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SHOX CNE9/10 Knockout in U2OS Osteosarcoma Cells and Its Effects on Cell Growth and Apoptosis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG Xue-Jiao Xu
ABCDEF Shi-Jie Xin
ABEF Hui-Ying Mao
BDE Hui-Jiao Zhang
DF Lan-Ni Chen
CD Li Li
CD Hua-Lei Bai
CD Hai-Hua Huang
ABCDEFG Min Zhu

Department of Endocrinology, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, Chongqing, P.R. China

Corresponding Author: Min Zhu, e-mail: zhumin@hospital.cqmu.edu.cn
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Background: Osteosarcoma is a common malignant tumor of musculoskeletal stromal cells. Osteosarcoma clinical behavior depends mostly on the histologic grade, the site of primary tumor, the response to chemotherapy, and the presence of pulmonary metastases. The aim of this study was to knockout SHOX CNE9/10 in U2OS osteosarcoma cells and to analyze the effects on cell growth and apoptosis.

Material/Methods: U2OS cells with CNE9 knockout and U2OS cells with CNE10 knockout were established via the CRISPR/Cas9 system. Sanger sequencing was used to detect the success of the knockdown experiment. Western blotting and quantitative polymerase chain reaction were used to detect the expression levels of short stature homeobox-containing gene (SHOX) protein and messenger RNA (mRNA) after knockdown of CNE9 and CNE10. The cell viability and apoptotic rate were detected by the Cell Counting Kit-8 method and by flow cytometry.

Results: The Sanger sequencing results showed that the knockdown experiment was successful. The levels of SHOX mRNA and protein were significantly reduced after knocking down CNE9 and CNE10. Knockdown of CNE9 and CNE10 significantly increased the growth and inhibited the apoptosis of U2OS osteosarcoma cells. CNE9/CNE10 knockdown U2OS cells were successfully constructed.

Conclusions: Knockdown of CNE9 and CNE10 promoted U2OS cell growth and inhibited apoptosis by decreasing SHOX expression. This CNE9/CNE10 knockout U2OS cell model could provide a bridge for the research on SHOX and CNEs in osteosarcoma.

MeSH Keywords: Apoptosis • Cell Growth Processes • Gene Knockdown Techniques • Osteosarcoma

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Background

Osteosarcoma is a common malignant tumor of musculoskeletal stromal cells. The incidence of osteosarcoma is approximately 3.1 per million, and the incidence is higher in males than in females [1]. Osteosarcoma occurs mostly at the medullary end of the long bone, and the tibia is the second most frequent site, followed by the proximal humerus [2]. Osteosarcoma clinical behavior depends mostly on the histologic grade, the site of the primary tumor, the response to chemotherapy and the presence of pulmonary metastases [3]. At present, the main treatment methods for osteosarcoma include surgery, chemotherapy, immunotherapy, and gene therapy, but some osteosarcoma patients still have poor prognoses [4]. Analysis of the occurrence and development of osteosarcoma is an important basis for the discovery of new methods for the diagnosis and treatment of osteosarcoma.

Short stature homeobox-containing gene (SHOX) is located in the pseudo-autosomal region 1 (PAR1) of the human sex chromosomes and plays an important role in promoting bone development and longitudinal growth [5,6]. The main mechanism of action described in the literature suggests that SHOX protein may control chondrocyte growth, apoptosis, and differentiation by regulating the expression of its target genes, such as BNP, Fgfr3, and Agc1 [7–10]. Recently, a study by Ni et al. [11] showed that the methylation level of SHOX is significantly increased in lung cancer patients and can be used as a potential biomarker for lung squamous cell carcinoma, and the increase in methylation level can affect the expression level of SHOX. In human mesenchymal stem cells, SHOX can regulate cell proliferation and osteogenic differentiation [12]. In addition, Weiss et al. [13] proposed that the methylation level of SHOX can be used to distinguish patients with malignant and non-malignant lung diseases. SHOX has a role in regulating apoptosis, which may be one of the reasons for the malignant growth of tumor cells [14]. It was also reported that the level of mSHOX2 DNA in the plasma of lung cancer patients can reflect the response to cytotoxic treatment and may be a tool to guide treatment [15]. *In vitro* results also showed that SHOX2 breast cancer cells are involved in epithelial-mesenchymal transition and receive targeted regulation of microRNA-375 [16]. These results suggest that the expression level of the SHOX gene may be related to tumor behaviors. These findings suggest that genetic defects in non-coding regions containing conserved non-coding elements (CNEs) can underlie cancer. Seven evolutionarily conserved non-coding elements located upstream (CNE5, CNE3, and CNE2) and downstream (CNE4, CNE5, ECR1, and ECS4/CNE9) of SHOX have been identified as enhancers [17,18].

