

## Original Article

# HOXA7 Impairs Osteogenic Differentiation via p38/JNK Signaling: Implications for Osteoporosis

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## Abstract

**Objective:** To elucidate the role and mechanism of HOXA7 in osteoporosis (OP), with the goal of informing future research directions in OP. **Methods:** We assessed HOXA7 gene and protein expression; the effect of HOXA7 on osteogenic differentiation in human bone marrow-derived mesenchymal stem cells (hBMSCs); and proliferation, apoptosis, and autophagy in MC3T3-E1 cells. We also evaluated p38 MAPK/JNK pathway-associated proteins in hBMSCs and MC3T3-E1 cells. Cells were transfected with si-HOXA7 and cultured with or without the JNK inhibitor SP600125 or the p38 inhibitor SB203580, after which cell viability, apoptosis, and autophagy were re-assessed. **Results:** HOXA7 overexpression inhibited osteogenic differentiation of hBMSCs, reduced OPG, OPN, and RUNX2 expression in hBMSCs, and decreased proliferation while promoting apoptosis in MC3T3-E1 cells. *In vitro*, HOXA7 modulated autophagy markers in MC3T3-E1 cells. Phosphorylated JNK and p38 (p-JNK, p-p38) were increased in hBMSCs following osteogenic induction, whereas HOXA7 upregulation significantly suppressed p-JNK and p-p38 in MC3T3-E1 cells. SP600125 and SB203580 attenuated the effects of HOXA7 silencing on proliferation, apoptosis, and autophagy in MC3T3-E1 cells. **Conclusion:** HOXA7 reduces osteogenesis and osteoblast proliferation and promotes osteoblast apoptosis via the p38 MAPK/JNK pathway, suggesting potential therapeutic avenues against OP.

**Keywords:** HOXA7, Osteogenic Differentiation, Osteoporosis, p38 MAPK/JNK pathway

## Introduction

Osteoporosis (OP) is a systemic skeletal disorder characterized by reduced bone mass, deterioration of bone microarchitecture, and decreased bone strength, resulting in increased fragility and fracture risk<sup>1,2</sup>. OP is a growing public health burden worldwide, with particularly

high impact in the Asia-Pacific region due to its large and aging population<sup>3,4</sup>. Globally, osteoporotic fractures are estimated to occur every 3 seconds, causing substantial patient morbidity and imposing significant economic costs<sup>5</sup>. Despite available therapies, important limitations remain. There is an urgent need to improve understanding of OP risk factors, identify new therapeutic targets, and strengthen targeted prevention and treatment strategies.

Homeobox (HOX) genes encode a family of transcription factors that are aberrantly expressed in various tumors. In humans, 39 HOX genes are organized into four clusters—HOXA, HOXB, HOXC, and HOXD<sup>6</sup>. These clusters occupy defined chromosomal loci; for example, the HOXA cluster resides at 7p15.3. In osteoblastogenesis, the HOXA cluster is of particular interest, and many HOXA-encoded proteins contribute to normal skeletal development<sup>7</sup>. Overexpression of HOXA7 (also known historically as HOX1.1) in transgenic animals provokes perinatal

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mortality and multiple craniofacial anomalies<sup>8</sup>. Other studies indicate that HOXA7 is an important regulator of cell fate and pattern formation during embryogenesis and is involved in stem-cell differentiation<sup>7</sup>. In addition, downregulation of HOXA7 as a target of miR-920 has been reported to promote osteogenic differentiation of human bone marrow mesenchymal stem cells in individuals with osteoporosis<sup>9</sup>. Based on this background, we hypothesized that HOXA7 is upregulated in OP and negatively regulates osteogenic differentiation through the p38 MAPK and JNK pathways.

To date, the role of HOXA7 in OP and its relationship with osteoblastic differentiation remain insufficiently characterized. In this study, we investigated the role of HOXA7 and its regulatory relationship with osteoblastic differentiation during OP progression, aiming to provide a foundation for the clinical development of new therapeutic targets for OP.

## Materials and Methods

### General information

Thirty-six postmenopausal women with osteoporosis (PMOP) treated at our hospital were enrolled as the OP group. An additional 30 postmenopausal women undergoing routine health examinations during the same period served as the non-osteoporotic (NOP) group. Based on bone mineral density (BMD) T-scores, participants were classified as OP (T-score  $\leq$  -2.5) or NOP (T-score  $\geq$  -1.0). Baseline characteristic data were not available for comparison; this limitation is noted in the Discussion.

### Blood sample collection

Fasting venous blood was collected in the early morning. Samples were allowed to clot for 30 minutes at room temperature, then centrifuged at 3000 rpm for 15 minutes at 4 °C. The serum supernatant was collected and stored at -80 °C until analysis. Serum HOXA7 levels were measured by ELISA using a commercial kit (BGT-KET-12191, Biogradetech, Wuhan, China). Absorbance was read at 450 nm using Synergy H1 microplate reader (Winooski, Vermont, USA). Each sample was measured in duplicate (triplicate when volume permitted), and the mean value was used for analysis; plate effects were controlled by running a pooled serum quality control on every plate and by fitting a standard curve per plate.

### Cell culture and osteoblast induction

MC3T3-E1 cells and human bone marrow-derived mesenchymal stem cells (hBMSCs) were obtained from Pricella (Wuhan, China) and cultured in  $\alpha$ -MEM with 10% fetal bovine serum (FBS; HyClone, USA) and 1% (v/v) penicillin-streptomycin (Beyotime, Shanghai, China) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. When cultures reached approximately 80% confluence, MC3T3-E1

cells were passaged using 0.25% trypsin, and passages 6-8 were used for experiments. hBMSCs were expanded to the third passage (P3) for subsequent assays. As previously described<sup>10</sup>, morphological identification of cultured hBMSCs was performed by transmission electron microscopy (TEM).

For osteogenic induction *in vitro*, osteogenic medium (OM) consisted of 50  $\mu$ g/mL ascorbic acid, 10 mM  $\beta$ -glycerophosphate, and 0.1  $\mu$ g/mL dexamethasone, as described previously<sup>11</sup>. hBMSCs maintained in growth medium without osteogenic supplements served as the control group.

To assess the p38 MAPK/JNK pathway, the JNK inhibitor SP600125 (cat. no. 8177; Cell Signaling Technology) and the p38 inhibitor SB203580 (cat. no. 5633; Cell Signaling Technology) were first dissolved in DMSO and then added to the MC3T3-E1 cells at a desired concentration of 20  $\mu$ M; the cells were then incubated at 37 °C in 5% CO<sub>2</sub> for 3 days.

