM containing 10% FBS, with MOI (multiplicity of infection) of 30. After 48 hours of incubation, cells were cultured in medium containing 5 mg/mL puromycin.

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

Transfected BMMs were inoculated in a 6-well plate with 5×10^3 cells per well. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and subjected to reverse transcription. The obtained complementary deoxyribose nucleic acid (cDNA) was amplified at 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s, for a total of 40 cycles. Primer sequences were as follows: Atp6v0d2, F: 5'-GTGAGACCTTGGAAGACCTGAA-3'; R: 5'-GAGAAATGTGCTCAGGGGCT-3'; Acp5, F: 5'-TGTGGCCATCTTTATGCT-3'; R: 5'-GTCATTTCTTTGGGGCTT-3'; Ctr, F: 5'-TGGTTGAGGTTGTGCCCA-3'; R: 5'-CTCGTGGGTTTGCCTCATC-3'; Mmp9, F: 5'-CGTGTCTGGAGATTCGACTTGA-3'; R: 5'-TTGGAAACTCACACGCCAGA-3'.

TRAP Staining

Pre-osteoclasts were washed with phosphate-buffered saline (PBS) twice, fixed in paraformaldehyde for 20 min, and dyed with TRAP solution (Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated at 37°C for 1 h and captured using an inverted microscope.

MicroCT Analyses

Mice were sacrificed by cervical vertebra dislocation. 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 used to reconstruct the 3D image of the microCT scan for analyzing the acquired data.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for all

statistical analysis. Data were expressed as mean ± SD (Standard Deviation). The *t*-test was used for analyzing differences between the two groups. $p<0.05$ indicated the significant difference.

Results

LncRNA Bmncr Expression Decreased During RANML-Induced Osteoclast Differentiation

Based on the stage of osteoclast differentiation, we determined the expression level of Bmncr at 0 h, 24 h (pre-osteoclasts), 72 h (mature osteoclasts), and 96 h (activated osteoclasts), respectively. QRT-PCR data showed that Bmncr expression markedly decreased at 24 h, 72 h, and 96 h, reaching the lowest level at 72 h (Figure 1A). Subsequently, BMMs were induced with RANKL (50 ng/mL) and M-CSF (50 ng/mL) for 120 h. TRAP staining showed that the amount of TRAP-positive osteoclasts decreased with the prolongation of RANKL and M-CSF induction (Figure 1B). QRT-PCR data revealed a gradual decrease in Bmncr expression with the prolonged induction of RANKL and M-CSF, reaching the lowest level at 72 h (Figure 1C). The above results indicated that lncRNA Bmncr exhibited a low expression during osteoclast differentiation.

LncRNA Bmncr Inhibited Osteoclast Differentiation and Bone Resorption

To evaluate the effect of Bmncr on osteoclast differentiation, we overexpressed or silenced Bmncr in RAW 264.7 cells by lentivirus transfection. Firstly, the transfection efficacy of LV-Bmncr and sh-Bmncr was verified by qRT-PCR (Figure 2A). TRAP staining was performed in transfected RAW 264.7 cells for 72 h. Bmncr overexpression markedly decreased the amount of osteoclasts, while knockdown of Bmncr increased osteoclast number (Figure 2B, 2C). Furthermore, transfected RAW 264.7 cells were cultured on bone sections for 5 days, and the bone resorption area was quantitatively analyzed. The results showed a lower bone resorption capacity in cells transfected with LV-Bmncr relative to controls. Conversely, cells transfected with sh-Bmncr had a higher capacity of bone resorption than controls (Figure 2D).

LncRNA Bmncr Inhibited Osteoclast-Related Gene Expressions

The expressions of osteoclast-related genes in transfected RAW 264.7 cells were examined by qRT-PCR. Overexpression of Bmncr markedly downregulated expressions of Atp6v0d2, Acp5, Ctr, and Mmp9. On the contrary, Bmncr knockdown upregulated their levels (Figure 3A-3D). These results indicated that Bmncr inhibited mRNA levels of osteoclast-related genes.