We constructed a SHOX CNE9/CNE10 knockout osteosarcoma U2OS cell line by using CRISPR/Cas9 to study the relationship between SHOX and its CNEs and to analyze the effects of CNEs and SHOX on osteosarcoma cells.

Material and Methods

Cell culture

The human osteosarcoma cell line U2OS was purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS, Thermo Fisher, Waltham, MA, USA). Cell culture conditions were 37°C, 5% carbon dioxide (CO_2) and 95% humidity.

Plasmid design and purification

The exact positions of CNEs on the X chromosome (NCBI build 36.1) are as follows: CNE9 (804746-805567) and CNE10 (853825-854598). The CNE9 and CNE10 gRNA (guide RNA) designations were based on CRISPR design (*http://crispr.mit.edu/*). The CRISPR-Cas9 GFP knockout plasmid was created by preparing and ligating gRNA GFP oligos to a pSpCas9(BB)-2A-GFP (PX459) plasmid (Addgene plasmid #48138). The forward and reverse oligos were prepared to be inserted into the pSpCas9(BB)-2A-GFP plasmid according to the protocol. Plasmids were then purified using QIAGEN EndoFree Plasmid Maxi Kit (Hilden). Target sequence designations are shown in Table 1.

Transfection

Transfection of CNE9 gRNA-Cas9-GFP, and CNE10 gRNA-Cas9-GFP into U2OS cells was performed with Lipofectamine™ 2000 transfection reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. At 48 hours after transfection, the cells were harvested for the follow-up experiments.

Sequencing verification

Next, the cells were expanded, and genomic DNA was extracted using a TAKARA kit. The oligo target site sequence was amplified by polymerase chain reaction (PCR), and the PCR amplification product was purified by using a TAKARA kit and ligated to a PMD18-T vector. After transformation and bacterial culture, 5 colonies were picked and placed in 3 mL LB (Luria-Bertani) liquid medium (containing 3 µL AMP) at 37°C at 200 rpm for 12 hours. PCR was performed using 1 µL of bacterial solution as a template. Then, the bacterial solution was sampled for Sanger sequencing.

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Table 1. Target sequence designations.

CNE	Target sequence	gRNA sequence (5'-3')
CNE9-up	GCCACCACCAGCTACTGGGG	Up-CACCGCCACCACCAGCTACTGGGG Down-AAACCCCCAGTAGCTGGTGGTGGC
CNE9-down	GAGGGTCCCCCTGGGACTGT	Up-CACCGAGGGTCCCCCTGGGACTGT Down-AAACACAGTCCCAGGGGGACCCTC
CNE10-up	GGGTCAGACGCAGCCACGCA	Up-CACCGGGTCAGACGCAGCCACGCA Down-AAACTGCGTGGCTGCGTCTGACCC
CNE10-down	GGATGCTAAGTCCATTAGCG	Up-CACCGGATGCTAAGTCCATTAGCG Down-AAACCGCTAATGGACTTAGCATCC

CNE9 – conserved non-coding elements 9; CNE10 – conserved non-coding elements 10.

Quantitative PCR

Total RNA of cells after transfection was extracted with TRIzol regent (Invitrogen, Carlsbad CA, USA) in accordance with the manufacturer's instruction. The messenger RNA (mRNA) was isolated and applied to generate complementary DNA (cDNA) by using the iScriptTM cDNA Synthesis Kit (Bio-Rad, CA, USA). TaqMan Universal Master Mix II was used for quantitative real-time PC (qRT-PCR) and the internal control was GAPDH. The primers were showed in Table 1.

Western blotting

Western blotting analyses were performed as described. The SHOX primary rabbit monoclonal antibody (1: 500, ab84804, 32 kD) and GAPDH monoclonal antibody (1: 1000, ab181602, 36 kD) were purchased from Abcam (San Francisco, CA, USA). The cell protein was extracted and separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the experimental voltage was 110 V and the duration of the experiment was 100 minutes. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes, the membranes were then blocked in 5% non-fat milk for 1 hour at room temperature. The primary antibody SHOX was added (1: 500 dilution) and then incubated overnight. The protein blot bands were detected using Pierce™ ECL plus western blotting substrate (Thermo Fisher, Waltham, MA, USA).