### Cell transfection

For HOXA7 silencing, small interfering RNA targeting HOXA7 (si-HOXA7; 5'-TCGCCACACGCTCTGTTT-3') and a corresponding negative control (si-NC) were synthesized by Ribobio (Guangzhou, China). For HOXA7 overexpression, the HOXA7 coding fragment (primers: forward 5'-TCATTCCTCCTCGTCC-3'; reverse 5'-ATGAGTTCTTCGTATTATGAACG-3') was amplified and cloned into the pcDNA3.1 plasmid to generate pcDNA3.1-HOXA7 (oe-HOXA7); empty pcDNA3.1 served as the negative control (oe-NC). Transient transfections were performed using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. Cells were used for osteogenic induction 24 h post-transfection. Transfection efficiency was verified by RT-qPCR.

### RT-qPCR

Total RNA was extracted using TRIzol reagent. Complementary DNA (cDNA) was synthesized by reverse transcription at 37 °C for 15 min, followed by enzyme inactivation at 95 °C for 5 min. Quantitative real-time PCR (RT-qPCR) was performed using the miScript SYBR Green PCR Kit on an ABI 7500 Fast Real-Time PCR System. Relative HOXA7 mRNA expression was calculated by the 2<sup>- $\Delta\Delta$ Ct</sup> method with GAPDH as the internal reference.

Primer sequences (5'→3'):

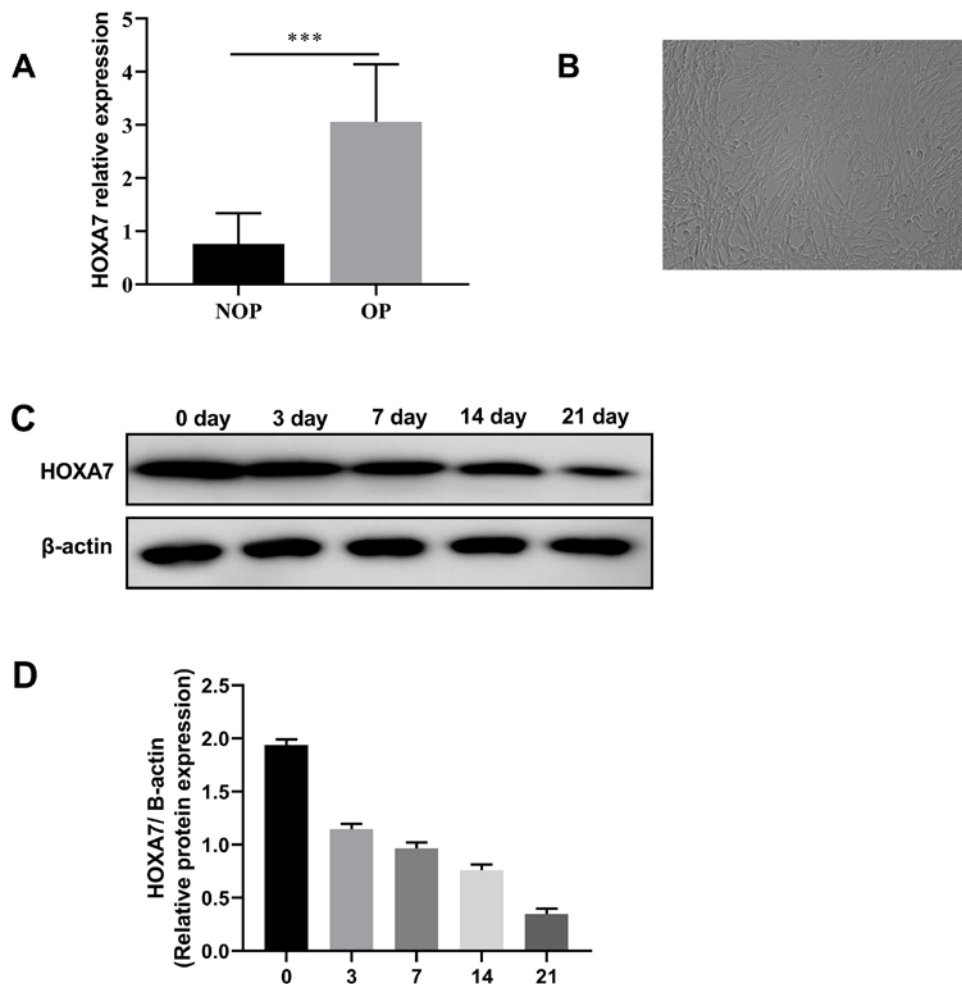
- GAPDH forward: AATCCCATCACCATCTTCCA; GAPDH reverse: CCTGCTTCACCACCTTCTTG
- HOXA7 forward: TTATACAATGTCAACAGCC; HOXA7 reverse: TCCTTATGCTCTTTCTTCC

### Alizarin red staining (ARS)

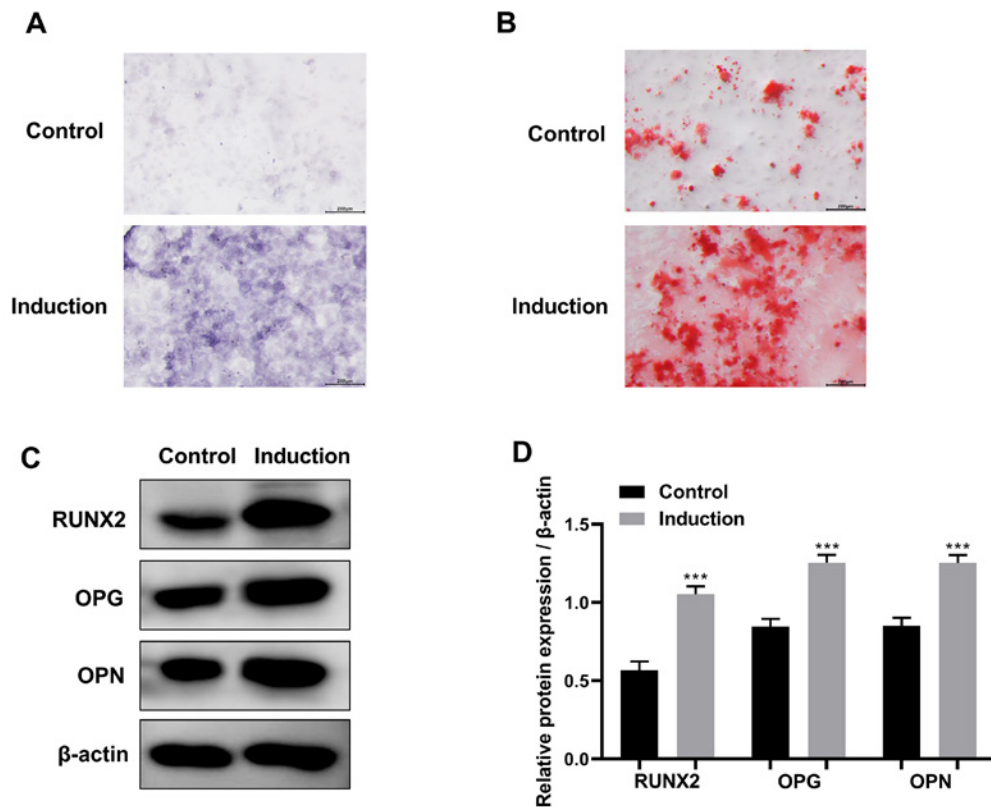
The ARS assay was performed as previously reported<sup>5</sup>. After 14 days of culture under osteogenic induction (OM) with the indicated HOXA7 transfections, cells were fixed with 4% ice-cold paraformaldehyde for 20 minutes and