Downregulation of LncRNA Bmncr in Marrow and Spleen of OVX Mice

Osteoporosis model in mice was established to determine the Bmncr expression further. Mi-

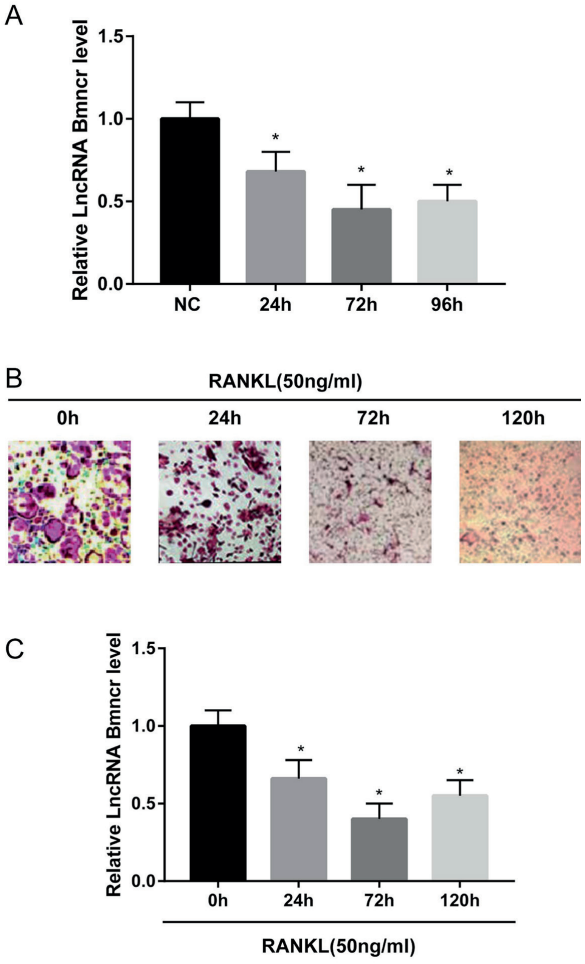

Figure 1. LncRNA Bmncr expression decreased during RANML-induced osteoclast differentiation. **A,** QRT-PCR data showed that Bmncr expression markedly decreased at 24 h, 72 h, and 96 h, reaching the lowest level at 72 h (Figure 1A). BMMs were induced with 50 ng/ml RANKL and 50 ng/ml M-CSF for 120 h. **B,** TRAP staining showed that the amount of TRAP-positive osteoclasts containing more than three nuclei decreased with the prolongation of RANKL and M-CSF induction (magnification: 100×). **C,** QRT-PCR data revealed the gradual decrease in Bmncr expression with the prolonged induction of RANKL and M-CSF, reaching the lowest level at 72 h.

