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# LncRNA UCA1 affects osteoblast proliferation and differentiation by regulating BMP-2 expression

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**Abstract. – OBJECTIVE: The aim of this study was to detect the expression of long non-coding ribonucleic acid (lncRNA) urothelial carcinoma associated 1 (UCA1) in the plasma of patients with osteoporosis (OST), and to investigate its influences on the proliferation and differentiation of osteoblasts and its mechanism.**

**PATIENTS AND METHODS: Plasma samples were collected from 52 OST patients treated in our hospital and 30 healthy subjects receiving a physical examination, respectively. The expression level of lncRNA UCA1 in OST patients and healthy subjects were detected *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Furthermore, osteoblast MC3T3-E1 cell lines with a stable knockout of UCA1 in mice were constructed using small-interfering RNA (siRNA). The influence of UCA1 knockout on the proliferation of osteoblasts was detected using cell counting kit-8 (CCK-8) assay. Meanwhile, the proportion of EdU-positive cells in osteoblasts of the control group and UCA1 knockout group was detected using EdU staining. Moreover, the messenger RNA (mRNA) levels of differentiation-related genes, including Runt-related transcription factor 2 (Runx2), Collagen1α1, osteoclast (OC), osteoprotegerin (OPG), osteopontin (OPN) and Osterix (OSX), were detected *via* RT-PCR. The protein expression level of Runx2 was detected *via* Western blotting. In addition, osteoblasts were cultured with a bone-derived medium for 14 d. Then, the differentiation status was detected *via* alizarin red staining and alkaline phosphatase staining. Finally, the expression of bone morphogenetic protein-2 (BMP-2)/(Smad1/5/8) signaling pathway was analyzed using Western blotting.**

**RESULTS: The expression of plasma lncRNA UCA1 was significantly increased in OST patients ($p<0.05$). Cell experiments revealed that UCA1 siRNA intervention could significantly promote the proliferation and differentiation of osteoblast MC3T3-E1 cell lines. In addition, Western blotting showed that the pro-apoptotic effect of UCA1 might be mediated by the BMP-2/(Smad1/5/8) signaling pathway in osteoblasts.**

**CONCLUSIONS: Inhibiting lncRNA UCA1 can promote the proliferation and differentiation of osteoblasts by activating the BMP-2/(Smad1/5/8) signaling pathway in osteoblasts. Therefore, UCA1 is expected to be a new therapeutic target for OST.**

*Key Words:*
LncRNA UCA1, Osteoporosis, Osteoblasts, Proliferation, Differentiation.

## Introduction

Osteoporosis (OST) is one of the most common bone metabolic diseases. OST is characterized by decreased bone mineral density (BMD) and increased fracture risk. Meanwhile, it is also a common complication of sarcopenia[1]. Currently, OST has been a major public health problem threatening the health of the elderly around the world[2]. It is recommended that physical exercise is the most effective non-drug preventive and therapeutic strategy for OST. This may be due to the reason that physical exercise can produce mechanical signals directly through muscle strength or indirectly exert a certain anabolic effect on the bone through endocrine regulation. However, its specific molecular mechanism remains unclear yet[3,4]. Therefore, searching for key genes and proteins leading to OST is of important significance for the early prevention and precise treatment of OST[5]. Bone morphogenetic protein-2 (BMP-2) plays a unique and important role in bone formation after birth. The main reason is that BMP-2 activates some important bone-derived transcription factors, such as Cbfa1, OSX and Msx2, by activating Smad1/5/8 and related downstream kinases. This may ultimately increase the ex-


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pression of bone matrix protein[6,7]. Studies have demonstrated that the expression level of BMP-2 is significantly declined in the bone tissues and osteoblasts of OST patients[8,9]. Furthermore, these results indicate that the up-regulation of BMP-2 expression in bone tissues of OST patients can exert a certain inhibitory effect on OST.

Long non-coding ribonucleic acid (lncRNA) is a kind of long-chain RNA molecule with more than 200 nucleotides in transcription length[10]. Although lncRNAs cannot encode corresponding proteins in cells, they can regulate the expression of corresponding target genes at the transcriptional/post-transcriptional level and epigenetic modification. This can ultimately affect the occurrence and development of diseases[11]. LncRNA urothelial carcinoma associated 1 (UCA1) is a member of the lncRNA family that plays an important role in various diseases, including tumors, cardiovascular diseases and endocrine diseases. For example, up-regulating the expression of lncRNA UCA1 in liver cancer cells promotes the progression of liver cancer by inhibiting miR-216b and activating the FGFR1/ERK signaling pathway[12]. However, no reports have explored the role of lncRNA UCA1 in OST yet.

