

LncRNA MALAT1 inhibits osteogenic differentiation of mesenchymal stem cells in osteoporosis rats through MAPK signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore whether long non-coding ribonucleic acid metastasis-associated lung adenocarcinoma transcript 1 (lncRNA MALAT1) could lead to osteoporosis (OP) by stimulating the activation of the mitogen-activated protein kinase (MAPK) signaling pathway.

MATERIALS AND METHODS: The OP model was first successfully established in rats. The expression of lncRNA MALAT1 in OP rats and normal rats was detected via quantitative Polymerase Chain Reaction (qPCR). Bone marrow mesenchymal stem cells (BMSCs) were cultured and transfected to establish the MALAT1 knockdown model. Subsequently, the apoptosis of mesenchymal stem cells in MALAT1 siRNA group and NC siRNA group was detected via flow cytometry. Meanwhile, the expressions of the MAPK signaling pathway proteins related to OP were detected via Western blotting. After alkaline phosphatase (ALP) staining in cells of both groups, early osteogenic differentiation of BMSCs was observed.

RESULTS: The results of qPCR showed that the expression of lncRNA MALAT1 in OP rats was significantly lower than that of normal rats. It was observed under a fluorescence microscope that there were a large number of siRNA particles in BMSCs. The expression of lncRNA MALAT1 in cells was detected via Real Time-fluorescence qPCR as well. The results indicated that siRNA transfection could effectively inhibit the expression of lncRNA MALAT1, indicating successful transfection.

Flow cytometry revealed that no significant difference was observed in the apoptosis of BMSCs between the MALAT1 siRNA group and NC siRNA group. Besides, the results of Western blotting showed that the expression levels of the MAPK signaling pathway-related proteins extracellular signal-regulated kinase 1/2 (ERK1/2) and P38 in MALAT1 siRNA group were significantly higher than those of the NC siRNA group. This indicated that inhibiting the expression of lncRNA MALAT1 might promote the activation of the OP-related MAPK pathway. According to the results of ALP staining, the depth of staining in MALAT1 siRNA group was markedly declined when compared with the NC siRNA group. Quantification of ALP activity demonstrated that ALP activity in the MALAT1 siRNA group was markedly declined compared with the NC siRNA group. The above results suggested that suppressing the expression of lncRNA MALAT1 could reduce the ALP activity of BMSCs. Furthermore, lncRNA MALAT1 inhibited osteogenic differentiation of BMSCs.

CONCLUSIONS: lncRNA MALAT1 was lowly expressed in OP rats. Moreover, it inhibited osteogenic differentiation of BMSCs by enhancing the activation of the MAPK signaling pathway, thereby promoting OP progression.

Key Words:

lncRNA MALAT1, MAPK signaling pathway, Osteoporosis, Mesenchymal stem cells.

Introduction

Osteoporosis (OP) is a systemic bone disease characterized by declined bone mass and bone tissue microstructural damage. OP can eventually lead to increased bone fragility and fragility fracture easily. At present, OP has been identified as one of the three major chronic diseases with diabetes and hypertension, which mostly occurs in post-menopause and elderly population. The pathogenesis of OP is mainly related to estrogen and age. Due to the decrease of estrogen after menopause, the balance between osteoblasts and osteoclasts is destroyed. This may lead to decreased bone mass and bone density as well as changes in bone tissue structure, thereby increasing bone fragility and the risk of fracture¹. In senile OP, oxidative stress is caused by aging. Various inflammatory mediators are elevated, such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1, IL-6, IL-7, and prostaglandin E2 (PGE2). The expressions of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL) are induced. Meanwhile, bone mass declines by stimulating osteoclasts and inhibiting osteoblasts. Currently, clinical therapeutic methods for OP mainly include hormone replacement², calcium supplement³, inhibition on bone resorption, etc. However, due to the toxic and side effects of drugs, malabsorption and poor patient compliance, the clinical therapeutic effect is far from satisfactory. Therefore, further studies are urgently needed to explore the pathogenesis of OP.