**Table 1.** Primary antibodies for Western Blot.

Primary antibody	Catalog number	Dilution ratio	Source
HOXA7	Ab211521	1:1,000	Abcam, Cambridge, UK
OPG	ab73400	1:1,000	Abcam, Cambridge, UK
OPN	Ab214050	1:1,000	Abcam, Cambridge, UK
RUNX2	ab192256	1:1,000	Abcam, Cambridge, UK
p62	Ab207305	1:1,000	Abcam, Cambridge, UK
LC3-II/I	Ab48364	1:1,000	Abcam, Cambridge, UK
ATG12	Ab303488	1:1,000	Abcam, Cambridge, UK
JNK	9252	1:1,000	CST, Danvers, MA, USA
p-JNK	9255	1:1,000	CST, Danvers, MA, USA
p38	9212	1:1,000	CST, Danvers, MA, USA
p-p38	9216	1:1,000	CST, Danvers, MA, USA
$\beta$ -actin	4967	1:1,000	CST, Danvers, MA, USA



**Figure 1.** HOXA7 expression in serum and hBMSCs. (A) Serum HOXA7 levels were higher in the OP group as measured by ELISA (OP group vs NOP group, n=36 vs 30). (B) Representative morphology of hBMSCs. (C–D) HOXA7 protein levels in hBMSCs were assessed by Western blotting. \*\*\*P < 0.001.



**Figure 2.** Osteogenic differentiation of hBMSCs. (A) ALP staining to assess early osteogenic differentiation. Scale bar = 200  $\mu$ m. (B) Alizarin Red S staining to assess matrix mineralization. Scale bar = 200  $\mu$ m. (C–D) Protein levels of osteogenic markers were evaluated by Western blotting. \*\*\* $P < 0.001$ .

washed three times with PBS. Fixed cells were stained with 40 mM Alizarin Red solution, then washed thoroughly with PBS. Orange-red deposits were observed under an inverted microscope, indicating calcified nodule formation.

#### Alkaline Phosphatase (ALP) Staining

To evaluate early osteogenic differentiation, ALP staining was performed on hBMSCs. After 7 days in OM with the indicated HOXA7 transfections, cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes and washed three times with double-distilled water (ddH<sub>2</sub>O). Cells were then stained with BCIP/NBT staining solution (Beyotime, China), rinsed with ddH<sub>2</sub>O, and gently washed with 1× PBS. Stained cells were imaged using an inverted microscope.

#### CCK-8 assay

Following transfection, MC3T3-E1 cells were dissociated with 0.25% trypsin, resuspended in complete medium,

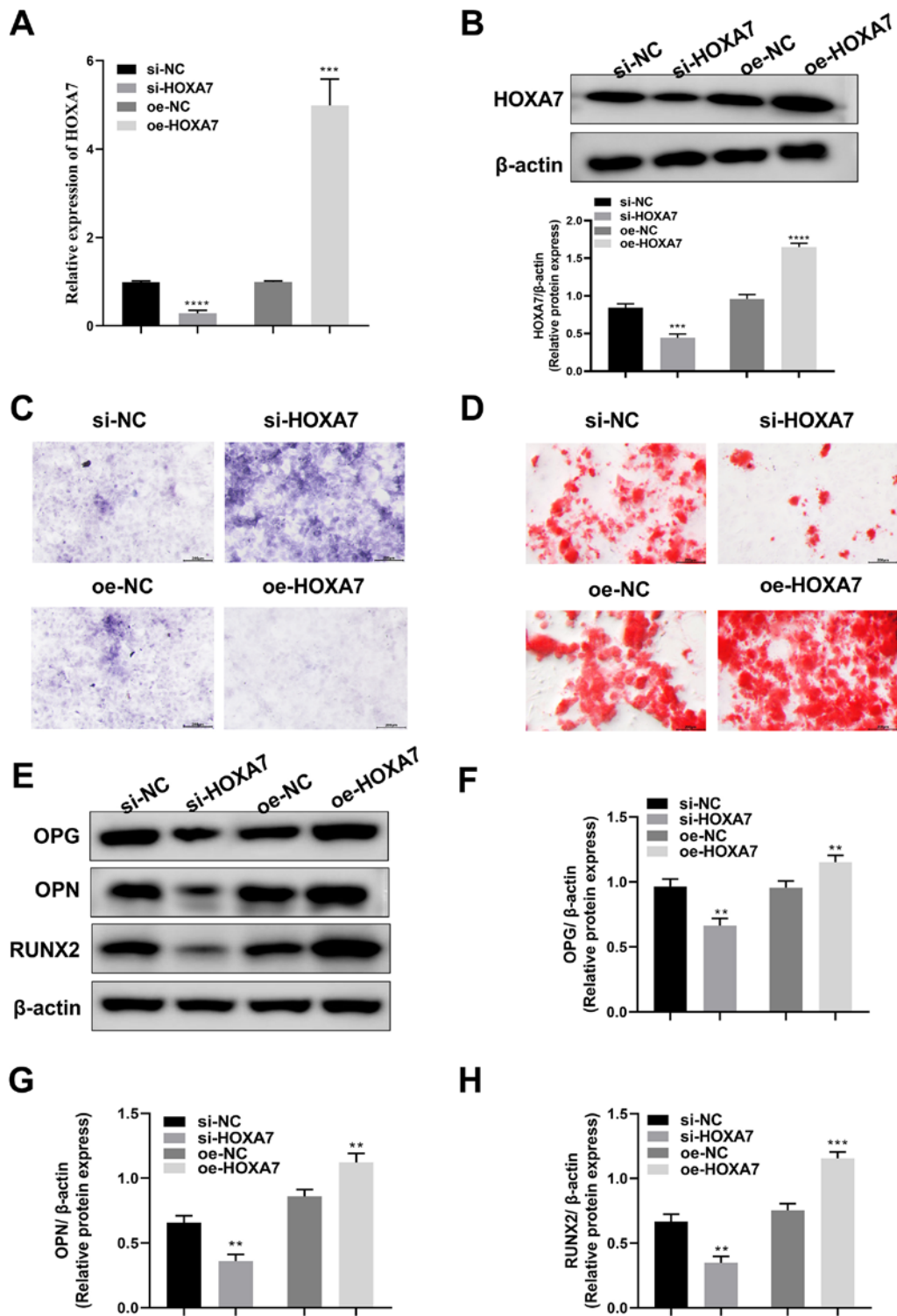
counted, and seeded into 96-well plates. At 24, 48, and 72 h post-transfection, 10  $\mu$ L of CCK-8 reagent was added to each well and incubated according to the manufacturer's instructions. Absorbance was then recorded using a microplate reader at the recommended wavelength.