In this study, the difference in the plasma level of UCA1 between healthy people and OST patients was detected. The influences of UCA1 knockout on the proliferation and differentiation of osteoblast MC3T3-E1 cell lines were further detected. In addition, we explored the possible underlying molecular mechanism.

## Patients and Methods

### Plasma Samples

52 OST patients aged (55.12±11.49) and treated in our hospital from December 2016 to March 2018 were enrolled in this study. Meanwhile, 30 healthy subjects aged (54.21±12.34) receiving a physical examination were selected as a control group. 4 mL of venous blood was collected, anticoagulated with sodium citrate and cryopreserved in a refrigerator at -20°C for subsequent experiments. This investigation was approved by the Ethics Committee of Southwest Hospital. Signed informed consent was obtained from each subject before the study.

### Cell Culture

The mouse osteoblast MC3T3-E1 cell line was purchased from BioLeaf Biotechnology (Shanghai, China). Cells were cultured in complete α-Modified Eagle medium (MEM; HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin in a humid environment with 95% air and 5% $CO_2$ at 37°C. The medium was replaced once every 2-3 d.

### UCA1 Knockout

In the logarithmic growth phase, MC3T3-E1 cells were immediately digested and inoculated into 6-well plates. After 12 h (60-80% cell fusion), the complete medium was discarded. Subsequently, the cells were washed with serum-free medium 2-3 times and starved in an incubator to realize synchronous growth. UCA1 siRNA was dissolved in RNase deionized water to prepare the transfection solution at a final concentration of 20 μmol/L. The cells were divided into two groups, including the control group and UCA1 knockout (UCA1 siRNA) group. Prepared transfection solution was added into each well and fully mixed, followed by cell culture for another 6 h. Then the solution was replaced with complete medium again. The base sequences of UCA1 siRNA were as follows: Forward: 5'-GAACUGUACAGGGAUUUAU-3', Reverse: 5'-AUAAUAAAAUGUUACACCU-3'.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) Total RNA was extracted from cells in each group according to the instructions of the TRIzol method (Invitrogen, Carlsbad, CA, USA). The concentration and purity of the extracted RNA were detected using an ultraviolet spectrophotometer. The RNA with absorbance $(A)_{260}/A_{280}$ of 1.8-2.0 could be used. (2) The extracted messenger RNA (mRNA) was synthesized into complementary deoxyribonucleic acid (cDNA) through RT and stored in a -80°C refrigerator. (3) RT-PCR system: 2.5 μL 10×Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq enzyme ($2\times10^6$ U/L) and 19 μL $ddH_2O$. The amplification system of RT-PCR was the same as above. The primer sequences of genes [Runt-related transcription factor 2 (Runx2), Collagen1α1, osteoclast (OC), osteoprotegerin (OPG), osteopontin (OPN) and OSX] were shown in Table I.

### Cell Counting Kit-8 (CCK-8) Assay

Cells in the logarithmic growth phase in each group were first inoculated into 96-well plates. Subsequently, the cells were cultured in a 5% $CO_2$,

**Table I.** Primer sequences in RT-PCR.

| Target gene | | Primer sequence |
|---|---|---|
| GAPDH | Forward | 5'-GACATGCCGCCTGGAGAAAC-3' |
| | Reverse | 5'-AGCCCAGGATGCCCTTTAGT-3' |
| UCA1 | Forward | 5'-TGCTGCCTTTTCTGTTCCTT-3' |
| | Reverse | 5'-AAGGTGCTGGGTAGGGAAGT-3' |
| Runx2 | Forward | 5'-GTCCAACCCGTAAGGT-3' |
| | Reverse | 5'-CGCTGCTGAGTCGATGCTAGCT-3' |
| Collagen1α1 | Forward | 5'-ACGTAGCTAGCTAGTCGGTATG-3' |
| | Reverse | 5'-AAAACGTGGCTAGTCGATCG-3' |
| OC | Forward | 5'-ATCGTAGCTAGCTAGTCGAGCA-3' |
| | Reverse | 5'-CCCCCTGTGCTAGCTAGCTAGC-3' |
| OPG | Forward | 5'-TTGTGTTAGCTTAGCCCGATCGTA-3' |
| | Reverse | 5'-ACCCGTGTGGCTAGTCGATC-3' |
| OPN | Forward | 5'-ACGATCGATCGTAGCTAGTC-3' |
| | Reverse | 5'-AAAACGATCGTAGCTAGTCGA-3' |
| OSX | Forward | 5'-GTGCTGATGTTAGCTAGCTAGCT-3' |
| | Reverse | 5'-AGCTAGTCGTAGCTAGCTGATCG-3' |