Long non-coding ribonucleic acids (lncRNAs) are a kind of non-coding RNAs with more than 200 nt in length. They have been found to participate in and regulate gene expression⁴⁻⁶. At first, lncRNAs were considered as by-products produced during RNA Polymerase II transcription, as well as a “noise” in the gene expression regulation without corresponding biological functions. With the deepening of research, the functions of lncRNAs have been gradually discovered. In recent years, researchers have explored the roles of lncRNAs in the occurrence and development of bone diseases. Several reports have shown that lncRNAs play vital roles in the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) and arthritis. For example, spinal deformity and abnormal osteogenesis occur in lncRNA HOTAIR knockout mice. Studies⁷ have found that lncRNA HOTAIR alters the

level of histone methylation in the promoter region of target genes to regulate its transcriptional activity. The expression of lncRNA H19 increases gradually during the osteogenic differentiation of BMSCs. As a ceRNA, lncRNA H19 regulates osteogenic differentiation of BMSCs, which can bind to miR-141 and miR-22 to inhibit their expressions in BMSCs. Meanwhile, the expression of p-catenin, as the target gene of miR-141 and miR-22, is indirectly elevated by increased expression of lncRNA H19. This can eventually promote osteogenic differentiation of BMSCs^{8,9}. Moreover, lncRNA DANCR regulates gene expression by regulating epigenetic modification. It can also inhibit the expression of RUNX2, thus reducing the osteogenic differentiation of BMSCs⁹. However, no reports have investigated the role of lncRNA MALAT1 in osteogenic differentiation of BMSCs in OP so far.

Mitogen-activated protein kinase (MAPK) signaling pathway is an important signal transduction system that commonly exists in eukaryotic cells and mediates cellular response. It includes extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, c-Jun N-terminal kinase (JNK) pathway, P38 pathway and ERKS pathway. During the imbalance of bone metabolism in OP, activated MAPK signaling pathway can promote the proliferation and differentiation of osteoclasts. RANKL secreted by osteoblasts in bone tissues acts on RANK in non-differentiated osteoclasts to activate the MAPK signaling pathway, ultimately inducing differentiation into osteoclasts. The differentiation of BMSCs is accurately regulated by mechanical stimuli and molecular signals in the extracellular environment. This process has been found to involve complex pathways at the transcriptional and post-transcriptional levels. Zhang et al¹⁰ studied the expression profile and function of lncRNAs during the differentiation of BMSCs into osteoblasts or osteoclasts. They have found that lncRNA xr-11050 regulates the differentiation of BMSCs into osteoblasts or osteoclasts by controlling the MAPK signaling pathway. The results suggest that BMSCs may become the most promising cell type for bone regeneration and repair after bone injury. Combined with the pathogenesis of OP, therefore, we hypothesized whether lncRNA MALAT1 could inhibit the osteogenic differentiation of BMSCs by stimulating the activation of the MAPK signaling pathway, eventually resulting in OP.

Materials and Methods

Grouping of Laboratory Rats and Establishment of OP Rat Model

This study was approved by the Animal Ethics Committee of Jilin University Animal Center. A total of 20 female Sprague-Dawley (SD) rats aged 12 weeks and weighing 250-300 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All rats were randomly divided into two groups, including group A ($n=10$) and group B ($n=10$). Before modeling, bone mineral density (BMD) was measured using dual-energy X-ray absorptiometry (NORLAND Corporation, Cranbury, NJ, USA). Ovariectomy was performed in rats of group A to establish the OP model. Briefly, the rats were anesthetized with 3% pentobarbital sodium (30 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) before the operation and fixed. The hair was shaved off and the skin was cut, followed by ovarian removal through the retroperitoneal approach. After the ligation of blood vessels for hemostasis, the skin was sutured layer by layer. After the operation, 80×10^4 U penicillin (Shanghai Xianfeng Pharmaceutical Co., Ltd. Shanghai, China, batch No.: S100824) was intramuscularly injected twice a day for 3 consecutive days. However, rats in group B underwent no operative treatment. After 8 weeks, BMD was measured again using dual-energy X-ray absorptiometry to confirm the successful establishment of the OP model in rats.