#### Flow cytometry

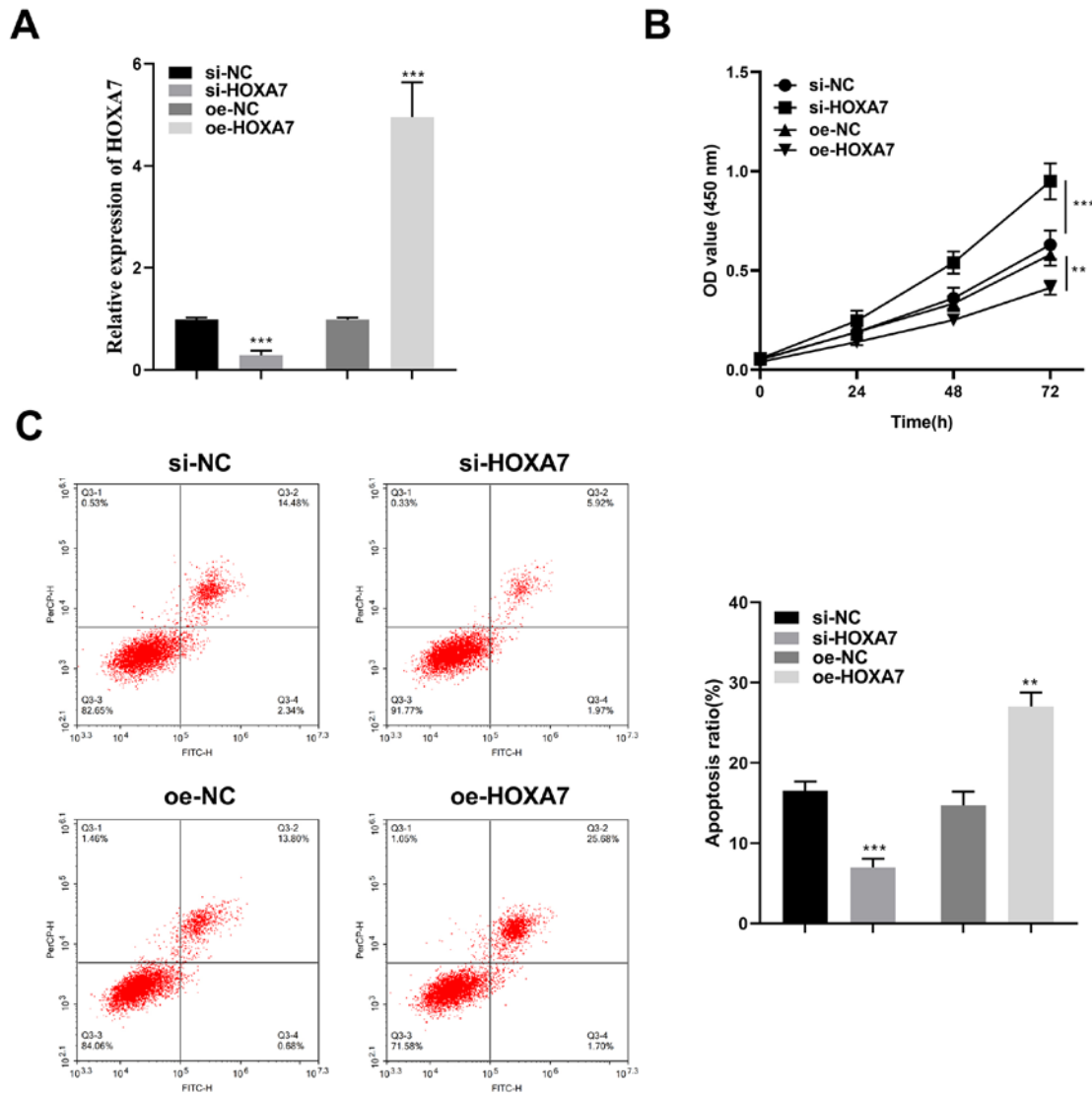
Transfected MC3T3-E1 cells ( $2 \times 10^5$  cells/well) were seeded into 6-well plates. Cells were washed with PBS and resuspended in 500  $\mu$ L Annexin V-FITC/PI staining buffer (Annexin V-FITC/PI kit, #45115, Cell Signaling Technology, MA, USA). After a 15-min incubation protected from light at room temperature, apoptosis was analyzed by flow cytometry.

