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LncRNA HOTAIR inhibited osteogenic differentiation of BMSCs by regulating Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: This study aims to investigate whether HOX transcript antisense RNA (HOTAIR) can participate in the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) by regulating the Wnt/β-catenin pathway, thereby participating in the pathogenesis of osteoporosis.

PATIENTS AND METHODS: We detected the expression level of HOTAIR in 60 osteoporosis patients and 60 normal controls by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Meanwhile, BMSCs derived from human or rats were subjected to determination of HOTAIR level. Subsequently, the effects of HOTAIR on osteogenic differentiation were evaluated by the activity of Alkaline Phosphatase (ALP), Alizarin Red S (ARS) staining, ALP staining and osteogenic-specific gene expression. The expression level of proteins related to the Wnt/β-catenin was determined by Western blot, and ALP activity was detected by ALP activity determination kit and alizarin red staining after knockdown or overexpression of HOTAIR, as well as the treatment of DKK1 or the Wnt pathway antagonist. Finally, osteoporosis model in rats was established by ovariectomy (OVX). We examined protein levels of HOTAIR, β-catenin, CyclinD, C-myc, and Runx2 in rat bone tissues. Bone morphology was observed in each group as well.

RESULTS: The serum and BMSCs levels of HOTAIR in patients with osteoporosis were remarkably higher than that in normal people. Inhibition of HOTAIR induced increased ALP activity increased osteogenic marker genes and enhanced number of calcified nodules in BMSCs. However, the overexpression of HOTAIR exhibited the opposite effects. HOTAIR inhibited the expression level of Wnt/β-catenin pathway-related protein. Also, Wnt pathway antagonist DKK1 partially reversed the regulatory effects of HOTAIR on Wnt/β-catenin. DKK1 treatment markedly reduced the promotive effect of HOTAIR knockdown on ALP activity, ALP content and calcification ability of BMSCs. DKK1 administration in rats undergoing OVX showed worse bone morphology relative to controls. Protein levels of HOTAIR, β-catenin, CyclinD, C-myc and Runx2 remarkably downregulated in OVX rats administrated with DKK1.

CONCLUSIONS: HOTAIR inhibits osteoblast differentiation of rat BMSCs. The underlying mechanism of which may be related to the mediation of Wnt/β-catenin pathway.

Key Words:
HOTAIR, Bone mesenchymal stem cells, Osteogenic differentiation, Wnt/β-catenin pathway.

Introduction

Osteoporosis is a systemic disease characterized by decreased production of bone, increased bone resorption, increased bone fragility and brittle fracture. The main clinical manifestations are somatic pain, shortening height, humpback, brittle fracture, which are especially vulnerable in the elderly as the proximal femoral fractures. Decreased bone mineral density is also one of the diagnostic criteria for osteoporosis[1]. Researches have shown that nearly 30 million women aged 50 and over in the United States present low bone mass or osteoporosis. The incidence of osteoporosis gradually increased with age. Osteoporotic fractures are the most serious consequences of senile osteoporosis, which easily leads to myelopathy fractures, vertebral compression fractures and other diseases in the elderly. Osteoporosis has a high incidence and great risk of surgery with

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poor outcomes. It is one of the important reasons for the disability and death of the elderly, which increases a heavy financial burden on society. Therefore, it is necessary to conduct an in-depth study on its pathogenesis.

Long noncoding ribonucleic acids (lncRNAs) are a family of non-coding RNA (ncRNA) transcripts produced by RNA polymerase II with 200 to 100 000 nt in length, which has no or little ability of protein coding. LncRNAs, as well as small interfering RNAs (siRNAs), Micro ribonucleic acids (miRNAs) and RNAs interact with Piwi protein, all belong to regulatory non-coding RNAs[2]. According to the location of lncRNA in the genome, it can be further divided into lncRNA in the intergenic region, natural antisense lncRNA and intronic lncRNA[3]. In contrast to the highly evolutionary conservation of miRNAs and snoRNAs, lncRNA sequences are less conserved and have greater variability among species, which is considered to be the result of species evolution, suggesting that lncRNAs have an important regulatory role in higher organisms. In the 1980s, studies have reported the presence of circulating RNA[4,5], which laid the foundation for the subsequent study of circulating lncRNAs. Since RNA is unstable and easily degraded by ribonuclease in the blood, the mechanism of how to secrete lncRNAs from the human blood and maintain its stability are not yet fully understood. According to the existing research and analysis, circulating lncRNAs may originate from living cells, that is, circulating lncRNAs may come from the active secretion of living cells, and most of them are presented as exosomes and microvesicles.

HOX transcript antisense RNA (HOTAIR) is the first lncRNA that was found to be trans-transcriptionally regulated. Its Deoxyribonucleic Acid (DNA) sequence is located between HOXC11 and HOXC12 on chromosome 12, including 5 short exons and 1 long exon[6]. As a member of lncRNA, HOTAIR has been proved to play an essential function in the development of tumors[7, 8]. However, its role in osteoporosis has not been reported.

Bone marrow mesenchymal stem cells (BMSCs), the origin of osteoblasts, have multi-directional differentiation potentials and can differentiate into various kinds of cells under certain conditions, including osteoblasts. BMSCs have many advantages, such as extensive source, convenient extraction, rapid expansion, strong plasticity, easy transfection, no immune rejection and no ethical debate, etc[9]. Therefore, based on the detection of HOTAIR expression in patients with osteoporosis, we investigated the effects of HOTAIR on the osteogenic differentiation of BMSCs and its downstream pathway, which provides a new idea for investigating the pathogenesis mechanism of osteoporosis.

Patients and Methods

Research Subjects and Sample Collection

A total of 60 patients diagnosed as osteoporosis from June 2015 to August 2017 and 60 normal volunteers as controls were selected, all of whom were female. No significant difference in age between groups was observed. Osteoporosis group inclusion criteria were as follows. 1. Complete clinical data and imaging information were needed for patients confirmed as osteoporosis. 2. All subjects signed informed consent. 3. Subjects in each group had no history of diabetes, hypertension, heart disease and other complications. 5 mL of venous blood was extracted from each subject in the morning under a fasting state. The blood sample was placed for 30 min, and centrifuged at 4°C, 3 000 g/min for 10 min, then the upper serum was harvested (non-hemolytic state), and centrifuged at 4°C, 13 500 g/min for another 15 min. The upper serum was collected into the Eppendorf (EP) tube and placed in a -80°C refrigerator for later experiments. This study was approved by the Ethics Committee of Gansu Provincial Hospital of Traditional Chinese Medicine. Signed written informed consents were obtained from all participants before the study.

Extraction of Serum RNA

0.25 mL of serum and 2-8 μL of Polyacryl Carrier were added to 0.75 mL of TRI Reagent BD, and another 0.2 mL of chloroform was added and shaken for 15 s. After incubation for 2-5 min, the mixture was centrifuged at 4°C, 12 000 g/min for 15 min. After centrifugation, the aqueous phase complex was transferred to another centrifuge tube. Then isopropanol was added to the mixture to extract RNA. Gel-like or white RNA was centrifuged a pellet was formed at the bottom of the tube. Ethanol was added and mixed to wash the RNA pellet. After centrifugation, the ethanol solution was removed, and the RNA precipitate was dried in the air for 5 min. The RNA was dissolved in RNase-free water and stored at -80°C for later use.

Determination of Bone Mineral Density

We used Toshiba Aquilion16 row CT scanner for spiral scanning. Scanning parameters were 120 kv, 125 MAS, 1.0 mm of each layer, 40 cm of field vision, 90 cm of scanning bed height. US Mindways Corporation 5 sample solid QCT phantom was accepted. The scanning method was as follows. The subject was in the supine position, the standard phantom was placed in the subject's medullary joint, the phantom should be as close as possible to the subject, and the midline of the phantom superimposed on the subject's midline. The spiral scan was performed on the 5 cm range above the acetabulum and below the trochanter of the femur.