Real Time-Polymerase Chain Reaction (RT-PCR)

Femoral tissues in both groups were first added with 2-3 mL of TRIzol (Invitrogen, Carlsbad, CA, USA), cut into pieces and fully ground into homogenate powder. Subsequently, the samples were transferred into 1.5 mL Eppendorf (EP; Hamburg, Germany) tubes, followed by incubation at room temperature for 5 min to be fully lysed. Total RNA was then extracted using the TRIzol Reagent. The absorbance (A_{260}/A_{280}) ratio was measured to determine RNA concentration. Finally, stepwise amplification was conducted according to relevant instructions, and reaction products were subjected to Real Time-Polymerase Chain Reaction (RT-PCR). Primer sequences used in this study were shown in Table I. The expression level of lncRNA MALAT1 was calculated by the $2^{-\Delta\Delta CT}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

Table I. Primer sequences.

Gene	Primer sequence
Gene	5'-3' TCAGTGTGGGGCAATCTT 3'-5' CGTTCTTCCGCTCAAATCC
GAPDH	5'-3' ACAACTTTGGTATCGTGAAGG 3'-5' GCCATCACGCCACAGTTTC

Cell Transfection

To study the role of lncRNA MALAT1 in the differentiation of BMSCs, it is necessary to regulate the expression of lncRNA MALAT1 in BMSCs. However, the length of lncRNA MALAT1 is long, and the lncRNA MALAT1 overexpression plasmid cannot be constructed successfully. Therefore, the expression of lncRNA MALAT1 in BMSCs was regulated using siRNA in a targeted manner. BMSCs were transfected with siRNA according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). MALAT1 siRNA and NC siRNA were purchased from Transheep (Shanghai, China). To detect the transfection efficiency, BMSCs were observed under a fluorescence microscope after Cy3-labeled siRNA transfection.

Apoptosis Assay

The apoptosis of BMSCs was detected *via* flow cytometry. After transfection, cells in MALAT1 siRNA group and NC siRNA group were collected, washed twice with cold Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) and resuspended in binding buffer. Subsequently, the cells were stained in strict accordance with fluorescein isothiocyanate (FITC) Annexin V apoptosis assay kit (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature in the dark for 30 min. Finally, apoptosis detection was performed using flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) within 1 h.

Western Blotting

Total cell lysates were lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China) to extract the total protein in tissues. The concentration of protein was detected according to the instructions of the bicinchoninic acid (BCA) protein assay kit (Pierce, Waltham, MA, USA). After separation, the proteins were transferred onto membranes and incubated with primary antibodies (1:1000) prepared with buffer (Beyotime Biotechnology, Shanghai, China) in an incubator at 4°C over-

night. GAPDH (D4C6R) antibody (97564, CST, USA) was used as an internal reference. The next day, the membranes were washed and incubated with the corresponding secondary antibody at room temperature for another 1 h. The membranes were washed again, followed by color development and fixation according to the instructions of SuperSignal West Dura Extended Duration Substrate Luminescent Substrate Kit (Millipore, Billerica, MA, USA). Finally, the optical density of immunoreactive bands was analyzed using BandScan 5.0 software. This experiment was repeated 3 times.

Osteogenic Induction and Alkaline Phosphatase (ALP) Staining

Cells in the MALAT1 siRNA group and NC siRNA group were first inoculated into 6-well plates at a density of 1×10^5 cells/well. When 80% of cell fusion, osteogenic induction solution was added to induce differentiation for 21 d. Osteogenic indexes were then detected. At 7 d after osteogenic induction, the cells were taken and washed with PBS 3 times. They were fixed with 4% paraformaldehyde (PFA) for 1 min and washed again with PBS 3 times. The reaction substrate (Naphthol AS-Bi Phosphate) was dissolved in dimethylformamide solution (400 μ L), while color developing agent fast violet B (40 mg) was dissolved in PBS (20 mL). After that, the substrate solution and developing solution were mixed evenly and added into the cells, followed by incubation at 37°C for 30 min. Finally, staining was observed under a microscope or camera.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Normality test and homogeneity test of variance were performed for all data before processing. In the case of normality and homogeneity of variance, *t*-test was adopted to compare the difference between the two groups. One-way ANOVA was applied to compare the differences among dif-

ferent groups, followed by Post-Hoc Test (Least Significant Difference). $p < 0.05$ suggested that the difference was statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Establishment of OP Rat Model

After 20 randomly-selected female SD rats were numbered, BMD was measured using dual-energy X-ray absorptiometry (Table II). All rats were randomly divided into two groups, including group A ($n=10$) and group B ($n=10$). Rats in group A underwent ovariectomy, while those in group B received no operative treatment. After 8 weeks, BMD was measured again using dual-energy X-ray absorptiometry (Table II). The results showed that BMD in group A after ovariectomy was significantly lower than that before ovariectomy. Meanwhile, BMD in group A after ovariectomy was markedly lower than that of group B, and the differences were statistically significant ($p < 0.05$). This suggested the successful establishment of OP model in rats.