#### Western blot analysis

Total protein was extracted using RIPA buffer (89901, Thermo, USA). Equal amounts of protein (30  $\mu$ g) were separated by 10% SDS-PAGE and transferred to PVDF



**Figure 3.** HOXA7 inhibits osteogenic differentiation of hBMSCs. (A) HOXA7 mRNA levels in hBMSCs transfected with si-HOXA7 or oe-HOXA7 were measured by qRT-PCR. (B) HOXA7 protein levels in hBMSCs with the indicated transfections were assessed by Western blotting. (C–D) ALP staining and Alizarin Red S (ARS) staining evaluating osteogenic differentiation and mineralization in transfected hBMSCs. (E–H) Protein levels of OPG, OPN, and RUNX2 were determined by Western blotting. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

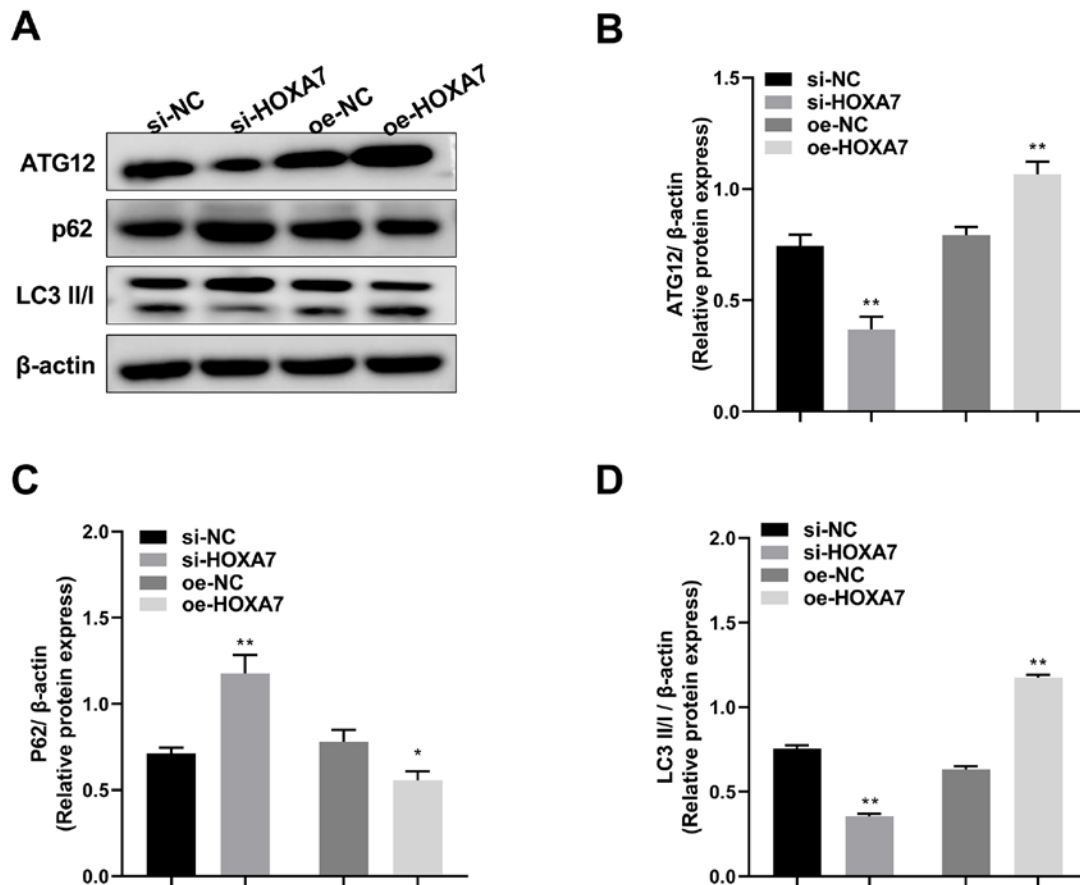


**Figure 4.** HOXA7 modulates proliferation and apoptosis of MC3T3-E1 cells. (A) HOXA7 mRNA levels in MC3T3-E1 cells transfected with si-HOXA7 or oe-HOXA7 were measured by qRT-PCR. (B) Cell viability in transfected MC3T3-E1 cells was assessed by CCK-8 assay. (C) Apoptosis in transfected MC3T3-E1 cells was analyzed by flow cytometry. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

membranes. After blocking with 5% non-fat milk, membranes were incubated overnight at 4 °C with primary antibodies against HOXA7, OPG, OPN, RUNX2, p62, LC3-II/I, ATG12, JNK, p-JNK, p38, p-p38, and  $\beta$ -actin. Membranes were then incubated with HRP-conjugated secondary antibody (1:2,000; 7074, Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Bands were visualized using an ECL detection system and quantified with ImageJ software. Signal intensities were normalized to  $\beta$ -actin. Primary antibody details are provided in Table 1.

## Statistical Analysis

Data are presented as mean  $\pm$  SD and were analyzed using SPSS version 21.0 (IBM). Differences between two groups were assessed with two-tailed Student's t-tests. Multiple-group comparisons were performed by one-way ANOVA followed by Tukey's post hoc test. Each independent experiment was performed at least three times (biological replicates), with triplicate technical replicates per experiment. For ELISA, duplicate/triplicate readings were



**Figure 5.** HOXA7 modulates autophagy markers in MC3T3-E1 cells. (A–D) Protein levels of ATG12, p62, and LC3-II/I in MC3T3-E1 cells with the indicated transfections were assessed by Western blotting. \* $P < 0.05$ , \*\* $P < 0.01$ .

averaged per subject prior to analysis. A value of  $P < 0.05$  was considered statistically significant.

## Results

### HOXA7 expression in serum and hBMSCs

Serum HOXA7 levels were significantly higher in the OP group as measured by ELISA (Figure 1A). Representative morphology of hBMSCs is shown in Figure 1B. Western blotting demonstrated that HOXA7 protein expression decreased following osteogenic induction of hBMSCs (Figure 1C–D). Together, these findings suggest that HOXA7 is associated with OP and is downregulated during osteogenic differentiation of hBMSCs.