37°C incubator for 0, 24, 48 and 72 h, respectively. The color-developing solution was prepared in the dark using 100 μL 1640 medium and 10 μL CCK-8 (10:1) (Dojindo, Kumamoto, Japan). Then the culture medium was discarded, and 110 μL/well solution was added into each well, followed by incubation at 37°C for 2 h. Finally, optical density (OD) at 450 nm was detected using an ultraviolet spectrophotometer.

### EdU (5-Ethynyl-2'-Deoxyuridine) Staining

24 h after UCA1 knockout in MC3T3-E1 cells using siRNA, the cells were stained with Click-iT EdU staining kit (Invitrogen, Carlsbad, CA, USA) according to the instructions. After staining, three visual fields were randomly selected in each glass slide and photographed using a fluorescence microscope. Finally, EdU positive-cells were counted.

### Western Blotting

(1) The culture medium was first discarded and the cells were washed with phosphate-buffered saline (PBS) 3 times. (2) 1000 μL lysis buffer was added into every dish and fully vibrated for 20 min. (3) The cells at the bottom of the dish were scraped off using a brush and placed into an Eppendorf tube (EP). (4) The collected cells were lysed using ultrasonic pyrolysis for about 15 s. (5) After standing for 15 min, the cells were centrifuged at 12000 rpm for 0.5 h. (6) The supernatant was taken and placed into an EP tube. The protein concentration was detected *via* ultraviolet spectrometry, and all protein samples were quantified to the same concentration. (7) The protein was sub-packaged and placed in a refrigerator at -80°C. The total protein extracted from osteoblasts was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubation with primary antibodies at 4°C overnight, the membranes were incubated again with goat anti-rabbit secondary antibody in the dark for 1 h. Protein bands were scanned and quantified using Odyssey scanner. The expression level of protein was detected, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

### Alkaline Phosphatase (ALP) Staining and Activity Determination

Cells in each group were washed twice with PBS, and fixed with 4% paraformaldehyde for 10 min. After washing with deionized water, the cells were stained according to the instructions of BCIP/NBT ALP staining kit (Beyotime, Shanghai, China) for 1 h. Images were acquired by a camera. The ALP activity was determined as follows: after culturing in the medium for 14 d, osteoblasts were washed with PBS and lysed, followed by centrifugation. The supernatant was retained and the ALP activity was determined according to instructions of the kit (Sigma-Aldrich, St. Louis, MO, USA) and quantified using bicinchoninic acid (BCA) protein assay kit (Pierce, Waltham, MA, USA).

### Alizarin Red Staining

The cells in each group were washed twice with pre-cooled PBS, fixed with 4% paraformaldehyde for 10 min and then incubated with 30 mM alizarin red S (pH=4.2, Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 10 min. Finally, images were acquired by a camera.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analyses. Measurement data were expressed as mean ± standard deviation. The *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

## Results

### Expression of Plasma LncRNA UCA1 in OST Patients

RT-PCR results revealed that the expression level of plasma lncRNA UCA1 in OST patients was about 11.54 times higher than that of the control group, showing a statistically significant difference ($p<0.05$) (Figure 1). This indicated that UCA1 might play an important role in the pathogenesis of OST.