Expression of lncRNA MALAT1 in Both Groups Detected via RT-PCR

Femoral tissues were extracted from 4 rats in each group, smashed and mixed evenly, followed by extraction of total RNA. The expression of lncRNA MALAT1 in group A and group B was detected via RT-PCR. As shown in Figure 1, the expression of lncRNA MALAT1 in group A was significantly declined when compared with group B, and the difference was statistically significant ($p < 0.05$). This indicated that the expression of lncRNA MALAT1 was significantly inhibited in OP rats.

Cell Transfection Results

BMSCs were transfected with siRNA according to the instructions of Lipofectamine 2000. To detect the transfection efficiency of Lipofectamine 2000, BMSCs were observed under a flu-

Table II. Measurement of BMD before and after ovariectomy in both groups.

Group	BMD before ovariectomy (g/cm ²)	BMD at 8 weeks after ovariectomy (g/cm ²)
Group A	0.3924 ± 0.0042	0.2104 ± 0.0036
Group B	0.3918 ± 0.0048	0.3920 ± 0.0038

$p < 0.05$: in the comparison of BMD in group A after ovariectomy with group B.

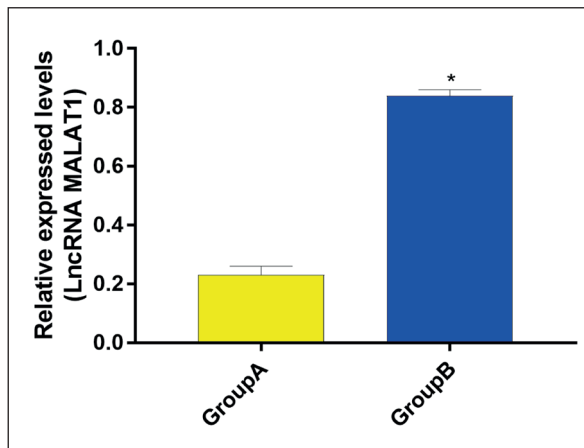


Figure 1. Expression of LncRNA MALAT1 in both groups detected *via* RT-PCR. * $p < 0.05$ vs. group A.

orescence microscope after Cy3-labeled siRNA transfection. The results found that there were a large number of siRNA particles in BMSCs (Figure 2). At the same time, the cells were collected at 72 h after transfection to extract total RNA. The expression of LncRNA MALAT1 in transfected cells was detected *via* Real Time-fluorescence qPCR. The results manifested that siRNA could effectively inhibit the expression of LncRNA MALAT1 (Figure 3).

Apoptosis Assay Results

BMSCs were first transfected with NC siRNA and MALAT1 siRNA, respectively. The cells were then collected after 72 h, and cell apoptosis was detected using flow cytometer. The results showed that no significant difference was observed in the apoptosis of BMSCs between

MALAT1 siRNA group and NC siRNA group. The above findings indicated that suppressing the expression of LncRNA MALAT1 had no influence on the apoptosis of BMSCs (Figure 4).

Western Blotting

The protein expressions of ERK1/2 and P38 in cell extracts of both groups were detected *via* Western blotting. As shown in Figure 5, the protein expressions of ERK1/2 and P38 in the MALAT1 siRNA group were markedly higher than those of the NC siRNA group ($p < 0.01$). The results suggested that the MAPK pathway was significantly enhanced in BMSCs after LncRNA MALAT1 was inhibited.