Cell Counting Kit-8 (CCK-8) assay

Cell viability was detected using the Cell Counting Kit-8 (CCK-8) assay. The U2OS cells (1×10^4 cells, 100 µL) were seeded in a 96-well culture plate. CCK-8 reagent (Beyotime, Beijing, China) was added in accordance with the manufacturer's instruction after the cells were cultured for a certain period of time. After added CCK-8 reagent, the samples were incubated for 2 hours under the original conditions. The cell viabilities were detected by Tecan Infinite M200 microplate reader (LabX, Switzerland).

Colony formation assay

Cells (5×10^3) were seeded in a 6-well plate. After 48 hours, the cell colonies were fixed with methanol and further stained with 0.5% crystal violet for 20 minutes. Colonies were detected under the microscope (Olympus IX71, Tokyo, Japan).

Flow cytometry

Cell apoptosis rates were detected by flow cytometry. The cells were washed and labelled with an apoptosis kit (Tianjin Biolite Biotech Co., Ltd., Tianjin, China.). The resulting fluorescence was measured by flow cytometry (BD FACSCanto II; BD Biosciences) and FlowJo 7.6.1 software (FlowJo LLC).

Statistical analysis

Statistical analysis was carried out with GraphPad Prism7.0 and data are shown as mean±standard deviation (SD). The comparison between the 2 groups were analyzed by Student's *t*-test. The *P* value less than 0.05 was considered statistically significant.

Results

CNE9 and CNE10 knockout U2OS cell model was successfully established.

We successfully established CNE9 and CNE10 knockout human osteosarcoma U2OS cell models. The genotypes of CNE9 and CNE10 are shown in Table 2. The sequencing results showed that there were 1088 bases of mutation/deletion in one allele of CNE9 and there were 1095 bases in another allele compared with those of the wild type CNE9 (Table 3). The sequencing results showed that there were 196 bases of mutation/deletion in one allele of CNE10 and there were 384 bases in another allele compared with the wild type of CNE10 (Table 4).

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Table 2. The genotype of CNE9 and CNE10.