Bone Volume Fraction and Trabecular Thickness

Femoral heads of patients or rats were collected, frozen, and reserved at -70°C. Before cutting, femoral heads were unfrozen at -20°C, 4°C and room temperature sequentially. 7 mm of specimens were taken within a maximum diameter perpendicular to the stress direction of the femoral head using a circular diamond cutting machine (Shenyang Kejing Equipment Manufacturing Co., Ltd. Model number, SXJ-2, Shenyang, China). The micro CT used in this experiment was GE eXplore LocusSP Specimen Scanner (GE Health Care Co., London, UK). Before scanning, specimens were stored in a 40% ethanol solution and treated specimens were placed vertically along the long axis. The sample holder was placed in a 70 KPa vacuum box for 20 min. The scanning parameters were as follows, 80 kV of voltage, 80 μA of current, scanning mode of 360° rotation, 270 min of scanning time. At the same time, the standard phantom was scanned for the preparation of CT correction. After the scan, the bone tissue from the specimen center (4.3 mm×4.3 mm×4.3 mm) was selected as the 3D reconstruction of 16.0 μm×16.0 μm ×16.0 μm voxels in the Region of Interest (ROI). Quantitative analysis was performed using MicroView 2.1.1 + Advanced Bone Analysis (GE Health Care Co., London, UK) software. Analytical parameters included bone volume fraction (BV/TV). The microstructure parameters of cancellous bone were measured by three-dimensional direct measurement and the structure was filled with the largest sphere by distance transformation to calculate the the mean trabecular thickness (Tb.Th.)

Establishment of Osteoporosis Model in Rats by OVX

A total of 40 6-week-old female SD rats (100-120 g) were selected. No significant difference in body weight was observed prior to animal procedures. Rats were habituated at an environment with temperature of 22 ± 5°C, humidity of 50 ± 10% and 12 h/12 h light/dark cycle. Rats had free access to food and water. After a one-week habituation, rats were intraperitoneally administrated with 50 mg/kg pentobarbital sodium for performing OVX. These rats were randomly assigned into four groups: PBS+sham group, DKK1+sham group, PBS+OVX group and DKK1+OVX group, with 10 in each group. Phosphate-buffer saline (PBS) or DKK1 was administrated in the tail vein of rats. This study was approved by the Animal Ethics Committee of Gansu Provincial Hospital of Traditional Chinese Medicine Animal Center.

BMSCs Isolation and Culture

Primary bone marrow MSCs were harvested from the femur and tibia of 3-week-old Sprague-Dawley (SD) rats. The soft tissue was removed, and both ends of the femur and tibia were resected. The bone marrow of the femoral and tibial bones was flushed out with 5 mL of Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, South Logan, UT, USA) (L) medium. The culture medium was collected and centrifuged at 900 g/min for 10 min. The supernatant was discarded. Precipitates were well mixed with DMEM (L) medium to prepare the cell suspension. The supernatant was gently overlaid on a Percoll separating a solution of 1.073 g/L and centrifuged at 900 g/min for 30 min to collect interface layer cells. DMEM (L) medium was centrifuged twice and finally cultured in DMEM (L) containing 10% fetal bovine serum (FBS, Hyclone, South Logan, UT, USA), 1% L-glutamine, 1% penicillin, 1% streptomycin and 1% 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES). The medium was resuspended and then homogenized and inoculated into a petri dish at 37°C and 5% CO_2. After 4 h, the culture medium was changed for the first time to remove the suspended cells. After that, the medium was replaced every 2 days. After 70-80% of the cells were fused, the cells were passaged with 2.5 g/L trypsin. Differentiation experiments were performed using mesenchymal stem cells in the third generation.

Extraction and Culture of BMSCs

After approval by the Ethics Committee, and acquisition of informed consent form, bone mar-

row samples were extracted from osteoporosis patients and healthy volunteers. Samples were centrifuged using the Ficoll-Hypaque method, and cells were suspended into α-MEM containing 10% FBS, 2 mM glutamine, and 100 U/mL penicillin and 100 μg/mL streptomycin. Medium was replaced every two days. Third-passage BMSCs were harvested for subsequent experiments.

Osteogenic Induction of BMSCs

The third generation of well-grown BMSCs from rats were seeded into 6-well plates at a density of 3.0×10^4/mL for osteogenic differentiation. The osteogenic induction medium was as follows: High glucose medium supplemented with 10% FBS, 1% L-glutamine, 10 nmol/L of dexamethasone, 10 mmol/L of β-glycerophosphate, 50 μg/ml of ascorbic acid, 1% penicillin-streptomycin and 1% HEPES. The cells were inducted for a total of 7 d -14 d.

Flow Cytometry Identification of BMSCs

The third generation of BMSCs (cell density was up to 80%) was trypsinized, centrifuged and the supernatant was discarded. Cells were resuspended and adjusted to the density to 3000-6000 cells/μL. CD29, CD90 and, CD45 antibody were used to label cells for 30-min incubation at room temperature, with untreated BMSCs and isotype-control as controls.

ARS Staining

After osteogenic induction for 14 days, the culture medium was discarded and washed 3 times with PBS. 60% isopropanol was used for fixing cells for 60 s and washed with PBS for 2 min. 10% ARS solution was utilized for staining for 3 min. After washed with PBS for 3 times, mineralized nodules were observed by optical microscope.

Construction of Lentiviral Vector and Cell Transfection

The plasmid complementary deoxyribonucleic acid -HOTAIR lentivirus shRNA vector and the control shRNA targeting GFP were designed and synthesized by the reagent company. Well-grown MSCs in the third generation were selected for lentiviral transfection. The interference sequences were sh-HOTAIR1 (CGAAGGTGAAAGCGAACCA), sh-HOTAIR2 (GGAACGGATTTAGAAGCCT), sh-HOTAIR3 (CAATATATCTGTTGGGCGT) and shRNA-NC (TTCTCCGAACGTGTCACGT).

Cell RNA Extraction and Quantitative Real-Time PCR detection

TRIzol (Invitrogen, Carlsbad, CA, USA) kit was used to extract total RNA of cells in different groups after culturing for 7 days. Extracted RNAs were reverse transcribed into complementary deoxyribonucleic acid (cDNA) and amplified for quantitative real-time PCR. The following osteogenic related genes were detected, ALP, Bglap and Runx2. Primer sequences were ALP (F: 5'-AAGGCTTCTTCTTGCTGGTG-3', R: 5'-GCCTTACCCTCATGATGTCC-3'), Bglap (F: 5'-AGCAAAGGTGCAGCCTTTGT-3', R: 5'- GCGCCTGGTCTCTTCACT-3' , Runx2 (F: 5'-ACTTCCTGTGCTCCGTGCTG-3', R: 5'-TCGTTGAACCTGGCTACTTGG-3'), GAPDH (F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3'), HOTAIR (5'-ATAGGCAAATGTCAGAGGGTT-3', R: 5'-ATTCTTAAATTGGGCTGGGTC-3').

Western Blot

Transfected cells in each group were lysed by the lysate solution. First, the cell lysate was added and centrifuged on ice. The supernatant was collected, and the protein concentration was determined according to the bicinchoninic acid (BCA) protein kit (Pierce, Rockford, IL, USA) instructions. Samples containing 50 μg of total protein were selected for dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis. Then, membranes were transferred and cut into the size of the polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) according to the molecular weight and blocked in 5% non-fat milk. A specific primary antibody was used for incubating overnight, and then, the second antibody was used. Finally, membranes were exposed following the instructions.

ALP Activity Assay

After culturing BMSCs for different treatments for 7 days, the medium was removed, and the plate was washed with PBS. 150 μL of 0.05% Triton X was added to each well for freezing, thawing, freezing and thawing, successively. After centrifugation at 15 000 rpm/min for 15 min at 4°C, the supernatant was transferred to a new auxiliary tube as a sample. ALP assay (Beyotime, Shanghai, China) kit was utilized to detect cell ALP activity.

ALP Staining

Differentially treated BMSCs for 7 days were selected to perform ALP staining experiments.

All staining experiments were carried out according to the instructions. Incubation solution was added in a 6-well plate with a glass slide at 37°C for 15 min and rinsed for 2 min. Counterstain hematoxylin counterstain was utilized for another 5 min, water rinsed for 2 min and dried in the air. The observation was performed under an optical microscope and taking photos.

Statistical Analysis

We used SPSS 22.0 software (SPSS, Chicago, IL, USA) for statistical analysis. The measurement data were expressed as mean ± standard deviation ($\bar{x}$±s), and the difference between the two groups was analyzed by *t*-test. $p<0.05$ was considered statistically significant.