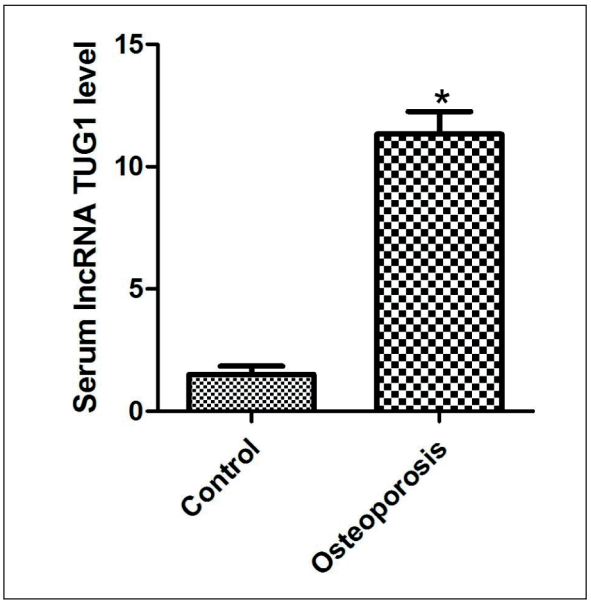


**Figure 1.** Expression of plasma lncRNA UCA1 in OST patients. Control: health controls, osteoporosis: OST. *$p<0.05$: There was a statistically significant difference compared with the control group.

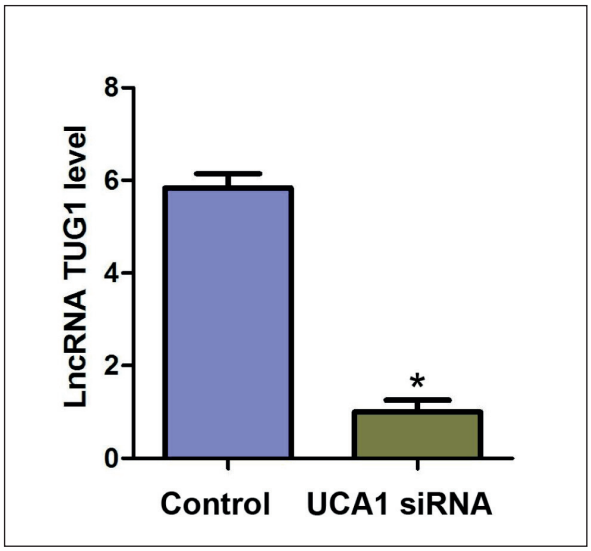


**Figure 2.** Detection of lncRNA UCA1 knockout. Control: blank control group, UCA1 siRNA: UCA1 knockout group. *$p<0.05$: There was a statistically significant difference compared with the control group.

### Construction of Cell Lines With Stable UCA1 Knockout

To deeply study the role of UCA1 in OST, siRNA was used to knock out UCA1 in MC3T3-E1 cells. The knockout efficiency of UCA1 in MC3T3-E1 cells was detected *via* RT-PCR. The results revealed that the expression level of UCA1 in UCA1 siRNA group was reduced by 79.86% compared with that of the control group ($p<0.05$) (Figure 2).

### Cell Proliferation Ability Detected Via CCK-8

The results of CCK-8 assay manifested that the proliferation ability of osteoblasts in UCA1 siRNA group was significantly stronger than that of the control group at 24, 48 and 72 h ($p<0.05$) (Figure 3). These results suggested that the proliferation ability of MC3T3-E1 cells was markedly increased after UCA1 inhibition.

### EdU Staining Results in Each Group

To further detect the influence of UCA1 knockout on cell proliferation in each group, EdU staining was performed to evaluate the cell proliferation ability. The results showed that the number of EdU-positive cells in UCA1 siRNA group was about 3.56 times higher than that of the control group ($p<0.05$) (Figure 4).

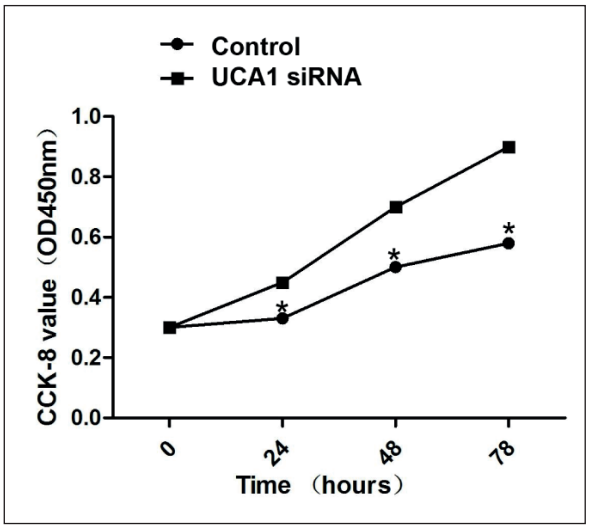


**Figure 3.** Cell proliferation ability detected via CCK-8. Control: blank control group, UCA1 siRNA: UCA1 knockout group. *$p$<0.05: There was a statistically significant difference compared with the control group.