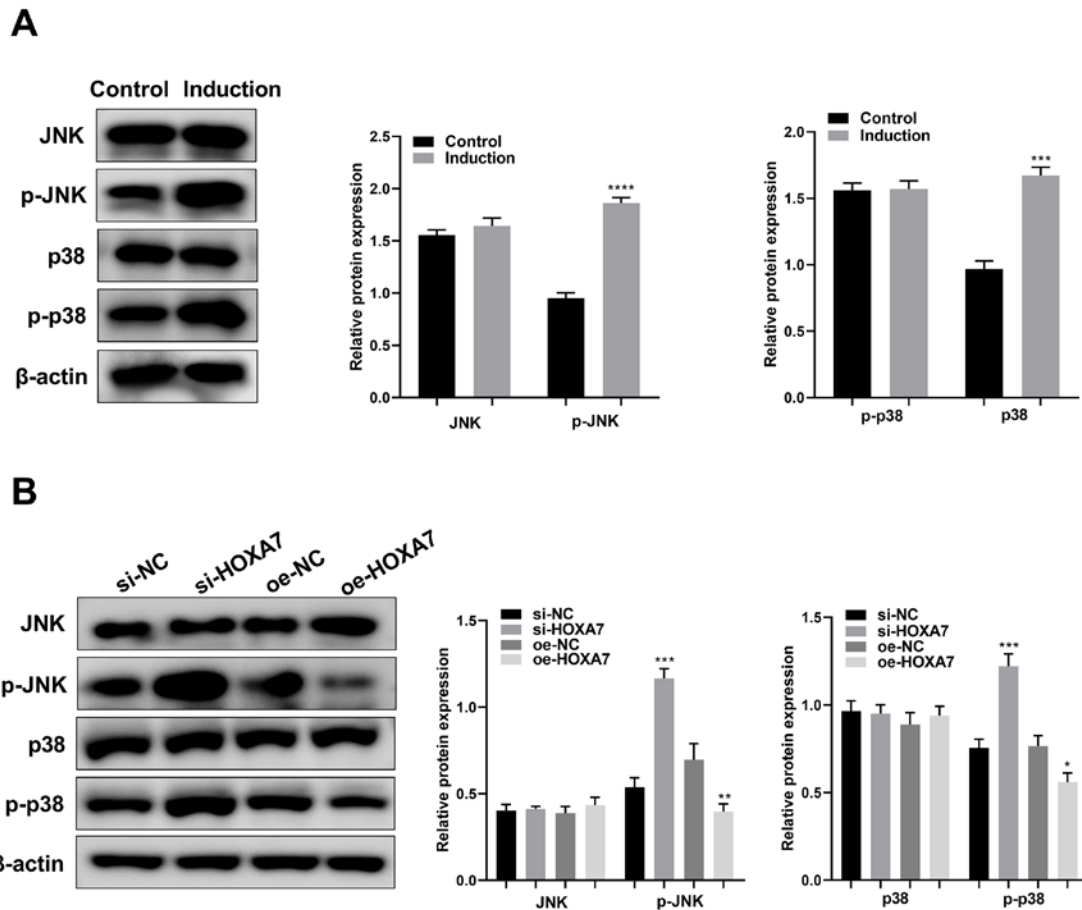
### Osteogenic differentiation of hBMSCs

In the induction group, ALP activity was increased (Figure 2A). Alizarin Red S staining revealed more intense mineral

deposition in induced hBMSCs than in controls (Figure 2B). Protein levels of RUNX2, OPG, and OPN were also elevated in induced hBMSCs (Figure 2C–D). These results confirm successful osteogenic differentiation and validate the use of the isolated hBMSCs for subsequent experiments.

### HOXA7 inhibits osteogenic differentiation of hBMSCs

We next evaluated the effect of HOXA7 on osteogenic differentiation. HOXA7 was silenced in hBMSCs using si-HOXA7 and overexpressed using oe-HOXA7. As expected, HOXA7 expression decreased in the si-HOXA7 group and increased in the oe-HOXA7 group (Figure 3A–B). ALP staining showed that HOXA7 overexpression inhibited osteogenic differentiation, whereas HOXA7 knockdown promoted it (Figure 3C). Consistently, Alizarin Red S staining indicated reduced calcium deposition with HOXA7 overexpression and increased deposition with HOXA7 knockdown (Figure 3D). Western blotting further



**Figure 6.** HOXA7 regulates the p38 MAPK/JNK pathway in hBMSCs and MC3T3-E1 cells. (A–B) Phosphorylated and total p38 MAPK and JNK (p-p38/p38, p-JNK/JNK) in hBMSCs and MC3T3-E1 cells with the indicated transfections were evaluated by Western blotting. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

demonstrated that HOXA7 overexpression decreased, while HOXA7 knockdown increased, the protein levels of OPG, OPN, and RUNX2 (Figure 3E–H). These data indicate that HOXA7 suppresses osteogenic differentiation of hBMSCs.

#### HOXA7 modulates proliferation and apoptosis of MC3T3-E1 cells

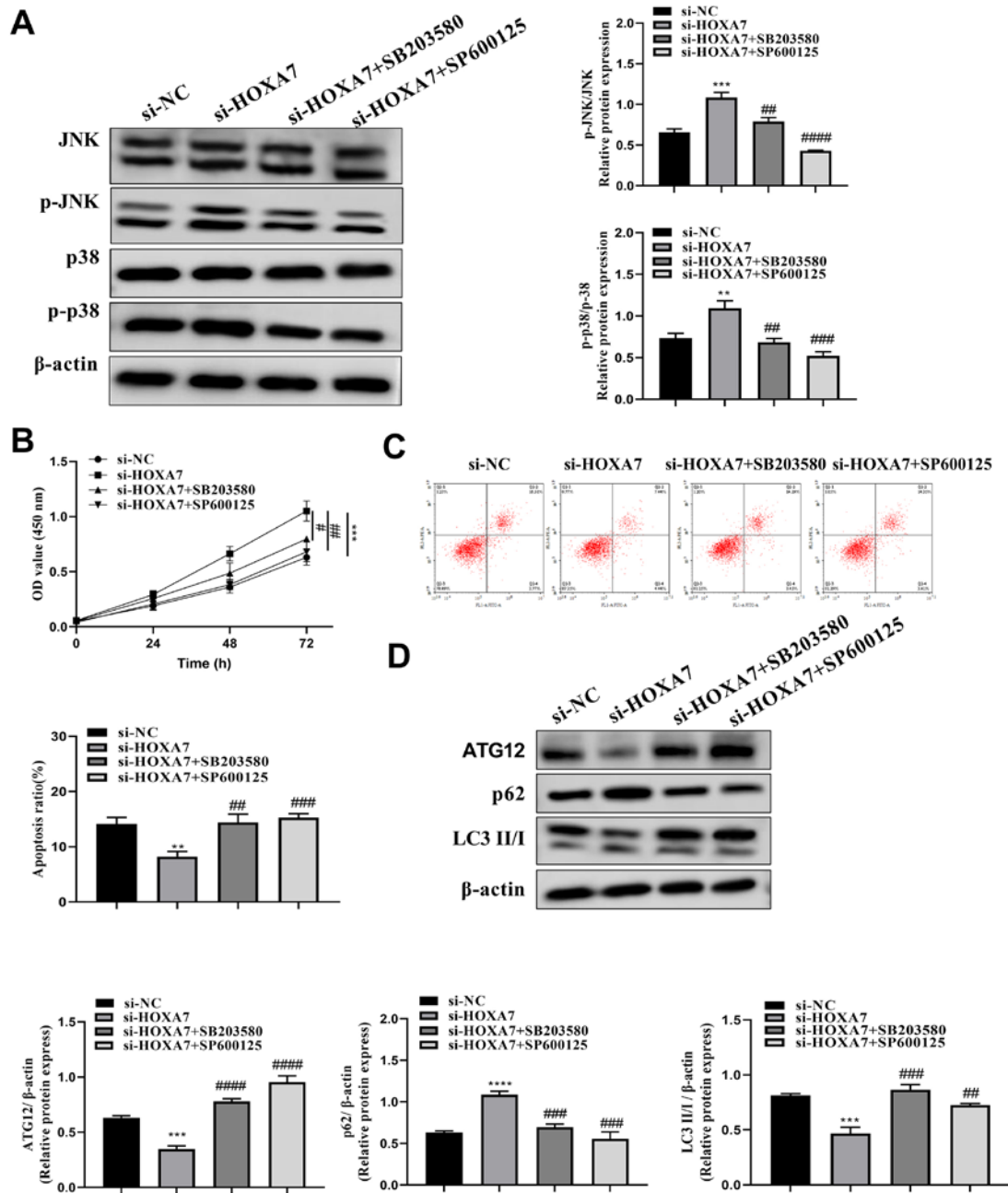
To investigate HOXA7-mediated effects on osteoblast lineage cells *in vitro*, MC3T3-E1 preosteoblasts were transfected with si-HOXA7 or oe-HOXA7 (Figure 4A). CCK-8 assays showed that HOXA7 overexpression significantly reduced cell viability, whereas HOXA7 knockdown increased viability (Figure 4B). Flow cytometry revealed a higher apoptotic rate in the oe-HOXA7 group, while HOXA7 knockdown significantly reduced apoptosis compared with controls (Figure 4C).