Osteogenic Induction Differentiation and ALP Staining

When 80% of cell fusion, osteogenic induction solution was added to induce differentiation for 21 d continuously. Osteogenic indexes were then detected. The mRNA expression levels of osteogenic differentiation-related genes (including OCN, Collagen I and RUNX2) were detected *via* Real Time-fluorescence qPCR. The results showed that the mRNA expression levels of OCN, Collagen I and RUNX2 in the MALAT1 siRNA group were remarkably declined when compared with those of the NC siRNA group. This suggested that inhibiting the expression of LncRNA MALAT1 could significantly reduce the mRNA expression levels of osteogenic differentiation-related genes (Figure 6A). Besides, BMSCs were stained using ALP staining kit after transfection with NC siRNA and MALAT1 siRNA, respective-

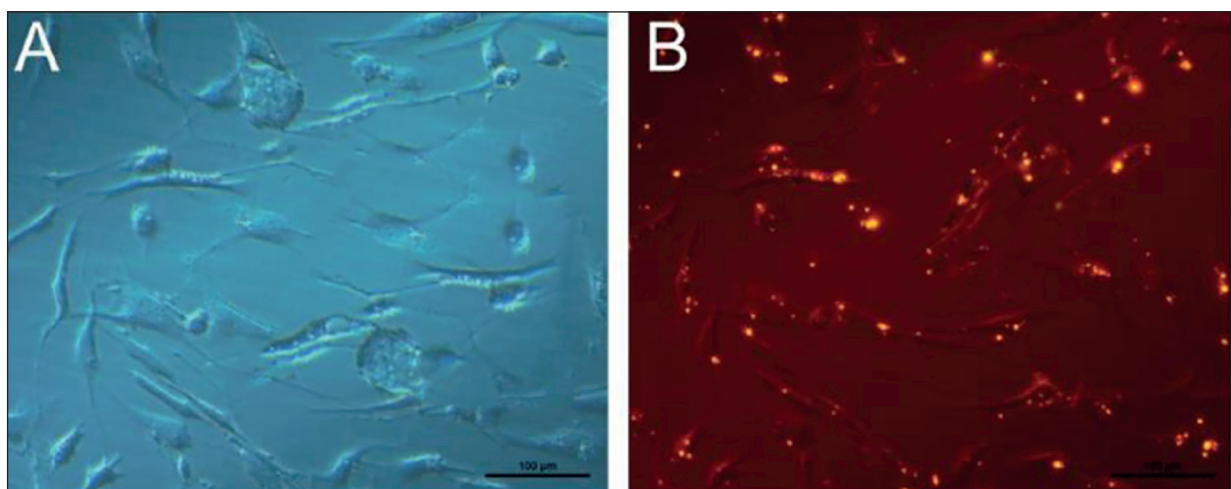


Figure 2. Transfection efficiency of siRNA (magnification $\times 200$). *A*, Light field. *B*, Dark field.

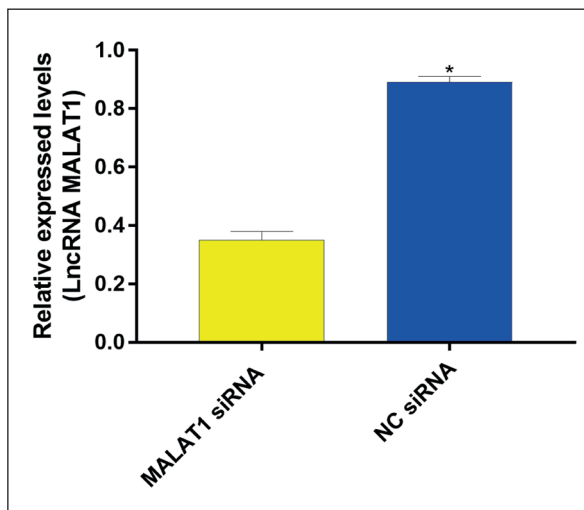


Figure 3. Knockdown efficiency of lncRNA MALAT1 detected *via* Real Time-fluorescence qPCR.

ly. It was found that the depth of staining in the MALAT1 siRNA group was markedly declined compared with the NC siRNA group (Figure 6B). ALP activity was quantified, and the results revealed that ALP activity in the MALAT1 siRNA group was significantly lower than that of the NC siRNA group (Figure 6C). The above results suggested that inhibiting

the expression of lncRNA MALAT1 reduced ALP activity of BMSCs. Furthermore, lncRNA MALAT1 could inhibit osteogenic differentiation of BMSCs.