### *Influence of UCA1 Knockout on Osteoblast Differentiation*

To evaluate the role of UCA1 in osteoblast differentiation, the mRNA expression levels of osteoblast differentiation-related genes were detected. RT-PCR results manifested that the mRNA levels of Runx2, Collagen1α1, OC, OPG, OPN and OSX in UCA1 siRNA group were significantly higher than those of the control group ($p$<0.05) (Figure 5), indicating that cell differentiation ability in UCA1 siRNA group was significantly enhanced. Furthermore, the protein level of Runx2 was detected *via* Western blotting. It was found that UCA1 siRNA also remarkably inhibited Runx2 expression at the protein level.

### *Influence of UCA1 Knockout on Osteoblast Differentiation Detected Via Alizarin Red and ALP Staining*

The cell differentiation ability in each group was detected using alizarin red and ALP staining. It was found that cell differentiation ability in UCA1 siRNA group was remarkably stronger than that of the control group. Furthermore, the ALP activity in UCA1 siRNA group was also remarkably increased ($p$<0.05) (Figure 6).

### *Influence of UCA1 Knockout on BMP-2/(Smad1/5/8) Signaling Pathway in Osteoblasts*

Considering the important role of the BMP-2/(Smad1/5/8) signaling pathway in the osteoblast proliferation and differentiation, the protein expression levels of BMP-2, phosphorylated-Smad1/5/8 and total Smad1/5/8 were detected *via* Western blotting in each group. The results revealed that the BMP-2/(Smad1/5/8) signaling pathway in osteoblasts was significantly activated after the UCA1 knockout ($p$<0.05) (Figure 7).

## Discussion

OST is a systemic skeletal disease characterized by loose bone structure and reduced bone mass. It will increase the risk of fracture if not treated in time[13]. As age increases, the risk of OST will also rapidly increase. In the aging society nowadays, OST has brought a heavy burden to patients' families and medical care department[14]. In recent years, great progress has been made in the prevention and treatment of female OST. The current treatment mainly focuses on increas-

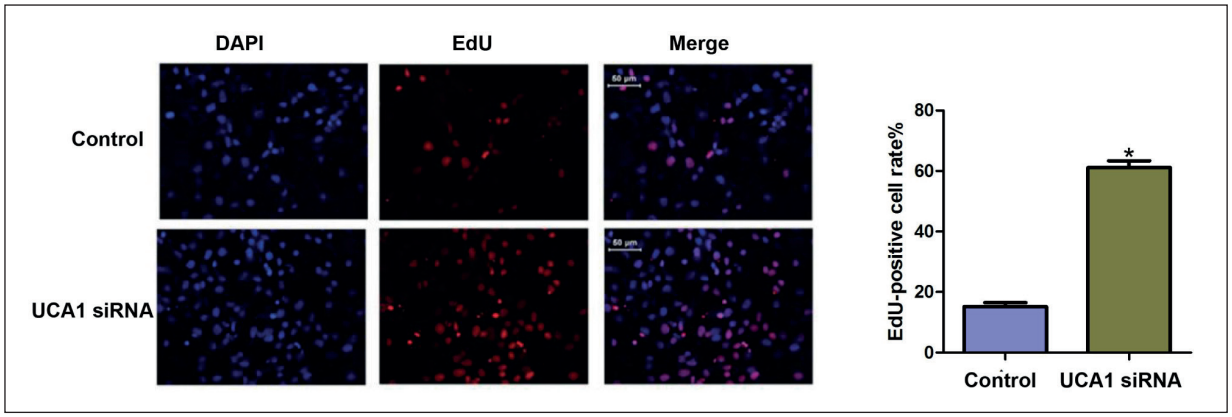


**Figure 4.** EdU staining results in each group. Control: blank control group, UCA1 siRNA: UCA1 knockout group (Magnification: 40×). *$p$<0.05: There was a statistically significant difference compared with the control group.