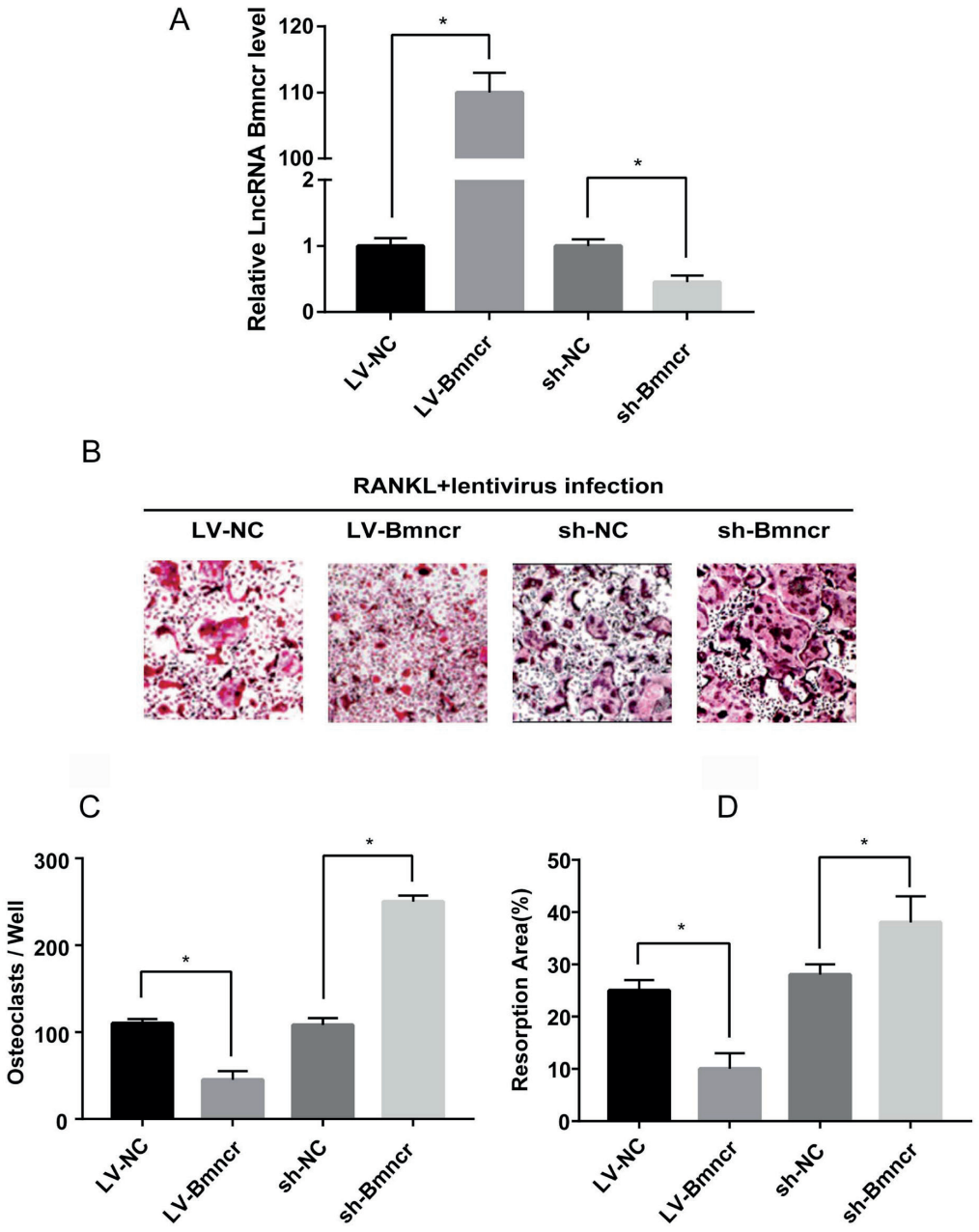

Figure 2. LncRNA Bmncr inhibited osteoclast differentiation and osteoclast bone resorption. **A,** Transfection efficacy of LV-Bmncr and sh-Bmncr in RAW 264.7 cells. Transfected RAW 264.7 cells were induced with 50 ng/mL RANKL and 50 ng/mL M-CSF for 72 h. **B-C,** Bmncr overexpression markedly decreased the amount of osteoclasts, while knockdown of Bmncr increased osteoclast number (magnification: 100×). **D,** Induced cells were cultured on bone sections for 5 days. Bmncr overexpression markedly inhibited bone resorption capacity, while knockdown of Bmncr improved bone resorption capacity.

croCT was applied to detect bone mineral density (BMD) and bone volume/structural parameters (BV/TV). Compared with mice in sham group, OVX mice showed lower BMD and BV/TV, indicating the successful establishment of osteoporosis model in mice (Figure 4A). Moreover, we found a lower level of Bmncr in bone marrow (left) and spleen tissue (right) of OVX mice than controls (Figure 4B).