Names	Genotype
CNE9	AATTATATATAATGTATACATATATTTAAATTATATATAATGTATACATATATTTATATATAATGTATACATATATTT AAATTATATATAATATACATATTTGGATAGTTTTATTTATTTATTTATTGGAGTCTTGCTCTGTCCCCCAGGCTGG AGTGCAGTGACATGATCTCTGCTCACTGCAACCTCCGCCTTCCAGGCTCAAGCGATCCTCCTGCCTAAGCCTC CCGAGTAGCTGGGATTACAGGCGCCCGCCACCACCAGCTACTGGGGAGGCTGAGGCAGGAGGATCGCTTGA CCCTCGGAGGCGGAGGTTGCAGTGAAGCGAGATTGCACCACTGCACTCCAGCCTGGGGGGCAGAGCCAGA CTCTGTCTAAAAAAAAAAAAGAAAGAAAAAGAAAAAAAAAACCTGCACGTTGCTGGATGAGGAAGGGCCTT TTTTCAAAAAGTTGCACTTTTTTTCCCCCTATACTTTACTTCTTTGCTGTCATTAGCTAGGACTTGAAAGGGCAG GAACTCTAATTGTGTTTCCTCTGTGGGGCACAGAGTCCTGTCTCGACCCCGTCGTGCTGCCAAAGAAAACGCC GGGTTCCCTTCCACGTTGTTCATTTTCCTTTTCCTGCCCACTTAACAAAATTATATTCAATGAGTTGTGGGGGGA ATTGACATCAACGGGCCGCGTCACGCAATTTCTGGTGAATATTCGAGAAGGGGGAAGCAAACAGTCCGGGTT CCTTCACATCTGTCAGAATCAATCACATTGTACCAAACCGAACGTAATCGCTTGCAAAGAAAATGAGGCATTTC CAGTGAAATCAGCCTCCCTTTACCCAGGAACATGTCCATAGTTAAGAGGTCTCTGTTTTAGGTTTTTTTTTTTT ATTTTAATCTTGCCTAATTAGTTTTTTTTTAATCACCCGTGCAACCGAGTCTCCAGACTGCATAATTGATGAGAT GCAGAAGCCAGTGTCAGAAATCAAATGTCGTAAATTAAAAATAACAAGTTTGAGACGAGTGATTCAATTAAA AATCCAGAAAACATTGGATTCTGCATAACTGAACGTTTGCATAGAGACACCGCCTGTCATCCGTATCACGTTG CACCCGAGGATTGGATTGGGGCTGTCACGGGTATTTC AATCAACTCAGTAAAAAAAAAAAAAAAAAAATTAGAAAACAAAGATGATATTTATTATTAACTTCCCGTGTTCAA AAACCACACGGAGACGTCTTTAGGTGGACTGCTTTACAGGACCAAGATTTATGTTAAGGGGACACTGCAGAC ACAAGAGGCCTGGCTGGCTGGGATGTAGGGAGGGTCCCCCTGGGACTGTTGGACCGTTTTCTGAGTACGTGA GGCTGCCAGGAGGACGGGGCTGCACAGAGCCGACTGGGACCCTCCAAGACGGACCCTGAGAAACCTCACTG GGTCCTCTCTCTCTTTTTTTTTTTTTTTTTTTTTTGAGACGGCTTTTCACTCTTGTTTCCCAGGCTGGAGTGCAGTG GTGCAATCTTGGCTTTGCAACCTCTGCCTCCCGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCGAGTAGCTGG GATGACAGGCGCGCGCCACCACGCCTGGCTAAGTTTTTGTATTTTTATTAGAGACAGAGCTTCACCATGTTGGC CAGGCTGGTCTCGAACTCCTGACCTCA
CNE10	GCCTGGAATAATCCCTCCTTTCCACGTCTTGTCCCGCCATGGAGTGCCCCAGAGTTCCGGGACGTGGAAGG TGCCGTCCCCTGCCTGACACGGTGCTGCCCAGCTCCCTCCTCATCCCGGTGGCTTCCCAAACTCCCCGGGCC TGCTGGAGGAAACACCTGTGCTGAAGACTCGGAGGCCCTTCCCTCCAAACCCTGACTCCGGGGTCAGCGAC GCCAACAGGTGTTTTTTCTCAGAATCGCCTTCTTGTCTGAATTGAGCCCCAGGATCAGAAGGCCAAGGGGCC AGGGGTCAGACGCAGCCACGCAGGGTTCCCCGGGGGCCAGGGCTTACCAGGTGGTTAATATTATCAGAGCT CATTTGCTGAAGCTGGACTGGCGTGTTCTCGGCGGTCCCAAATAAATTTAACGAGAGCGACTTTCAAAGCCCA CCTATTAGGACCGTCTGTTCAATGGACAGGAGGGTTAACCGTAATTCGAGGAGGCAGCTTGGCCGTCTCCAAA TAGGAACACATGGACTGTGAGGCTTGGCCCCATTTGTGGTAATTTTAAGGTCTCGTTTCCATTTGTAAGAAACAT AACTACTTACATATGGAAAGAATGAATAAGAGAAGTCATTAAAATTTCTCTAATTTAGGAAAAAAATAAAAAAAAA AAGATGTAACGAAGTAATATCTTTACCAGGCCAGGCAGAAATGTAAATTAGACATTAGAAGAAAAACCTATTAACA GGATGGCTGGATGGCTGCGTGGCTTGCCTGTTTTTTTTTTTTTTTAATCTAATGAATTTATGAATTGCTAGTTGTGGG GGTCACAGATTTTGTTAATTTCCTATGTGTTTAGCTCTGCAGTAAGAAGGGAGGATTTGCTTATGAACCACGGATGCT AAGTCCATTAGCGGGGCGGTTGCTAATCAGAACACAAATGGTAATACGTACGGTCCAGCATTGAAAAAAAAAAAGA CAGAAAAACACATGAATTATTTCAAGTTGGGGTTTTTCCAACTTCTGTCCGTTGTCCCCACAGGTCATCAGAATATTTA AACATCAGGGAGTAAGAGGCGACCTAGCTAGGTAGCTGCCTGATTATAAAATATATGCAAAAGCTTTCTTTTTTTTTT TTTTTTTAAACAGAGTCTTGCTTTGTCGCCCAGGCTGGAGTGCAATGGTGAGATCTCGGCTCACTGCAACCTCCAG CTCCTGGGTTCCAGCGATTCTCCTGCCTCAGCCTCCCAAGGAGCTGGGATTACAAGCGCCCGCCACCACACCCAGC TCATTTTTGTATTTTTAGTAGAAACGGGGTTTCACCATGTTGGCCAGGCTGGTCTCGAACTCCTGACCTTAAGTGATCC ACCTGCCTCCACTTCCCAAAGTGCTGGGATGACAGGCTTGAGCCACCGTG.

CNE9 – conserved non-coding elements 9; CNE10 – conserved non-coding elements 10.