Results

The Expression of HOTAIR in Peripheral Blood of Patients With Osteoporosis Was Significantly Higher Than That in Normal Group

We detected the expression of HOTAIR in 60 patients with osteoporosis and 60 normal controls by qRT-PCR. No significant differences in age, gender, height and weight were exerted between the two groups (Table I). The results demonstrated that the serum expression of HOTAIR in patients with osteoporosis was remarkably higher than that of normal people ($p<0.001$) (Figure 1A). Furthermore, we extracted BMSCs from osteoporosis patients and healthy controls. HOTAIR was highly expressed in BMSCs extracted from osteoporosis patients relative to controls (Figure 1B). Patients with osteoporosis were assigned into high expression group and low expression group according to the median expression of HOTAIR. The HOTAIR high expression group had a lower bone mineral density ($p<0.01$) (Figure 1C). Lower bone volume fraction was observed in the HOTAIR high expression group ($p<0.05$) (Figure1D). Also, lower Tb.Th. was seen in HOTAIR high expression group ($p<0.05$) (Figure 1E). These results suggested that HOTAIR may be involved in the development of osteoporosis.

Identification of BMSCs

We selected BMSCs as the cell model to study osteoporosis. On the fourth day, we observed that the cell body of BMSCs was long spindle shaped and had a strong refraction under the inverted microscope. Cells were passaged, BMSCs induced by osteoblast presented morphological changes. They began to differentiate as well as grew by static adherence, and calcified nodules were visible to the naked eye within a week with over 80% cell fusion (Figure 2A). Flow cytometry results illustrated the positive rate of CD29 (99.51%), CD90 (99.89%) and the negative rate of CD45 (0.28%) in the third generation of BMSCs, which were in line with the immunophenotypic characteristics of BMSCs, rather than hematopoietic stem cells (Figure 2B). Subsequently, ARS staining revealed obvious calcified nodules of BMSCs cultured in osteogenic induction medium for 14 days, whereas the control group did not show such results (Figure 2C). The expression of osteoblast marker genes ALP, Runx2 and Bglap were detected at day 1, 3, 7 and 14 after induction of differentiation. Only expression levels of ALP and Runx2 were found to be elevated on the first day. On day 3, expression levels of all the

Table I. Baseline characteristics.

Items	Control	Osteoporosis	*p*
n	60	60	
Age (years)	62±9	63±11	Matched
Gender	Female	Female	Matched
Trauma	Low energy	Low energy	Matched
Height (cm)	156±5	154±4	0.726[a]
Weight (kg)	57±13	53±15	0.322[a]
BMI (kg/m^2)	23.5±3.2	21.3±4.9	0.067[a]
Smoker (n)	3	2	0.862[b]
Alcohol (n)	1	2	1.000[b]
Rheumatoid arthritis (n)	5	6	1.000[b]
Steroid (n)	4	5	1.000[b]

[a]Paired-sample *t*-test; [b]McNemar test

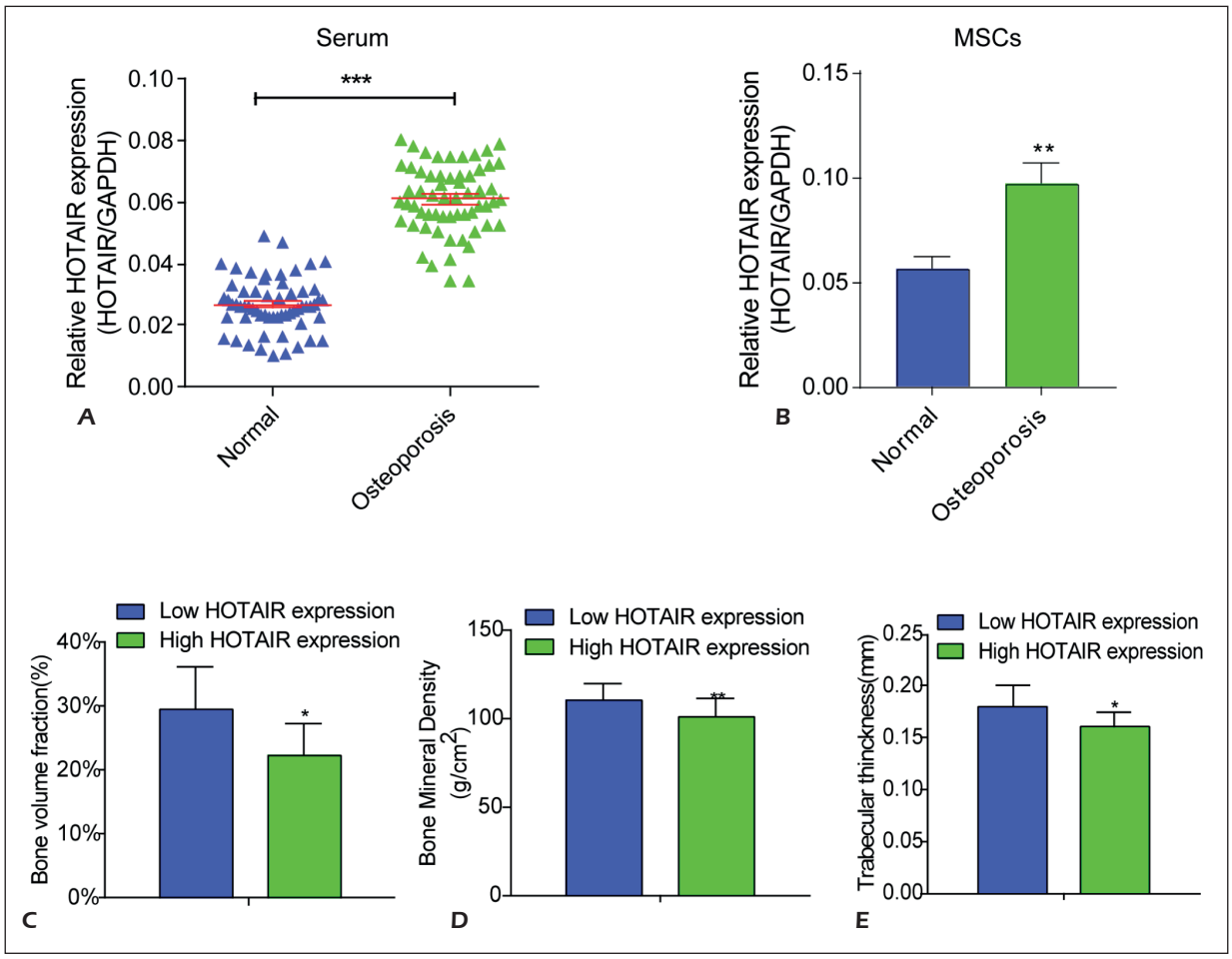

Figure 1. HOTAIR expression in peripheral blood of patients with osteoporosis was significantly higher than the normal group. ***A,*** The expression level of HOTAIR in peripheral blood of 60 patients with osteoporosis was significantly higher than that in the normal group of 60 people. ***B,*** HOTAIR was highly expressed in BMSCs extracted from osteoporosis patients relative to healthy controls. ***C,*** Among patients with osteoporosis, bone mineral density in HOTAIR high expression group was significantly lower than the low expression group. ***D,*** Among patients with osteoporosis, bone volume fraction in the HOTAIR overexpression group was significantly lower than the low expression group. ***E,*** Among patients with osteoporosis, Tb.Th. in HOTAIR high expression group was significantly lower than the low expression group.

above-mentioned genes were significantly enhanced than those before induction of differentiation, which were gradually increased with time passed (Figure 2D). These results indicated that BMSCs culture method was effective and could induce osteogenic differentiation. We also found that the expression of HOTAIR decreased as the induction days increased (Figure 2E).