Discussion

OP is a systemic metabolic bone disease, which is mostly caused by the destruction of balance between bone formation of osteoblasts and bone resorption of osteoclasts due to estrogen deficiency. OP may eventually result in bone remodeling disorders¹¹. Previous studies have mainly focused on the increase in bone resorption induced by osteoclasts. However, few reports have investigated the defect of bone formation induced by osteoblasts. BMSCs are the source of osteoblasts in bone tissues. Abnormal osteogenic differentiation of BMSCs has been found in both OP patients and mouse models^{12,13}. These findings suggest that abnormal osteogenic differentiation of BMSCs may be related to the occurrence and development of postmenopausal OP. However, its specific regulatory mechanism remains unclear. In this experiment, therefore, this mechanism was explored at the lncRNA and signaling pathway molecule levels.

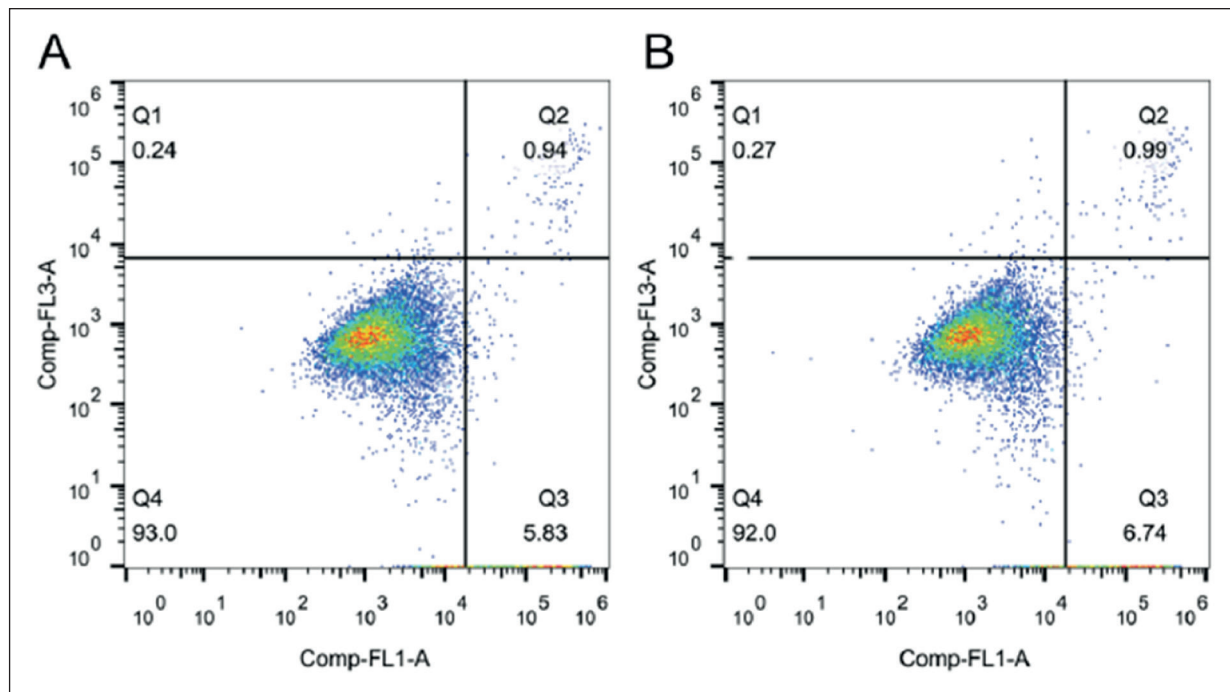


Figure 4. Apoptosis of BMSCs after knockdown of lncRNA MALAT1 detected *via* flow cytometry.