Knockdown of CNE9 and CNE10 could inhibit SHOX

The results of this study showed that the expression levels of SHOX2 mRNA and protein were significantly lower in the CNE9 or CNE10 knockout groups than in the control group (Figure 1A, 1B). This suggested that knockdown of CNE9 or CNE10 could inhibit the expression of the SHOX gene.

Knocking down CNE9 and CNE10 promoted cell growth and proliferation

These studies showed that knockdown of CNE9 and CNE10 significantly promoted U2OS cell viability and cell proliferation (Figure 2A, 2B). This suggested that downregulation of CNE9 and CNE10 promoted osteosarcoma cell proliferation by inhibiting SHOX levels.

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Table 3. Sequence of CNE9 after knockdown.

Names	Sequence
One allele	AATTATATATAATGTATACATATATTTAAATTATATATAATGTATACATATATTTATATATAATGTATACATATATTTAA ATTATATATAATATACATATTTGGATAGTTTTATTTATTTATTTATTGGAGTCTTGCTCTGTCCCCCAGGCTGGAGT GCAGTGACATGATCTCTGCTCACTGAAACCTCCGCCTTCCAGGCTCAAGCGATCCTCCTGCCTAAGCCTCCCGAGTA GCTGGGATTACAGGCGCGTAGGCTGACTGGGCTAAATCCGGATTGATGAGGAAGGGCCTTTTTTCAAAAAGTTGG TCAGTCGGCTACCGTTTTCTGAGTACTTTGAGGCTGCCAGGAGGACGGGGATGCACAGAGCCGACTGGGACCCTC CAAGACGGACCCTGAGAAACCTCACTGGGTCCTCTCTCTCTTTTTTTTTTTTTTTTTTTTTGAGACGGCTTTTCACTCT TGTTTCCCAGGCTGGAGTGCAGTGGTGCAATCTTGGCTTTGCAACCTCTGCCTCCCGGGTTCAAGCGATTCTCCTGC CTCAGCCTCCCGAGTAGCTGGGATGACAGGCGCGCGCCACCACGCCTGGCTAAGTTTTTGTATTTTTATTAGAGACA GAGCTTCACCATGTTGGCCAGGCTGGTCTCGAACTCCTGACCTC
Another allele	AATTATATATAATGTATACATATATTTAAATTATATATAATGTATACATATATTTATATATAATGTATACATATATTTAAATTAT ATATAATATACATATTTGGATAGTTTTATTTATTTATTTATTGGAGTCTTGCTCTGTCCCCCAGGCTGGAGTGCAGTGAC ATGGTCTCTGCTCACTGCAACCTCCGCCTTCCAGGCTCAAGCGATCCTCCTGCCTAAGCCTCCCGAGTAGCTGGGAT TACAGGCGCGTAGGCTGAGCTGGTGATCGGAGGACGGCTACCGTTTTCTGAGTACGTGAGGCTGCCAGGAGGAC GGGGCTGCACAGAGCCGACTGGGACCCTCGAACTCGTACCCTGAGGAACCTCACTGGGTCCTCTCTCTCTTTTTTTT TTTTTTTTTTTTTGAGACGGCTTTTCACTCTTGTTTCCCAGGCTGGAGTGCAGTGGTGCAATCTTGGCTTTGCAACCT CTGCCTCCCGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCGAGTAGCTGGGATGACAGGCGCGCGCCACCACGCC TGGCTAAGTTTTTGTATTTTTATTAGAGACAGAGCTTCACCATGTTGGCCAGGCTGGTCTCGAACTCCTGACCTCA

CNE9 – conserved non-coding elements 9.

Table 4. Sequence of CNE10 after knockdown.