HOTAIR Inhibited the Expression of Osteogenic Genes

The expression of HOTAIR was detected after transfection of lentiviral sh-NC, sh-HOTAIR1, sh-HOTAIR2 and sh-HOTAIR3 in MSCs, respectively. We found that all three lentiviruses could inhibit the expression of HOTAIR, while sh-HOTAIR2 had the most significant effect, therefore, sh-HOTAIR2 was chosen for subsequent experiments (Figure 3A). We then constructed the HOTAIR overexpression vector (pcDNA-HOTAIR), which exhibited a significant increase in HOTAIR expression after MSCs transfection (Figure 3B). After detecting the viability of sh-HOTAIR2-treated cells and HOTAIR-overexpressing pcDNA-HOTAIR-treated cells, the results indicated that lowly expressed HOTAIR increased the ALP activity and highly expressed HOTAIR inhibited ALP activity (Figure 3C, 3D). Osteogenic differentiation of BMSCs was induced and mRNA levels of osteoblast marker genes ALP, Runx2 and Bglap were significantly increased after the knockdown of HOTAIR. The overexpres-

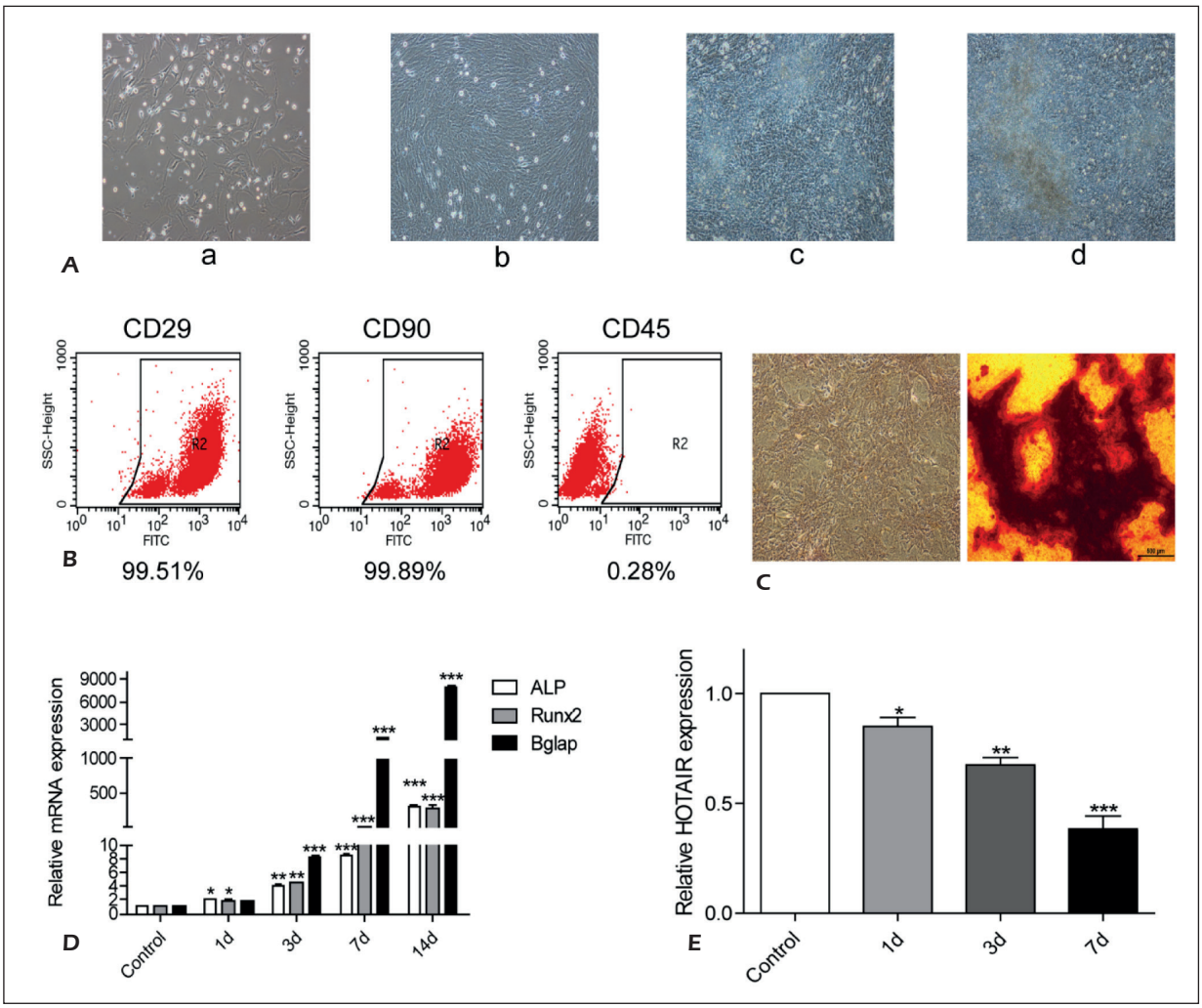

Figure 2. Phenotypic characterization of bone marrow MSCs. ***A,*** (a) Morphology of normally MSCs on day 4, showing a long spindle shape. (b) Morphology of MSCs cultured in osteogenic induction medium for 1 day. (c) Morphology of MSCs cultured in osteogenic induction medium for 7 days. (d) Morphology of MSCs cultured in osteogenic induction medium for 14 days. ***B,*** Flow cytometry identification of MSCs specific surface antigens, including positive CD29, CD90, negative CD45. ***C,*** Calcified nodules were shown in MSCs cultured in osteogenic induction medium for 14 days by alizarin red staining, while the control group did not appear. ***D,*** The expression levels of osteoblast marker gene ALP, Runx2 and Bglap at different days of induction, which was significantly increased on the third day of induction, and all were increased significantly on the seventh day. ***E,*** The expression of HOTAIR became lower as the induction days increased.

sion of HOTAIR resulted in the opposite results (Figure 3E, 3F). These results indicated that HOTAIR inhibits the expression of osteogenic genes and may inhibit osteogenic differentiation.

HOTAIR Inhibited the Expression of Osteoblast-Related Proteins and Formation of Osteogenic Calcified Nodules

After knockdown or overexpression of HOTAIR, MSCs were induced to differentiate into osteoblasts. We next detected the protein expressions of ALP, Runx2, OCN and OPN. Knockdown of HOTAIR led to the elevated protein expressions of ALP, Runx2, OCN and OPN. Meanwhile, overexpression of HOTAIR resulted in the opposite results (Figure 4A, 4B). ALP staining was performed on differentially treated MSCs for 14 days. ALP staining showed that inhibition of HOTAIR expression resulted in deeper color, indicating that ALP activity was increased, and the degree of bone differentiation was higher. Overexpression of HOTAIR achieved the opposite conclusion (Figure 4C). ARS staining revealed that the mineralized nodules were observed under inverted micro-