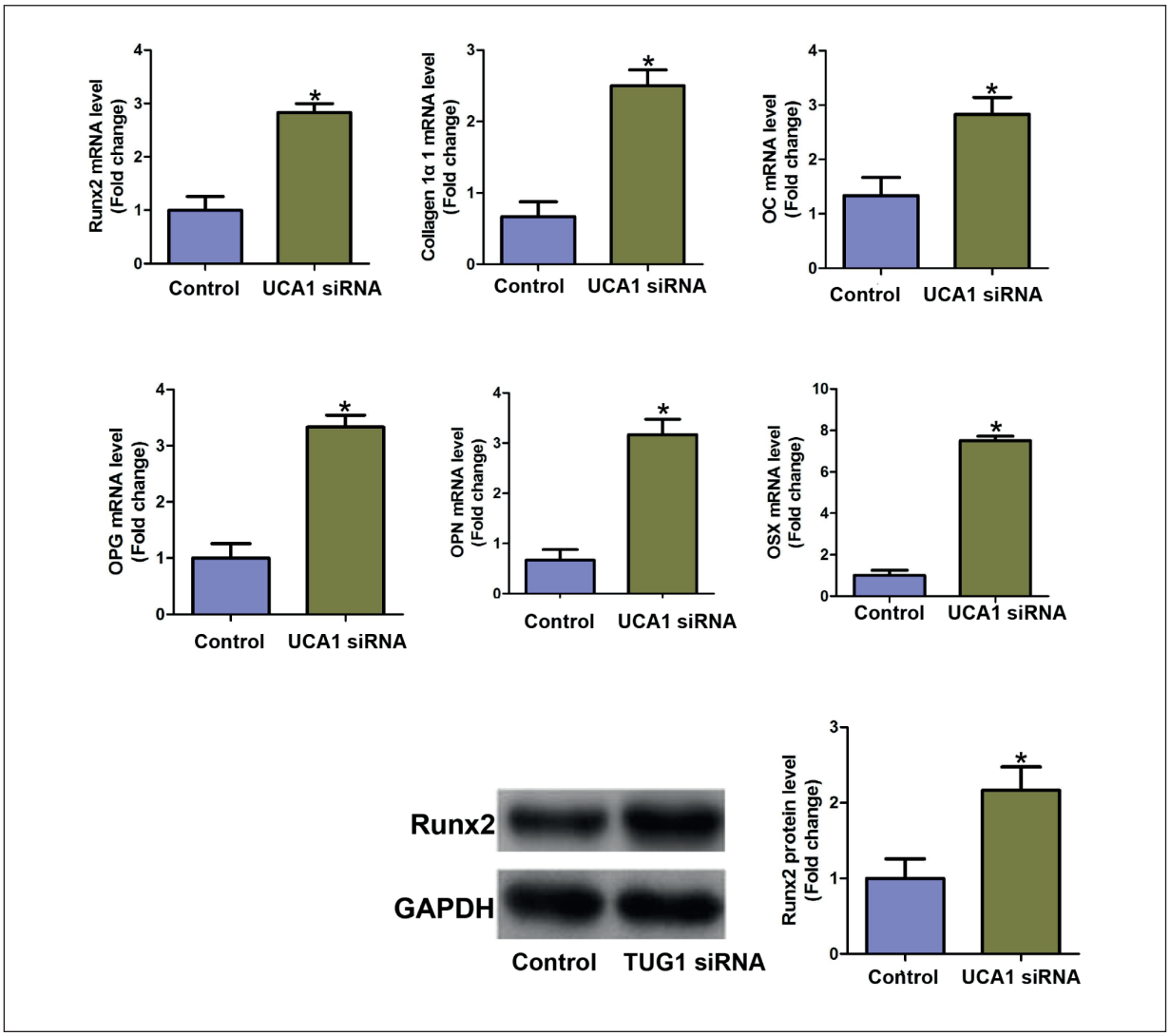


**Figure 5.** Influence of UCA1 knockout on osteoblast differentiation. Control: blank control group, UCA1 siRNA: UCA1 knockout group. *$p$<0.05: There was a statistically significant difference compared with the control group.

ing bone formation or reducing bone resorption, thus lowering the occurrence rate of OST-induced fractures[15].