#### HOXA7 modulates autophagy markers in MC3T3-E1 cells

To evaluate the impact of HOXA7 on autophagy, Western blotting was used to assess autophagy-related proteins (p62, LC3-II/I, and ATG12) in MC3T3-E1 cells. As shown in Figure 5A–B, HOXA7 overexpression increased LC3-II/I and ATG12, while decreasing p62 (an autophagy-associated substrate). Conversely, HOXA7 knockdown produced the opposite pattern. These findings indicate that HOXA7 modulates autophagy-related protein levels in MC3T3-E1 cells.

#### HOXA7 regulates the p38 MAPK/JNK pathway in hBMSCs and MC3T3-E1 cells

To investigate signaling pathways associated with HOXA7 function in OP, proteins in the p38 MAPK/JNK pathway were examined in hBMSCs and MC3T3-E1 cells. In hBMSCs, phosphorylated JNK and p38 (p-JNK and p-p38)



**Figure 7.** Effects of SP600125 and SB203580 on proliferation, apoptosis, and autophagy in MC3T3-E1 cells after HOXA7 knockdown. (A) Western blotting of p-JNK/JNK and p-p38/p38 in MC3T3-E1 cells under the indicated conditions. (B) CCK-8 assay assessing cell viability. (C) Flow cytometry analysis of apoptosis. (D) Western blotting of ATG12, p62, and LC3-II/I. Significance vs. si-NC: \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001. Significance vs. si-HOXA7: ##P < 0.01, ###P < 0.001, ####P < 0.0001.

increased following osteogenic induction (Figure 6A). In MC3T3-E1 cells, HOXA7 overexpression reduced pathway activation, reflected by lower p-JNK/JNK and p-p38/p38 ratios, whereas HOXA7 knockdown produced the opposite effect (Figure 6B).

To test whether p38 MAPK/JNK activity mediates HOXA7-dependent cellular effects, MC3T3-E1 cells were treated with the JNK inhibitor SP600125 and the p38 inhibitor SB203580. Western blotting showed that both inhibitors attenuated the HOXA7-knockdown-induced

increases in p-JNK and p-p38 (Figure 7A). Functionally, SP600125 and SB203580 reduced the HOXA7-silencing-associated increase in cell viability (Figure 7B) and partially reversed the decrease in apoptosis (Figure 7C). In addition, both inhibitors counteracted the HOXA7-knockdown effects on autophagy-related protein levels (Figure 7D). Collectively, these results support a model in which HOXA7 modulates OP-relevant phenotypes by suppressing p38 MAPK/JNK signaling in osteoblast-lineage cells.

## Discussion

HOXA7 has been implicated in the regulatory network of osteoporosis (OP), but its mechanistic role in OP pathogenesis remains insufficiently defined. In this study, serum HOXA7 levels were significantly higher in the OP group, whereas HOXA7 mRNA and protein expression decreased during osteogenic induction of hBMSCs. Functional assays demonstrated that HOXA7 suppresses osteogenic differentiation in hBMSCs. In MC3T3-E1 cells, HOXA7 also modulated proliferation, apoptosis, and autophagy. Moreover, our data indicate that HOXA7 influences these phenotypes at least in part by regulating the p38 MAPK and JNK signaling pathways, thereby contributing to OP-relevant cellular dysfunction.

Mesenchymal stem cells (MSCs) derived from bone marrow are readily *expanded in vitro* and, under osteogenic induction, can differentiate into osteoblasts<sup>12</sup>. Consequently, they are widely used as seed cells in bone tissue engineering and play important roles in promoting bone repair and reconstruction in patients with osteoporosis (OP)<sup>13</sup>. Osteoblasts are essential for bone formation and remodeling and are responsible for the synthesis, secretion, and mineralization of bone matrix<sup>14</sup>. Several studies have associated HOXA7 with OP<sup>7,9,15</sup>, but its mechanism of action in OP has not been fully elucidated.

Alkaline phosphatase (ALP) activity and matrix mineralization are key readouts of osteogenesis. ALP is a canonical early marker of osteoblast differentiation that hydrolyzes phosphate esters and supports maturation and mineral deposition<sup>16</sup>. Alizarin Red S (ARS) staining detects calcium-containing mineralized nodules and is commonly used to assess late-stage osteogenesis<sup>17</sup>. RUNX2, OPN, and OPG are frequently evaluated as osteogenesis-associated markers in osteoblast differentiation studies<sup>18</sup>.

A study by da Silva et al.<sup>7</sup> reported increased expression of HOXA cluster genes during osteoblast differentiation and highlighted their roles as mediators of positional identity in the skeletal system, whereas in our study HOXA7 decreased during induction, suggesting gene-specific regulation within the HOXA cluster. Prior work also showed that NSD2 inhibits osteogenic differentiation of hBMSCs by transcriptionally downregulating HOXA2 via H3K36me2 dimethylation<sup>19</sup>, and that silencing HOXA2 increases bone alkaline phosphatase in rats<sup>20</sup>. Substantial evidence implicates HOXA10 in regulating osteoblast differentiation and osteoporosis<sup>21,22</sup>,

and HOXA13 overexpression has been reported to enhance osteogenic differentiation in OP models<sup>23,24</sup>. With respect to HOXA7, downregulation via miR-920 promotes osteogenic differentiation of hBMSCs in OP populations<sup>9</sup>, and HOXA7 overexpression can attenuate the pro-osteogenic effect of miR-196a-5p in aged bone marrow MSCs (O-BMSCs)<sup>25</sup>.