Discussion

As a systemic bone metabolic disease, osteoporosis is characterized by bone mass reduction,

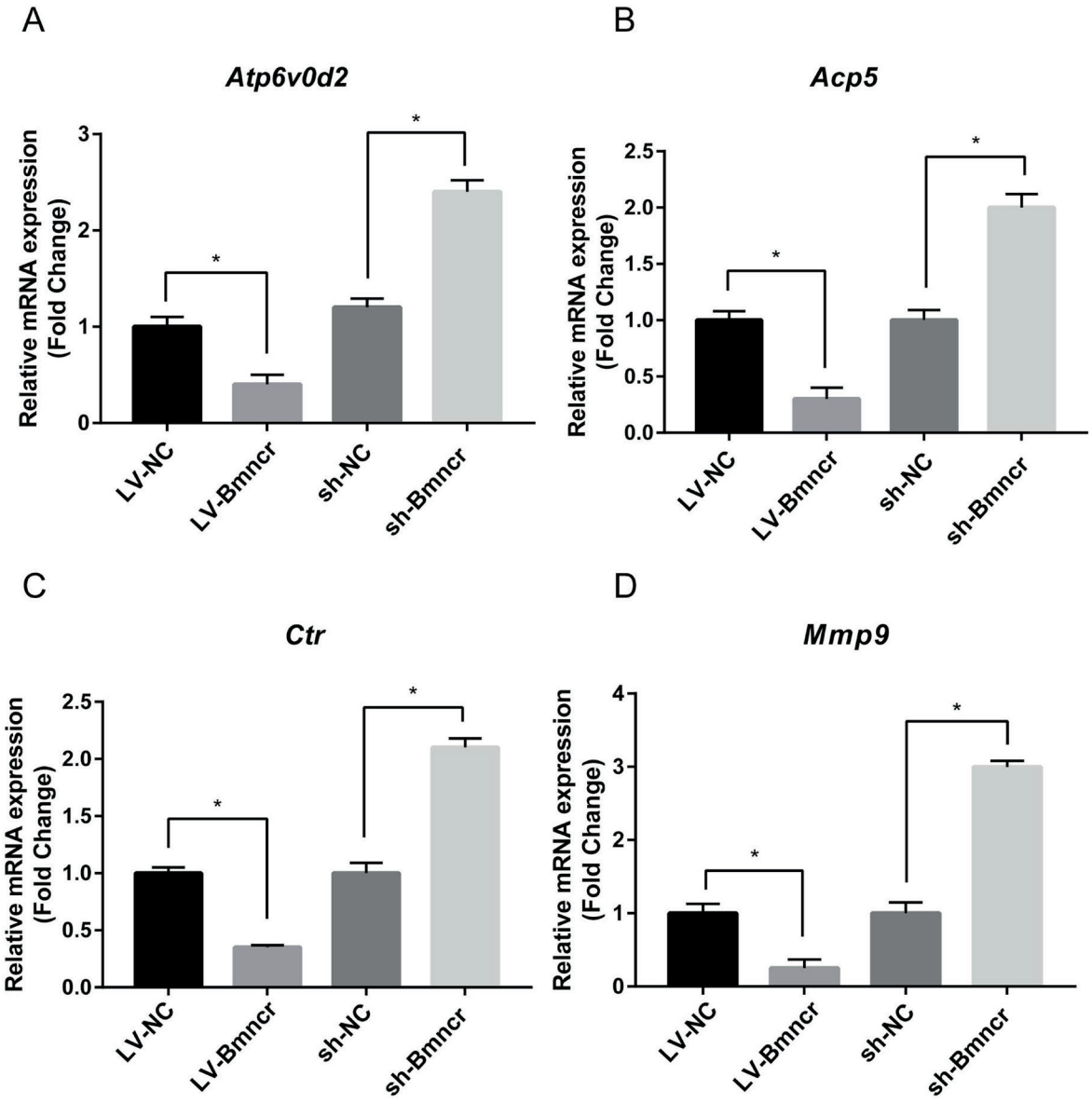

Figure 3. LncRNA Bmncr inhibited osteoclast-related gene expressions. Overexpression of Bmncr markedly downregulated expressions of Atp6v0d2 (**A**), Acp5 (**B**), Ctr (**C**) and Mmp9 (**D**) in RAW 264.7 cells. On the contrary, Bmncr knockdown upregulated their levels.