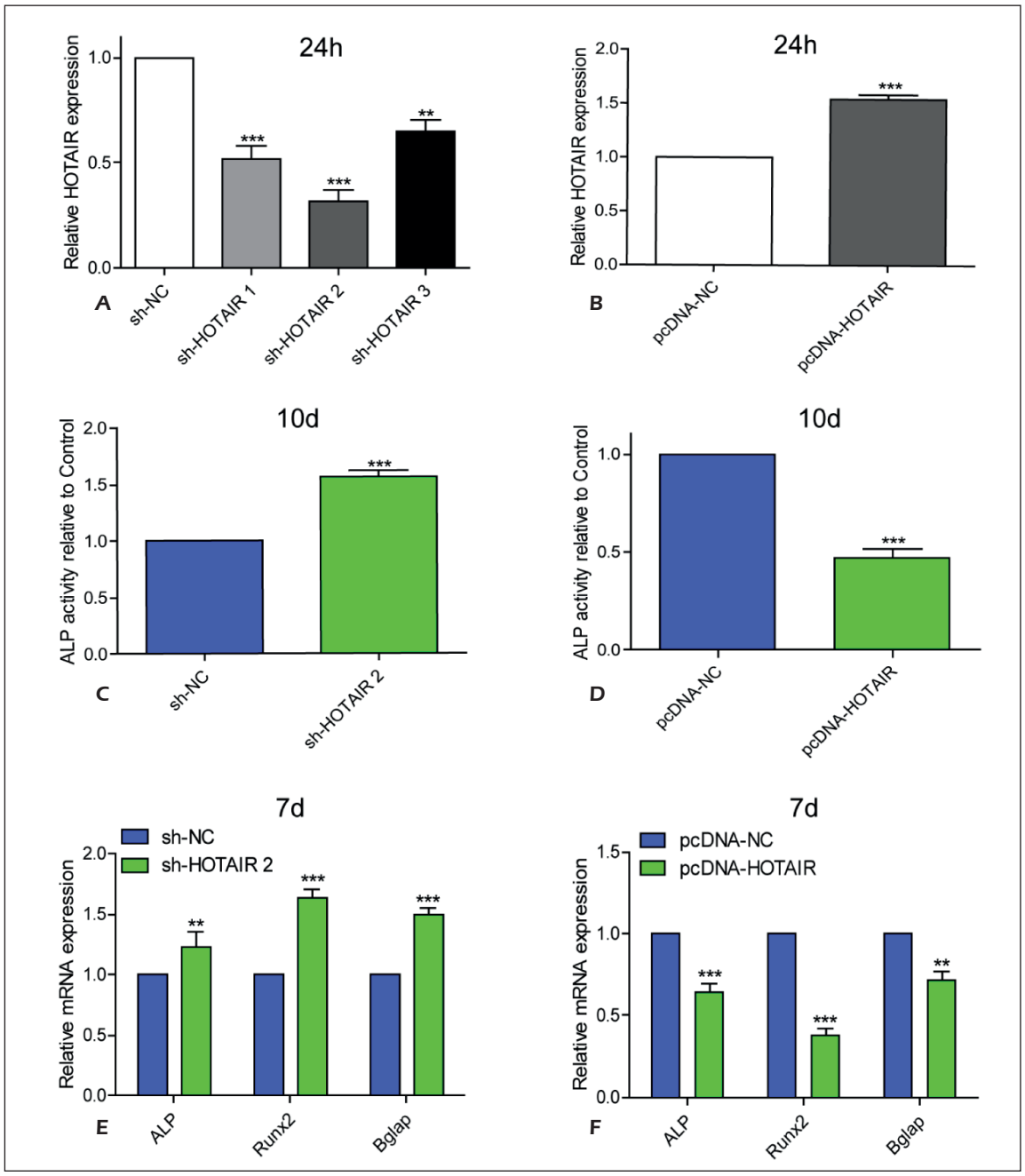

Figure 3. HOTAIR inhibited osteogenic gene expression. ***A,*** Transfection of three HOTAIR sequences, of which the effect of sh-HOTAIR 2 interference was most significant. ***B,*** After transfection of pcDNA-HOTAIR cells, HOTAIR expression was significantly increased. ***C,*** After knockdown of HOTAIR, cell ALP activity was significantly increased. ***D,*** After overexpression of HOTAIR, cell ALP activity decreased significantly. ***E,*** After knockdown of HOTAIR, expression levels of osteoblast marker genes ALP, Runx2, Bglap were significantly increased. ***F,*** After overexpression of HOTAIR, expression levels of osteoblast marker genes ALP, Runx2, Bglap were significantly decreased.

scope 14 days after osteogenic induction. Inhibition of HOTAIR expression led to a deeper color of ARS staining, indicating more calcium deposition and a higher degree of bone differentiation. However, the result was reversed after overexpression of HOTAIR (Figure 4D). These data indicated that HOTAIR inhibits the formation of osteogenic calcified nodules.

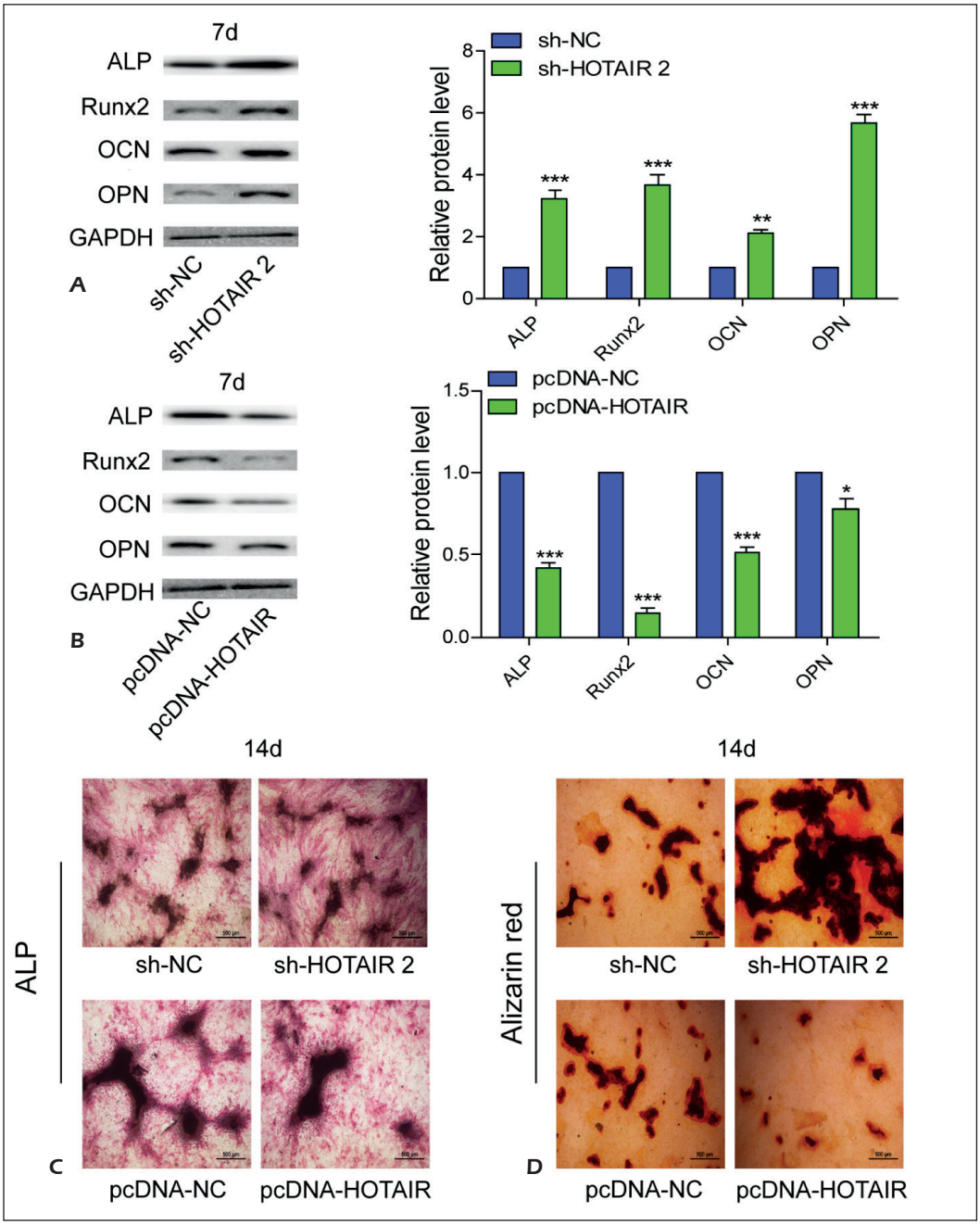

Figure 4. HOTAIR inhibited the expression levels of osteoblast-associated proteins and formation of osteogenic calcified nodules. ***A,*** After knockdown of HOTAIR, osteoblast-related genes ALP, Runx2, OCN, OPN expressions were significantly increased. ***B,*** After overexpression of HOTAIR, osteoblast-related genes ALP, Runx2, OCN, OPN expressions were significantly lower. ***C,*** Results of ALP staining of cells after knockdown or overexpression of HOTAIR. D, Results of alizarin red staining after HOTAIR knockdown or overexpression.