The proliferation and differentiation of osteoblasts are complex processes mediated by various extracellular growth factors, including BMPs and transforming growth factor-β (TGF-β). Some cytokines related to osteoblast proliferation and differentiation are activated by these growth factors. For example, the activation of the TGF-β/BMP signaling pathway activates osteogenic induction. Meanwhile, the downstream transcription factor Smad in the pathway is crucial for osteogenic induction. The binding of BMP or TGF-β ligands to serine/threonine kinase receptor can lead to the phosphorylation and activation of Smad2/3. Once BMPs are activated, Smad1/5/8 can be phosphorylated. After that, they can bind to Smad4 to form a complex, ultimately activating proteins closely related to osteoblast proliferation and differentiation[15-17]. Therefore, many genes or proteins that can regulate this pathway are expected to be potential therapeutic targets for OST. For example, tripartite motif-containing 33 (TRIM33) positively and negatively regulate the TGF-β/BMP signaling pathway in tumors. Up-regulating TRIM33 in osteoblasts can significantly activate the TGF-β/BMP signaling pathway, thereby promoting the osteoblast proliferation and differentiation[18]. According to a recent study, BMP-2/Smad signaling

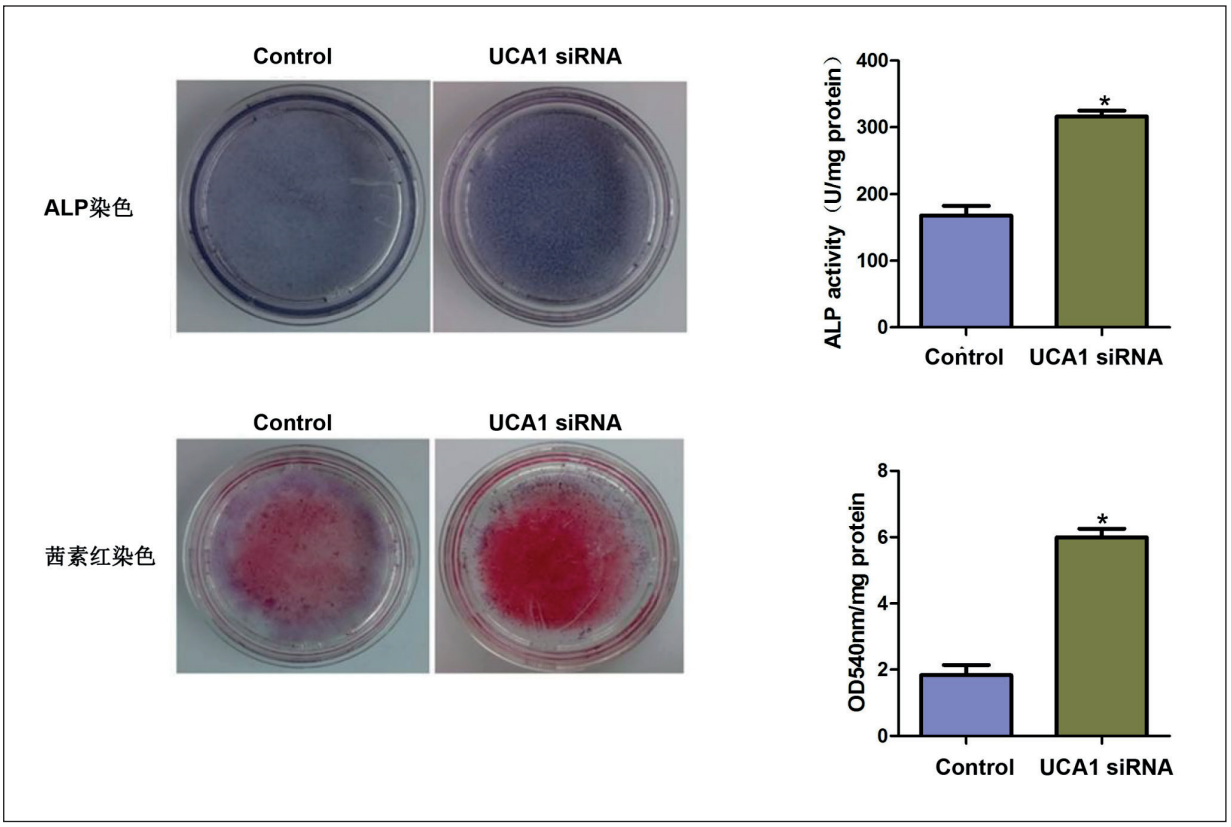


**Figure 6.** Influence of UCA1 knockout on osteoblast differentiation detected via alizarin red and ALP staining. Control: blank control group, UCA1 siRNA: UCA1 knockout group. *$p$<0.05: There was a statistically significant difference compared with the control group.

pathway can be affected by the destruction of actin microfilament. Moreover, a kind of connexin CCN1 is found in pre-osteoblasts. CCN1 has the ability to connect Smad protein and actin microfilament. In addition, its knockout can significantly reduce the transcriptional activity of Smad protein[19].