bone microarchitecture damage, decreased bone strength, and increased fracture risk. Osteoporosis is more common in the elderly, which is the main reason for disability in the elderly[22]. Osteoclasts are large, multinucleated hematopoietic cells derived from the monocyte/macrophage lineage[23]. Osteoclasts are formed by mononuclear precursor cell fusion, which are specific in resorbing mineralized bone matrix. In the pathogenesis of osteoporosis, dysregulated osteoclasts exert a vital role. The over activation of osteoclasts leads to higher bone resorption rate than bone formation rate, resulting in the imbalanced bone metabolism[24]. The important role of osteoclasts in the process of bone remodeling allows it to be the research focus in osteoporosis.

RANKL is produced by osteogenic lineage cells and osteoclasts, which initiates differentiation signaling pathway through binding to the RANK receptor expressed on the surface of monocyte/macrophage hematopoietic cells[25,26]. After osteoclast precursor cells are stimulated by

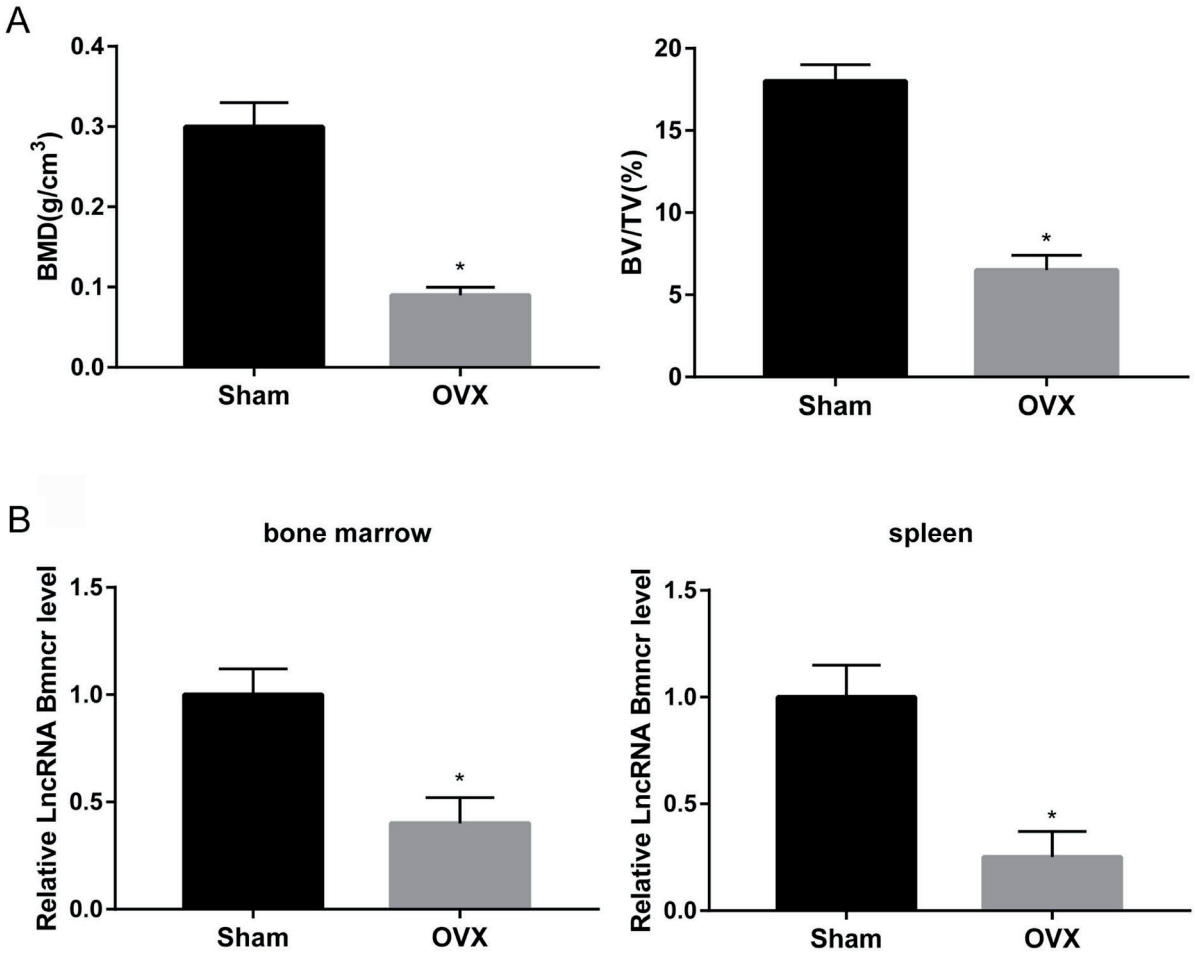

Figure 4. Downregulation of lncRNA Bmncr in bone marrow and spleen tissues of OVX mice. **A,** Compared with mice in sham group, OVX mice showed lower BMD (left) and BV/TV (right). **B,** Compared with mice in sham group, OVX mice showed a lower level of Bmncr in bone marrow (left) and spleen tissue (right).

RANKL factor, RANKL promotes proliferation and growth of osteoclast precursor cells, and it inhibits osteoclast apoptosis. Activation of osteoclast-related genes results in the maturation of osteoclast precursor cells and bone resorption[27]. RANKL and M-CSF can replace osteoblasts to induce osteoclast differentiation and maturation. Meanwhile, the differentiation and maturation of osteoclast precursor cells are inseparable from RANKL and M-CSF[28], indicating the positive regulatory effect of RANKL on osteoclast differentiation. Onal et al[29] have shown that RANKL is a key cytokine that activates the bone remodeling, which is considered as a therapeutic target in bone metabolic diseases. We investigated the biological mechanisms of RANKL-induced osteoclast differentiation.