HOTAIR Inhibited the Osteogenic Differentiation of BMSCs by Inhibiting the Wnt/β-Catenin Signaling Pathway

To explore how HOTAIR inhibits the osteogenic differentiation of BMSCs, we found HOTAIR may participate in the development of various diseases through Wnt/β-catenin signaling pathway by literature reviews. In this study, the expression levels of β-catenin, CyclinD and C-myc were significantly decreased after overexpressing HOTAIR, while the expression level of DKK1, the signal inhibitor of Wnt/β-catenin,

was significantly increased (Figure 5A). Subsequently, we examined the effect of DKK1 on osteogenic differentiation of MSCs. After 0.5 μg/ml of DKK1 was added in the induction medium, the expression levels of ALP and Runx2 decreased significantly (Figure 5B). Moreover, we detected the expression levels of HOTAIR transfected with shRNA and relative genes in Wnt/β-catenin signaling pathway, and found that all of them were significantly increased, as well as the osteoblast differentiation marker Runx2 (Figure 5C). DKK1 treatment remarkably inhibited the elevated ALP activity due to HOTAIR knockdown (Figure 5D). Meanwhile, the enhanced ALP content and calcification ability resulted from HOTAIR knockdown were also reversed by DKK1 treatment (Figure 5E). All these results have been reversed after treatment with Wnt/β-catenin signal pathway inhibitor DKK1. These results indicated that HOTAIR inhibits osteogenic differentiation of BMSCs by inhibiting the Wnt/β-catenin signaling pathway.

DKK1 Accelerated the Progression of Osteoporosis

To determine the expression pattern of HOTAIR and the effect of DKK1 on osteoporosis animal model, we established *in vivo* osteoporosis model in rats by OVX. Our results demonstrated that HOTAIR level was higher in bone tissues of control rats administrated with DKK1 than controls. DKK1 administration also upregulated HOTAIR level in OVX group. Besides, OVX rats presented higher abundance of HOTAIR than controls, which was consistent with the *in vitro* results (Figure 6A). Subsequently, bone morphology in OVX rats was observed. It is shown that bone density, bone volume fraction and Tb.Th were lower in control rats administrated with DKK1. Similar trends were identified after DKK1 administration in OVX rats. Moreover, OVX rats had lower bone density, bone volume fraction and Tb.Th then controls (Figure 6B-6D). Protein levels of β-catenin, CyclinD, C-myc and Runx2 wer downregulated after DKK1 administration in both control rats and OVX rats. At the same time, OVX rats presented lower levels of these genes relative to controls (Figure 6E). The above results demonstrated that HOTAIR was upregulated in OVX rats, and tail vein administration of DKK1 could accelerate the progression of osteoporosis. In summary, HOTAIR inhibited osteogenic differentiation of BMSCs by regulating Wnt/β-catenin pathway (Figure 7).

Discussion

Osteoporosis is an age-related skeletal disease characterized by decreased bone mass, destruction of the bone microarchitecture, increased bone fragility, and the occurrence of fractures. Osteoporosis is a common disease that seriously endangers the physical and mental health of middle-aged and elderly people. With the prolongation of human life and the aging of the world's population, the incidence of osteoporosis is rising day by day, along with cardiovascular and cerebrovascular diseases[10], which brings a huge economic burden. In our country, the prevalence of osteoporosis is up to 50% -60% in older women and about 20% -30% in men. Our country is not only a country with a large population, but also with a large population of older people. Bones are metabolically active tissues that are repaired and renewed by the ongoing bone remodeling mechanism due to damage, fatigue, aging, inflammation, or metabolic loss such as a low-calcium environment. Osteoclasts and osteoblasts dominate bone resorption and bone formation during the process of old bone resorption and new bone remodeling. They are regulated by the systemic hormonal system (PTH, 1-25$(OH)_2D_3$) and local cytokines (IL-1, IL-6, PGE2, TGF-β, IGF-1, RANKL, OPG, etc.)[11, 12].

BMSCs are a group of pluripotent stem cells located in the mesoderm, which can differentiate into many kinds of cells such as adipocytes, osteoblasts, chondrocytes and myocytes under different conditions[13-15]. It was originally isolated from the bone marrow by Friedenstein et al[16], and was found to be widespread in connective tissues such as fat, muscle and blood. Because BMSCs are easy to be cultured *in vitro* and can differentiate into osteoblasts under osteogenic conditions, they have become the ideal cells for bone tissue engineering and have a huge effect on promoting the repair and reconstruction of bone in patients with osteoporosis[17]. The key to bone regeneration is to improve the osteogenic differentiation of human BMSCs.

LncRNAs are non-coding RNAs longer than 200 nucleotides[18,19]. Earlier studies showed that lncRNAs are ineffective, but recent studies have found that lncRNAs regulate the expression of genes at epigenetic, transcriptional and post-transcriptional levels. LncRNA not only functions in physiological processes but also has been demonstrated to be dysfuncional in various tumor tissues. LncRNA is involved in the occurrence and development of tumor tissue, which is expected to become a new target for cancer diagnosis and treatment[20].

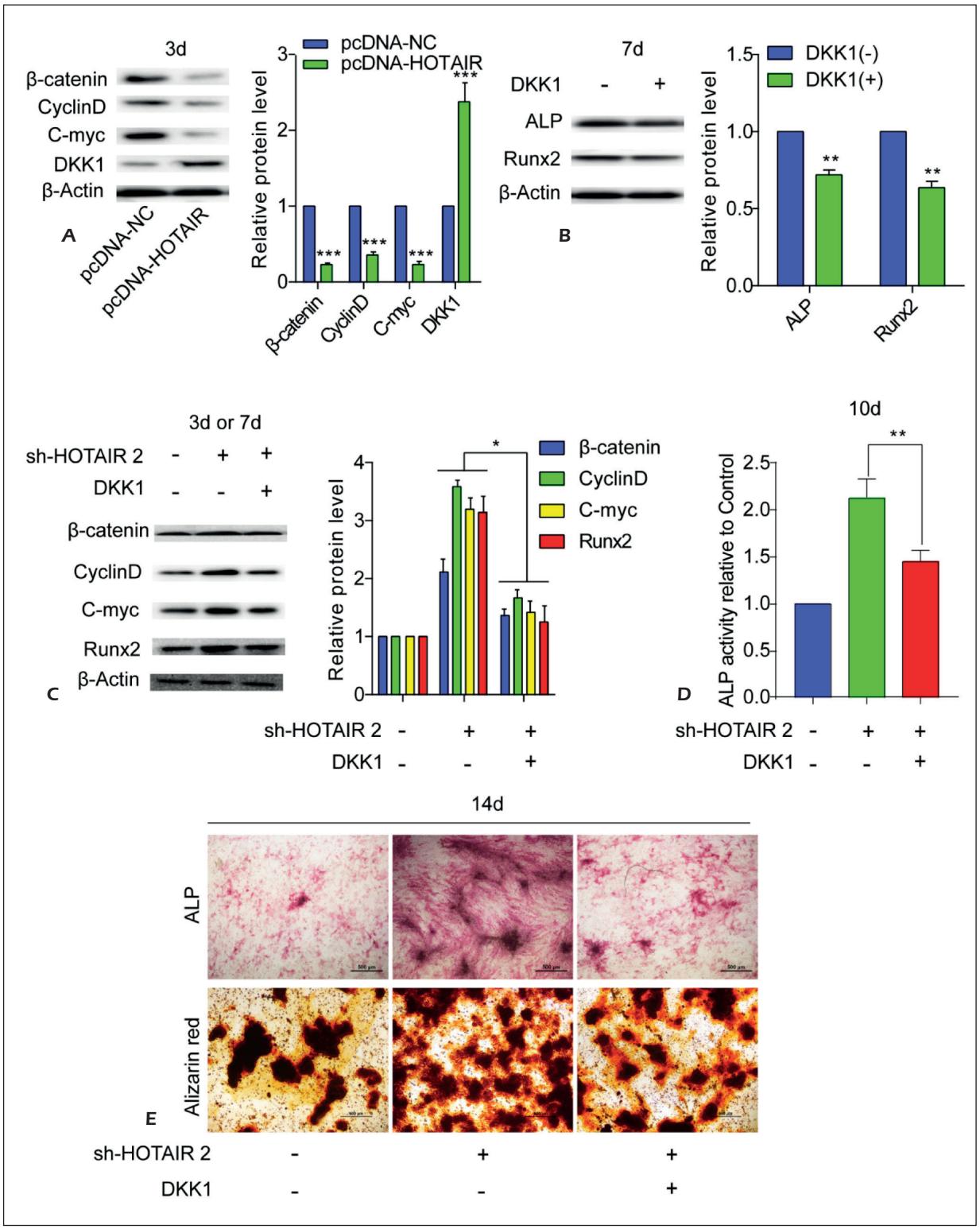

Figure 5. HOTAIR inhibited osteogenic differentiation of MSCs by suppressing the Wnt/β-catenin signaling pathway. ***A,*** Expression levels of Wnt/β-catenin signaling pathway related proteins after overexpression of HOTAIR. ***B,*** After 0.5 ug / ml of DKK1 induction, the protein expressions of ALP and Runx2 were decreased significantly. ***C,*** The expression levels of β-catenin, CyclinD, C-myc and Runx2 after DKK1 treatment and knockdown of HOTAIR, respectively. ***D,*** DKK1 treatment decreased ALP activity elevation due to HOTAIR knockdown. ***E,*** DKK1 treatment decreased the elevation of ALP content and calcification ability due to HOTAIR knockdown.