Xiao C. et al[20] have indicated that lncRNA UCA1 regulates a variety of life activities. For example, UCA1 promotes an epithelial-mesenchymal transition in breast cancer cells by activating the Wnt/β-catenin signaling pathway[20]. The expression level of UCA1 is significantly increased in osteosarcoma tissues and cells. Mean-

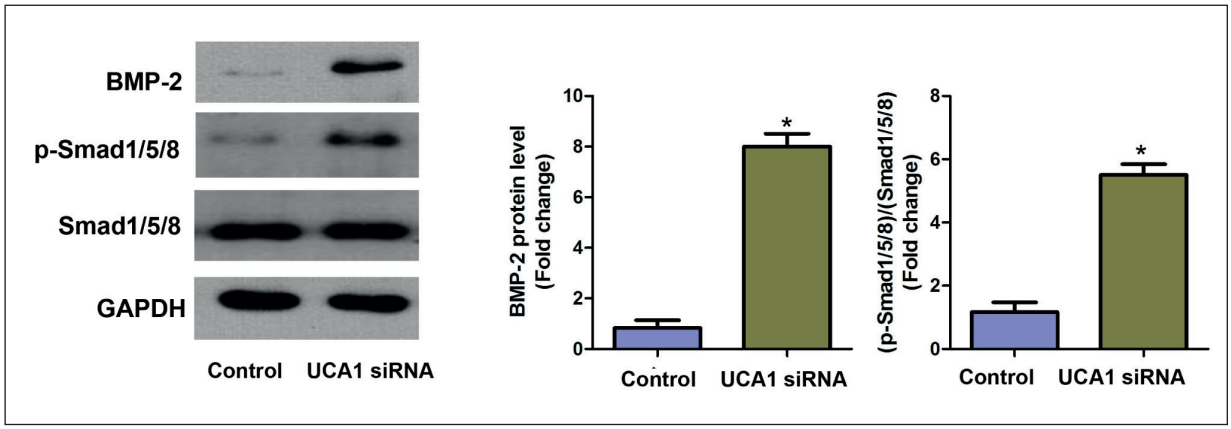


**Figure 7.** Influence of UCA1 knockout on BMP-2/(Smad1/5/8) signaling pathway in osteoblasts. Control: blank control group, UCA1 siRNA: UCA1 knockout group. *$p$<0.05: There was a statistically significant difference compared with the control group.

while, its expression in osteosarcoma has a positive correlation with tumor size, tumor grade, distant metastasis and clinical stage[21]. Moreover, UCA1 can promote the invasion and metastasis of pancreatic cancer by mediating the Hippo signaling pathway[22]. In this study, our results revealed for the first time that the expression level of plasma UCA1 was abnormally increased in OST patients. Subsequent *in-vitro* experiments showed that UCA1 knockout could markedly promote the proliferation ability of osteoblasts and up-regulate the expression levels of classical differentiation-promoting genes and proteins in osteoblasts. Finally, it was further found in this study that the proliferation- and differentiation-promoting effects on osteoblasts after UCA1 knockout might be mediated by the BMP-2/Smad signaling pathway. However, there were still some deficiencies in this experiment as follows: (1) only one kind of cell line was used, and neither primary cells nor other cell lines were used. (2) The direct target gene of UCA1 was not found. (3) The conclusion was not verified *via* cell experiments. (4) Only the classical BMP/Smad signaling pathway was detected. The non-classical pathway also plays an important role in the osteoblast proliferation and differentiation. Therefore, whether UCA1 affects the non-classical pathway needs to be further verified.

## Conclusions

We clarified for the first time that inhibiting UCA1 can promote the osteoblast proliferation and differentiation in mice by mediating the BMP-2/Smad signaling pathway. Therefore, drugs can be designed with UCA1 as a target in the prevention and treatment of OST in the future.

### Conflict of Interests

The authors declare that they have no conflict of interest.

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