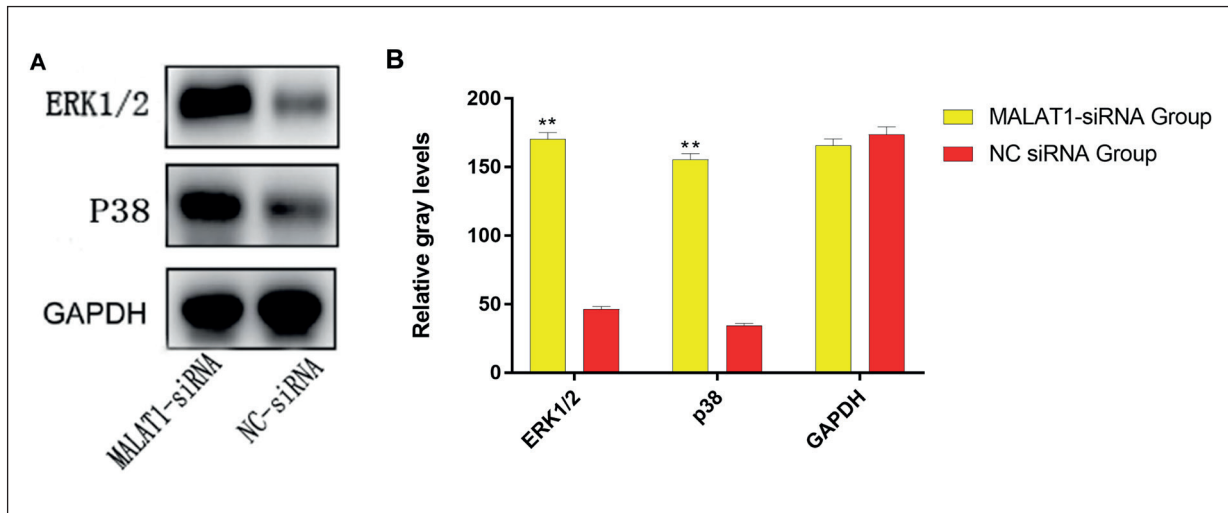


Figure 5. Protein expressions of ERK1/2 and P38 analyzed *via* Western blotting. The protein expressions of ERK1/2 and P38 in MALAT1 siRNA group were markedly higher than those of the NC siRNA group, and there were statistically significant differences ($p < 0.01$).