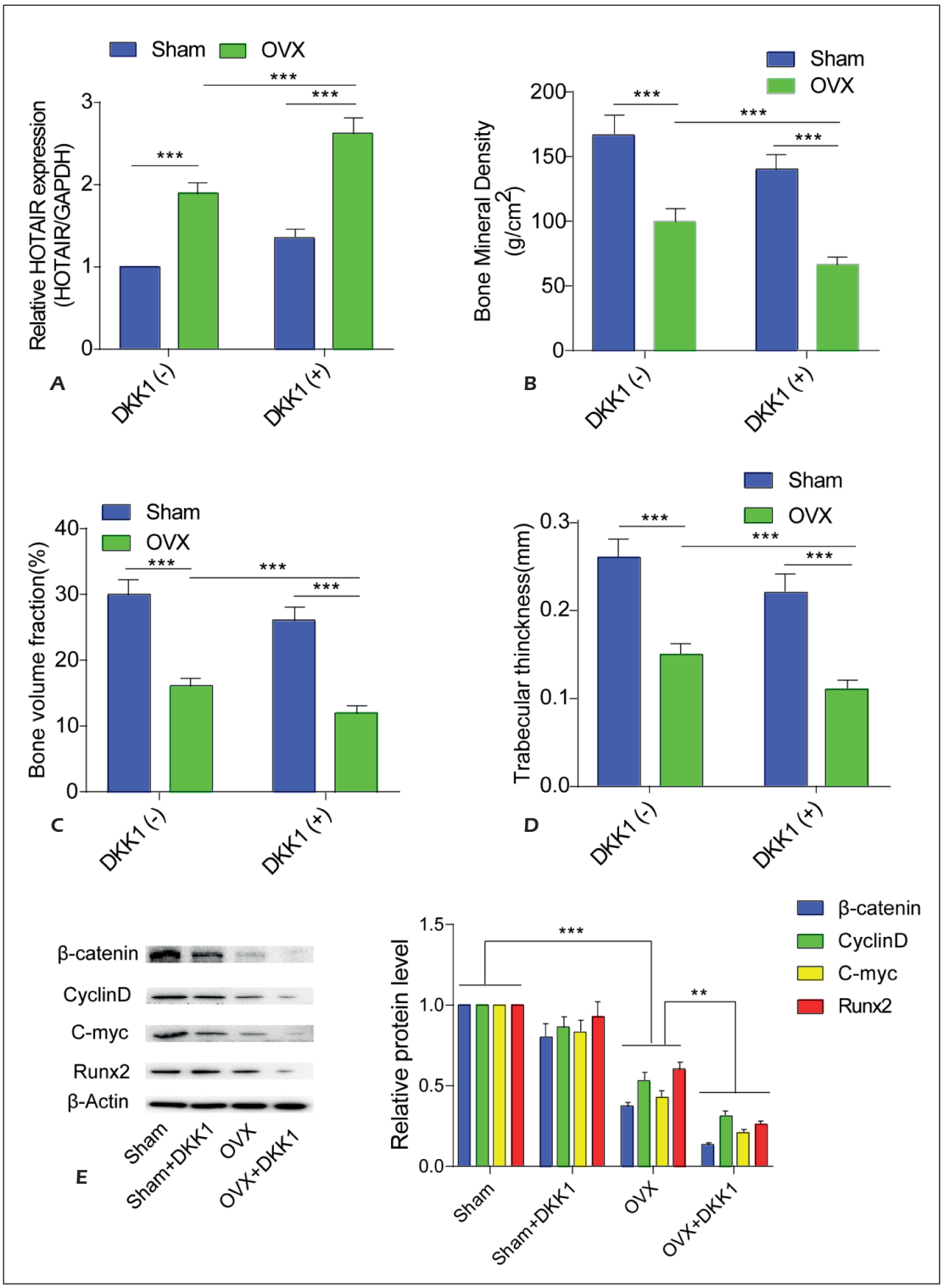

Figure 6. DKK1 administration aggravated osteoporosis severity. ***A,*** DKK1 administration upregulated HOTAIR level in control rats and OVX rats. OVX rats had higher level of HOTAIR than controls. ***B,*** DKK1 administration decreased bone density in control rats and OVX rats. OVX rats had lower level of bone density than controls. ***C,*** DKK1 administration decreased bone volume fraction in control rats and OVX rats. OVX rats had lower level of bone volume fraction than controls. ***D,*** DKK1 administration decreased Tb.Th in control rats and OVX rats. OVX rats had lower level of Tb.Th than controls. ***E,*** DKK1 administration downregulated protein levels ofβ-catenin, CyclinD, C-myc, and Runx2 in control rats and OVX rats. OVX rats had lower levels of β-catenin, CyclinD, C-myc, and Runx2 than controls.

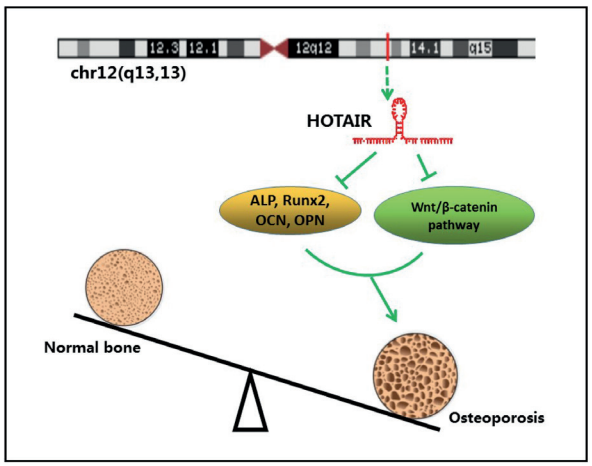

Figure 7. Summary of the Regulation and Mechanism of HOTAIR in osteoporosis.

LncRNA may enter the human circulatory system as microbubbles, exosomes, or protein complexes, forming circulating lncRNAs that are stable and widely present in body fluids such as blood and urine. Arita et al[21] found that lncRNA remains stable in plasma following repeated freeze-thaw cycles, which is consistent with the findings of Tong et al[22] and Ren et al[23]. Even in the presence of ribonucleases (RNase), circulating lncRNA is also stable. Meanwhile, with the promotion of real-time quantitative PCR technology, detection of circulating lncRNA in patients becomes easier. Because of the characteristics of minimally invasive, easy drawing and simple testing, more and more researchers focus on the lncRNA study[24]. However, there are few studies on lncRNA in osteoporosis. The role of lncRNA in osteoporosis is still worth exploring.

HOTAIR is the first lncRNA found to be trans-transcriptionally regulated. HOTAIR, a member of lncRNA, has been proved to be involved in the development of tumors, but its role in osteoporosis has not been reported. This study detected elevated HOTAIR expression in patients with osteoporosis, which may provide a new idea for the discovery of the pathogenesis of osteoporosis. LncRNA DANCR is the ncRNA located on chromosome 4, and DANCR was first found to be highly expressed in tumor cells. Some studies[25-27] have shown that DANCR is also involved in the regulation of the differentiation of synovial MSCs, osteoblasts and other precursor cells. In BMSCs, Zhu et al[28] found that DANCR can recruit EZH2 to Runx2 promoter and catalyze the methylation of H3K27, resulting in inhibition of Runx2 and osteogenic differentiation. In addition to DANCR, lncRNA HoxA-AS3 has also been shown to inhibit osteogenic differentiation. LncRNA HoxA-AS3[29] is an overexpressed lncRNA during adipogenic differentiation. HOTAIR was highly expressed in serum samples and BMSCs of osteoporosis patients than controls. Besides, overexpression of HOTAIR inhibited osteogenic differentiation and expression of osteogenic differentiation marker in this study.