LncRNA could not encode proteins, which accounts for 80% of non-coding RNAs[30]. Some authors[31,32] have identified the role of lncRNAs in cell differentiation and tumorigenesis. Multiple lncRNAs are differentially expressed in tumor diseases, neurodegenerative diseases, and others[33,34]. Kretz et al[35] found that lncRNA ANCR could regulate osteoblast differentiation. Wei et al[36] showed that lncRNA HOTAIR regulates osteoblasts to differentiate and proliferate by sponging miR-17-5p and its target Smad7 *via* BMPs/TGF-β pathway. LncRNA AK077216 can promote osteoclast differentiation and bone resorption[20]. This study explored the role of lncRNA Bmncr in osteoclast differentiation. LncRNA Bmncr was lowly expressed in the marrow and spleen of osteoporosis mice. Besides, its expression gradually decreased

during RANML-induced osteoclast differentiation, indicating its inhibitory role in the process of osteoclast differentiation. Furthermore, the role of Bmncr in regulating RANKL-induced osteoclastic differentiation was explored by lentivirus transfection in RAW 264.7 cells. The overexpression of Bmncr inhibited osteoclast differentiation and osteoclast resorption, and it downregulated expressions of Atp6v0d2, Acp5, Ctr, and Mmp9. It is suggested that lncRNA Bmncr exerts an inhibitory role in osteoclast differentiation and can be used as a potential indicator for osteoporosis treatment.

Conclusions

We showed that lncRNA Bmncr expression remains low in bone marrow and spleen of osteoporosis mice. LncRNA Bmncr inhibits the RANKL-induced osteoclast differentiation, thus alleviating the progression of osteoporosis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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