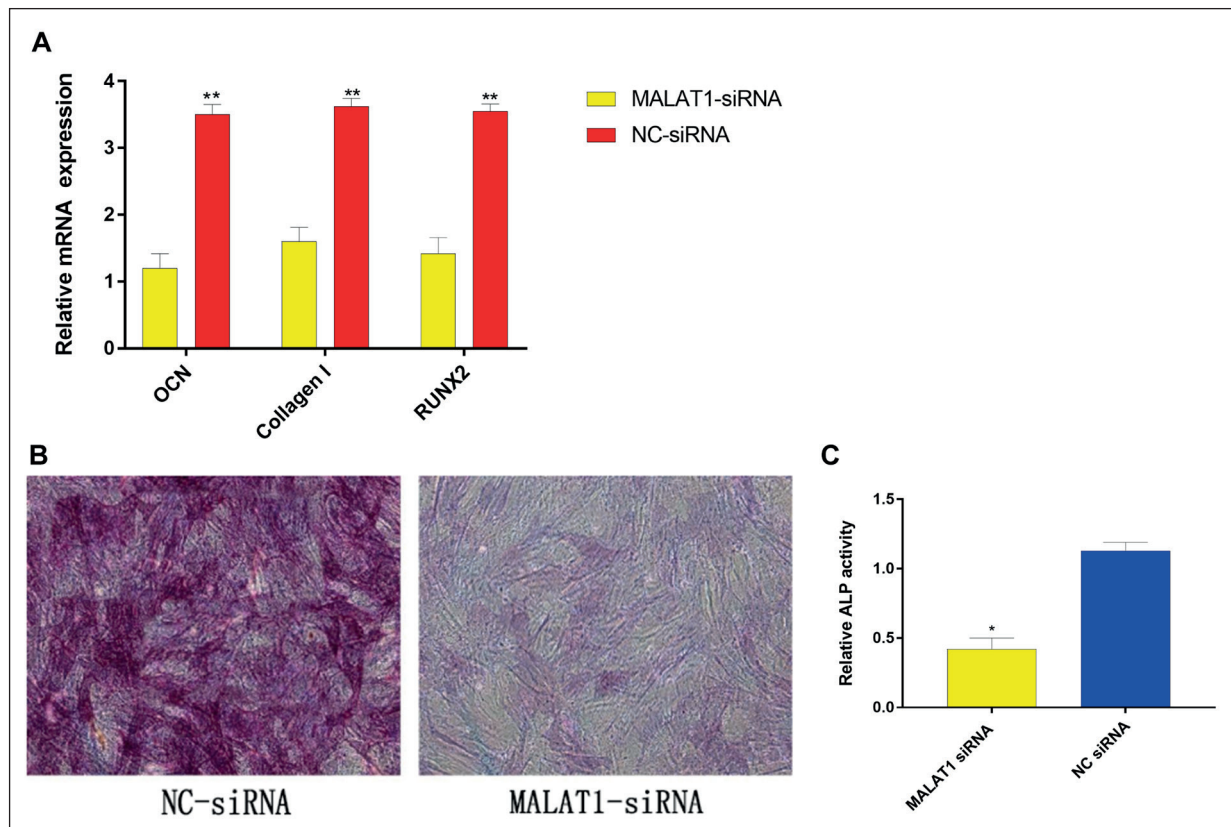


Figure 6. *A*, The mRNA expression levels of osteogenic differentiation-related genes after knockdown of lncRNA MALAT1 detected *via* Real Time-fluorescence qPCR (** $p < 0.01$). *B*, Osteogenic differentiation of BMSCs after knockdown of lncRNA MALAT1 detected *via* ALP staining (magnification $\times 200$). *C*, Quantitative detection of ALP activity of BMSCs after knockdown of lncRNA MALAT1.

At present, lncRNAs have become an interesting research field. It has been demonstrated that they not only exist in patients with hematological tumors, but also stably exist in human plasma or serum or some other non-neoplastic diseases¹⁴. Therefore, specific lncRNAs have been widely applied in the diagnosis and treatment of various diseases. Meanwhile, they possess potential values in clinical application^{15,16}. In the present work, the OP model was successfully established in rats. The expression of lncRNA MALAT1 in OP rats and normal rats was detected *via* RT-PCR, to determine whether lncRNA MALAT1 promoted OP through a specific pathway. Meanwhile, BMSCs were cultured and transfected to establish the model of lncRNA MALAT1 knockdown *in vitro*. This might help to further study the mechanism of lncRNA MALAT1 in OP. Flow cytometry indicated that there was no significant difference in the apoptosis of BMSCs between MALAT1 siRNA group and NC siRNA group. This indicated that lncRNA MALAT1 in OP model could not promote OP by affecting the apoptosis of BMSCs. Subsequently, Western blotting was performed to detect the expressions of proteins in OP-related MAPK signaling pathway. The results showed that the levels of the MAPK signaling pathway-related proteins (ERK1/2 and P38) in MALAT1 siRNA group were significantly higher than those of the NC siRNA group. These findings indicated that inhibiting the expression of lncRNA MALAT1 might promote the activation of OP-related MAPK pathway. In addition, ALP staining was conducted for cells in both groups. ALP is an important marker for early osteogenic differentiation, which plays a key role in the calcification process *in vitro*¹⁷⁻¹⁹. Therefore, early osteogenic differentiation of BMSCs can be observed by detecting ALP synthesis²⁰. According to the results of ALP staining, the depth of staining in MALAT1 siRNA group was significantly declined when compared with that of the NC siRNA group. ALP activity was then quantified, and it was found that ALP activity in the MALAT1 siRNA group was markedly lower than the NC siRNA group. This suggested that suppressing the expression of lncRNA MALAT1 could reduce the ALP activity of BMSCs. Furthermore, lncRNA MALAT1 could inhibit osteogenic differentiation of BMSCs.

Conclusions

We found that lncRNA MALAT1 was lowly expressed in OP rats. Meanwhile, it inhibited os-

teogenic differentiation of BMSCs by enhancing the activation of the MAPK signaling pathway, thereby promoting OP progression. Our findings might provide a living model for the further study on the mechanism of OP, and could lay a foundation for an in-depth study on the human OP. In the future, more experiments are still needed to deeply explore the mechanism of OP.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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