Wnt is a type of glycoprotein rich in L-cysteine, which is about 3946 kDa. Stably expressed Wnt1 and Wnt3a can promote the proliferation of C3H10T1/2 cell line and induce the activity of ALP, indicating that Wnt can promote the formation of precursor osteogenesis growth and osteoblast differentiation in the early stage. In addition, Wnt also has an effect on the osteoblast orientation. Wnt1 and Wnt10b can inhibit the differentiation of adipocytes or preadipocytes. Overexpression of Wnt3a in C3H10T1/2 can inhibit the expression of adipocyte marker PPART2, thus promoting its differentiation to osteocytes. However, Mbalaviele et al[30] suggested that the effect of β-catenin on osteogenic differentiation of BMSCs is mediated by the mechanism of Tcf/Lef, and the increased response of T cells to osteogenic factors such as BMP- 2 is also responsible for the differentiation. Through the induction signal, β-catenin and osteogenic factors promote T cell differentiation into osteoblasts in a synergistic manner. In the present study, we accessed the expression levels of key proteins of Wnt/β-catenin by overexpression or knockdown of HOTAIR and found that HOTAIR inhibited osteogenic differentiation by inhibiting the activation of Wnt/β-catenin.

Conclusions

Lnc RNA HOTAIR is highly expressed in patients with osteoporosis and inhibits the differentiation of MSCs into osteoblasts by suppressing the activation of Wnt/β-catenin signaling pathway. Our study provides theoretical basis and new research direction to explore the mechanism of osteoporosis.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Hu BT, Chen WZ. MOTS-c improves osteoporosis by promoting osteogenic differentiation of bone marrow mesenchymal stem cells via TGF-beta/Smad pathway. Eur Rev Med Pharmacol Sci 2018; 22: 7156-7163.
2) Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. Cell 2009; 136: 629-641.
3) Moran VA, Perera RJ, Khalil AM. Emerging functional and mechanistic paradigms of mammalian long non-coding RNAs. Nucleic Acids Res 2012; 40: 6391-6400.
4) Kopreski MS, Benko FA, Kwak LW, Gocke CD. Detection of tumor messenger RNA in the serum of patients with malignant melanoma. Clin Cancer Res 1999; 5: 1961-1965.
5) Bazanova NV, Seits IF. [Can the presence of an RNA-lipoprotein complex in human blood serum give evidence of a cancerous disease?]. Eksp Onkol 1989; 11: 37-39.
6) Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 2007; 129: 1311-1323.
7) Wan Y, Chang HY. HOTAIR: Flight of noncoding RNAs in cancer metastasis. Cell Cycle 2010; 9: 3391-3392.
8) Gutschner T, Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. RNA Biol 2012; 9: 703-719.
9) Hessert D, Tanzer D, Brunstetter T, Kaupp S, Murdoch D, Mirzaoff M. Topical cyclosporine A for postoperative photorefractive keratectomy and laser in situ keratomileusis. J Cataract Refract Surg 2013; 39: 539-547.
10) Utian WH, Archer DF, Bachmann GA, Gallagher C, Grodstein F, Heiman JR, Henderson VW, Hodis HN, Karas RH, Lobo RA, Manson JE, Reid RL, Schmidt PJ, Stuenkel CA. Estrogen and progestogen use in postmenopausal women: July 2008 position statement of The North American Menopause Society. Menopause 2008; 15: 584-602.
11) Jacquin C, Gran DE, Lee SK, Lorenzo JA, Aguila HL. Identification of multiple osteoclast precursor populations in murine bone marrow. J Bone Miner Res 2006; 21: 67-77.
12) Hansen T, Otto M, Gaumann A, Eckardt A, Petrow PK, Delank KS, Kirkpatrick CJ, Kriegsmann J. Cathepsin K in aseptic hip prosthesis loosening: expression in osteoclasts without polyethylene wear particles. J Rheumatol 2001; 28: 1615-1619.
13) Jackson WM, Nesti LJ, Tuan RS. Concise review: clinical translation of wound healing therapies based on mesenchymal stem cells. Stem Cells Transl Med 2012; 1: 44-50.
14) Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1966; 16: 381-390.
15) Knight MN, Hankenson KD. Mesenchymal stem cells in bone regeneration. Adv Wound Care (New Rochelle) 2013; 2: 306-316.
16) Friedenstein AJ, Chailakhyan RK, Gerasimov UV. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet 1987; 20: 263-272.
17) Rodda SJ, McMahon AP. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development 2006; 133: 3231-3244.
18) Feng YZ, Shiozawa T, Miyamoto T, Kashima H, Kurai M, Suzuki A, Konishi I. BRAF mutation in endometrial carcinoma and hyperplasia: correlation with KRAS and p53 mutations and mismatch repair protein expression. Clin Cancer Res 2005; 11: 6133-6138.
19) Maher CA, Kumar-Sinha C, Cao X, Kalyana-Sundaram S, Han B, Jing X, Sam L, Barrette T, Palanisamy N, Chinnaiyan AM. Transcriptome sequencing to detect gene fusions in cancer. Nature 2009; 458: 97-101.
20) Chandra GS, Nandan TY. Potential of long non-coding RNAs in cancer patients: from biomarkers to therapeutic targets. Int J Cancer 2017; 140: 1955-1967.
21) Xian HP, Zhuo ZL, Sun YJ, Liang B, Zhao XT. Circulating long non-coding RNAs HULC and ZNFX1-AS1 are potential biomarkers in patients with gastric cancer. Oncol Lett 2018; 16: 4689-4698.
22) Tong YS, Wang XW, Zhou XL, Liu ZH, Yang TX, Shi WH, Xie HW, Lv J, Wu QQ, Cao XF. Identification of the long non-coding RNA POU3F3 in plasma as a novel biomarker for diagnosis of esophageal squamous cell carcinoma. Mol Cancer 2015; 14: 3.
23) Ren S, Wang F, Shen J, Sun Y, Xu W, Lu J, Wei M, Xu C, Wu C, Zhang Z, Gao X, Liu Z, Hou J, Huang J, Sun Y. Long non-coding RNA metastasis associated in lung adenocarcinoma transcript 1 derived miniRNA as a novel plasma-based biomarker for diagnosing prostate cancer. Eur J Cancer 2013; 49: 2949-2959.
24) Tsui NB, Ng EK, Lo YM. Stability of endogenous and added RNA in blood specimens, serum, and plasma. Clin Chem 2002; 48: 1647-1653.
25) Kretz M, Webster DE, Flockhart RJ, Lee CS, Zehnder A, Lopez-Pajares V, Qu K, Zheng GX, Chow J, Kim GE, Rinn JL, Chang HY, Siprashvili Z, Khavari PA. Suppression of progenitor differentiation requires the long noncoding RNA ANCR. Genes Dev 2012; 26: 338-343.
26) Zhang L, Chen S, Bao N, Yang C, Ti Y, Zhou L, Zhao J. Sox4 enhances chondrogenic differentiation and proliferation of human synovium-derived stem cell via activation of long noncoding RNA DANCR. J Mol Histol 2015; 46: 467-473.
27) Tong X, Gu PC, Xu SZ, Lin XJ. Long non-coding RNA-DANCR in human circulating monocytes: a potential biomarker associated with postmenopausal osteoporosis. Biosci Biotechnol Biochem 2015; 79: 732-737.
28) Zhu L, Xu PC. Downregulated LncRNA-ANCR promotes osteoblast differentiation by targeting EZH2 and regulating Runx2 expression. Biochem Biophys Res Commun 2013; 432: 612-617.

29) Zhu XX, Yan YW, Chen D, Ai CZ, Lu X, Xu SS, Jiang S, Zhong GS, Chen DB, Jiang YZ. Long non-coding RNA HoxA-AS3 interacts with EZH2 to regulate lineage commitment of mesenchymal stem cells. Oncotarget 2016; 7: 63561-63570.

30) Mbalaviele G, Sheikh S, Stains JP, Salazar VS, Cheng SL, Chen D, Civitelli R. Beta-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation. J Cell Biochem 2005; 94: 403-418.