In our study, HOXA7 was more highly expressed in the serum of patients with osteoporosis. Moreover, HOXA7 expression progressively decreased during osteogenic induction of hBMSCs, suggesting a negative association with osteogenic differentiation. Functional experiments confirmed that HOXA7 markedly inhibited osteogenic mineralization and differentiation, and changes in osteogenesis-related proteins further supported these findings. Collectively, these results indicate that HOXA7 inhibits osteogenic differentiation of hBMSCs.

Osteoblasts play a critical role in the pathogenesis of OP. Enhancing osteoblast proliferation and limiting apoptosis may contribute to prevention and clinical management of OP. In this study, mouse preosteoblast MC3T3-E1 cells were transfected with si-HOXA7 or oe-HOXA7 to investigate HOXA7's role in osteoblastic proliferation, apoptosis, and autophagy *in vitro*. The *in vitro* data showed that HOXA7 knockdown significantly increased osteoblast proliferation and reduced apoptosis.

Autophagy is a conserved lysosomal degradation pathway that delivers intracellular components to lysosomes for turnover and is essential for recycling unnecessary or damaged organelles and proteins to maintain cellular homeostasis<sup>26</sup>. Dysregulated autophagy has been linked to tumorigenesis, immune disorders, cardiovascular disease, and osteoporosis<sup>27-30</sup>. During osteogenic differentiation of hBMSCs, autophagy can supply energy and nutrients by clearing damaged organelles and misfolded proteins, thereby promoting the expression of osteogenesis-related genes and osteogenic differentiation<sup>31,32</sup>. For example, under osteogenic induction, autophagy increases in periodontal ligament stem cells, and its activation enhances osteogenic readouts such as ALP activity and osteocalcin expression<sup>33,34</sup>. Prior studies indicate that autophagy protects against osteoblast death<sup>35</sup>, whereas autophagy inhibition exacerbates apoptosis in MC3T3-E1 osteoblasts<sup>36</sup>. Small molecules can modulate these processes; for instance, leonurine promotes hBMSC proliferation and osteogenic differentiation via autophagy activation through the PI3K/Akt/mTOR pathway in rats<sup>37</sup>, and asperuloside alleviates OP by promoting autophagy and regulating Nrf2 activation<sup>38</sup>. These observations highlight complex interrelationships between autophagy and osteogenic differentiation that jointly maintain cellular function and bone homeostasis, supporting autophagy activation as a potential therapeutic approach in OP.

HOXA7 has been reported to promote autophagy in HTR-8/SVneo trophoblast cells and to be important for trophoblast biology<sup>39</sup>. Consistent with an autophagy-related role, we observed that HOXA7 overexpression increased LC3-II/I and ATG12 and decreased p62 in MC3T3-E1 cells,

indicating that HOXA7 modulates autophagy markers in this osteoblast-lineage model. Whether these autophagy changes are ultimately protective or detrimental in the osteoporotic context warrants further investigation.

The mitogen-activated protein kinase (MAPK) pathway is a key signaling cascade that transmits extracellular cues from the cell surface to the nucleus and regulates fundamental cellular processes, including differentiation, growth, inflammatory responses, and stress adaptation<sup>40,41</sup>. The c-Jun N-terminal kinase (JNK) pathway is one of the three canonical MAPK branches. Prior studies have shown that hBMSC-derived exosomes promote osteoblast proliferation via MAPK signaling to ameliorate OP<sup>25</sup>; caffeine has been reported to influence bone homeostasis by affecting both osteoclasts and osteoblasts through MAPK pathways<sup>42</sup>; and HDAC9 knockdown inhibits hBMSC osteogenic differentiation, in part by suppressing MAPK signaling<sup>43</sup>. Small-molecule studies further indicate that kaempferol counteracts dexamethasone-induced osteogenic inhibition via JNK and p38 pathways in MC3T3-E1 cells<sup>44</sup>, and dendrobine reverses dexamethasone-mediated suppression of osteogenic differentiation through JNK and p38 signaling, suggesting therapeutic potential for glucocorticoid-induced osteoporosis<sup>45</sup>.

To probe pathway involvement in our model, we treated MC3T3-E1 cells with the JNK inhibitor SP600125 and the p38 inhibitor SB203580 and evaluated the effects of HOXA7 on proliferation, apoptosis, and autophagy. HOXA7 upregulation significantly reduced phosphorylation of JNK and p38 MAPK, whereas HOXA7 silencing increased phosphorylation, indicating pathway activation upon HOXA7 knockdown. Consistently, SP600125 and SB203580 attenuated the HOXA7-silencing-induced changes in cell viability, apoptosis, and autophagy, supporting a role for p38/JNK signaling in mediating HOXA7-dependent phenotypes.

### Limitations

This study has several limitations. First, we did not analyze associations between clinical characteristics and serum HOXA7 levels in OP patients. Second, the work lacks *in vivo* validation. Third, the cellular experiments relied on two species (human hBMSCs and mouse MC3T3-E1 cells), which may introduce interspecies differences. Fourth, direct transcriptional targets of HOXA7 were not investigated. Finally, additional studies are needed to delineate more precisely how HOXA7 interfaces with p38 MAPK/JNK signaling during OP progression.

### Conclusion

Our findings suggest that HOXA7 influences osteogenic differentiation of hBMSCs and regulates proliferation, apoptosis, and autophagy in MC3T3-E1 cells, at least in part via the p38 MAPK and JNK pathways. However, further studies are needed to fully define HOXA7's role in

OP pathophysiology, which may provide insights to guide future therapeutic strategies.

### Ethics Approval

*This study was approved by the Ethics Committee of Wendeng Orthopaedic Hospital of Shandong Province (No. LL20231201). All procedures were conducted in accordance with the Declaration of Helsinki (1964) and its later amendments.*

### Consent to Participate

*Written informed consent was obtained from all participants prior to enrollment.*

### Authors' contributions

*Yijun Wang and Jingjing Zhang contributed to the conception of the study. Bo Wang and Ziwei Wang contributed significantly to the data analysis and study preparation. Peirui Miao performed the data analyses and drafted the manuscript. Yanke Hao helped perform the analysis and provided constructive discussion. All authors read and approved the final study.*

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