Names	Sequence
One allele	GCCTGGAATAATCCCTCCTTTCCACGTCTTGTCCCGCCATGGAGTGCCCCAGAGTTCCGGGACGTGGAAGGTGCCGTCC CCTGCCTGACACGGTGCTGCCCAGCTCCCTCCTCATCCCGGTGGCTTCCCAAACTCCCCGGGCCTGCTGGAGGAAACAC CTGTGCTGAAGACTCGGAGGCCCTTCCCTCCAAACCCTGACTCCGGGGTCAGCGACGCCAACAGGTGTTTTTTCTCAGA ATCGCCTTCTTGTCTGAATTGAGCCCCAGGATCAGAAGGCCAAGGGGCCAGGGGTCACATAGTCTCCTAATAGGAACAC ATGGACCGTGATAGCTTGGCCCCATTTGTGGTAATTTTAAGGTCTCGTTTCCATTTGTAAGAAACATAACTACTTACATATG GAAAGAATGAATAAGAGAAGTCATTAAAATTTCTCTAATTTAGGAAAAAAATAAAAAAAAAAAAGATGTAACGAAGTAATAT CTTTACCAGGCCAGGCAGAAATGTAAATTAGACATTAGAAGAAAAACCTATTAACAGGATGGCTGGATGGCTGCGTGGCTT GCCTGTTTTTTTTTTTTTTTTAATCTAATGAATTTATGAATTGCTAGTTGTGGGGGTCACAGATTTTGTTAATTTCCTATGTGTTTA GCTCTGCAGTAAGAAGGGAGGATTTGCTTATGAACCACGGATGCTAAGTCCATTAGCGGGGCGGTTGCTAATCAGAACACA AATGGTAATACGTACGGTCCAGCATTGAAAAAAAAAAAGACAGAAAAACACATGAATTATTTCAAGTTGGGGTTTTTCCAACT TCTGTCCGTTGTCCCCACAGGTCATCAGAATATTTAAACATCAGGGAGTAAGAGGCGACCTAGCTAGGTAGCTGCCTGATTATA AAATATATGCAAAAGCTTTCTTTTTTTTTTTTTTTTTTAAACAGAGTCTTGCTTTGTCGCCCAGGCTGGAGTGCAATGGTGAGATCT CGGCTCACTGCAACCTCCAGCTCCTGGGTTCCAGCGATTCTCCTGCCTCAGCCTCCCAAGGAGCTGGGATTACAAGCGCCCGCC ACCACACCCAGCTCATTTTTGTATTTTTAGTAGAAACGGGGTTTCACCATGTTGGCCAGGCTGGTCTCGAACTCCTGACCTTAAGT GATCCACCTGCCTCCACTTCCCAAAGTGCTGGGATGACAGGCTTGAGCCACCGTG
Another allele	GCCTGGAATAATCCCTCCTTTCCACGTCTTGTCCCGCCATGGAGTGCCCCAGAGTTCCGGGACGTGGAAGGTGCC GTCCCCTGCCTGACACGGTGCTGCCCAGCTCCCTCCTCATCCCGGTGGCTTCCCAAACTCCCCGGGCCTGCTGGA GGAAACACCTGTGCTGAAGACTCGGAGGCCCTTCCCTCCAAACCCTG ACTCCGGGGTCAGCGACGCCAACAGGT GTTTTTTCTCAGAATCGCCTTCTTGTCTGAATTGAGCCCCAGGATCAGAAGGCCAAGGGGCCACCTACCTGATGTG ATTGATTTTCTCTAATTTAGGAAAAAAATAAAAAAAAAAAAGATGTAACGAAGTAATATCTTTACCAGGCCAGGCAG AAATGTAAATTAGACATTAGAAGA AAAACCTATTAACAGGATGGCTGGATGGCTGCGTGGCTTGCCTGTTTTTTTTT TTTTTTAATCTAATGAATTTATGAATTGCTAGTTGTGGGGGTCACAGATTTTGTTAATTTCCTATGTGTTTAGCTCTGCA GTAAGAA GGGAGGATTTGCTTATGAACCACGGATGCTAAGTCCATTAGCGGGGCGGTTGCTAATCAGAACACAAATG GTAATACGTACGGTCCAGCATTGAAAAAAAAAAAGACAGAAAAACACATGAATTATTTCAAGTTGGGGTTTTTCCAAC TTCTGTCCGTTGTCCCCACAGGTCATCAGAATATTTAAACATCAGGGAGTAAGAGGCGACCTAGCTAGGTAGCTGCCT GATTATAAAATATATGCAAAAGCTTTCTTTTTTTTTTTTTTTTTTAAACAGAGTCTTGCTTTGTCGCCCAGGCTGGAGTG CAATGGTGAGATCTCGGCTCACTGCAACCTCCAGCTCCTGGGTTCCAGCGATTCTCCTGCCTCAGCCTCCCAAGGA GCTGGGATTACAAGCGCCCGCCACCACACCCAGCTCATTTTTGTATTTTTAGTAGAAAC GGGGTTTCACCATGTTGG CCAGGCTGGTCTCGAACTCCTGACCTTAAGTGATCCACCTGCCTCCACTTCCCAAAGTGCTGGGATGACAGGC TTGAGCCACCGTG

CNE10 – conserved non-coding elements 10.

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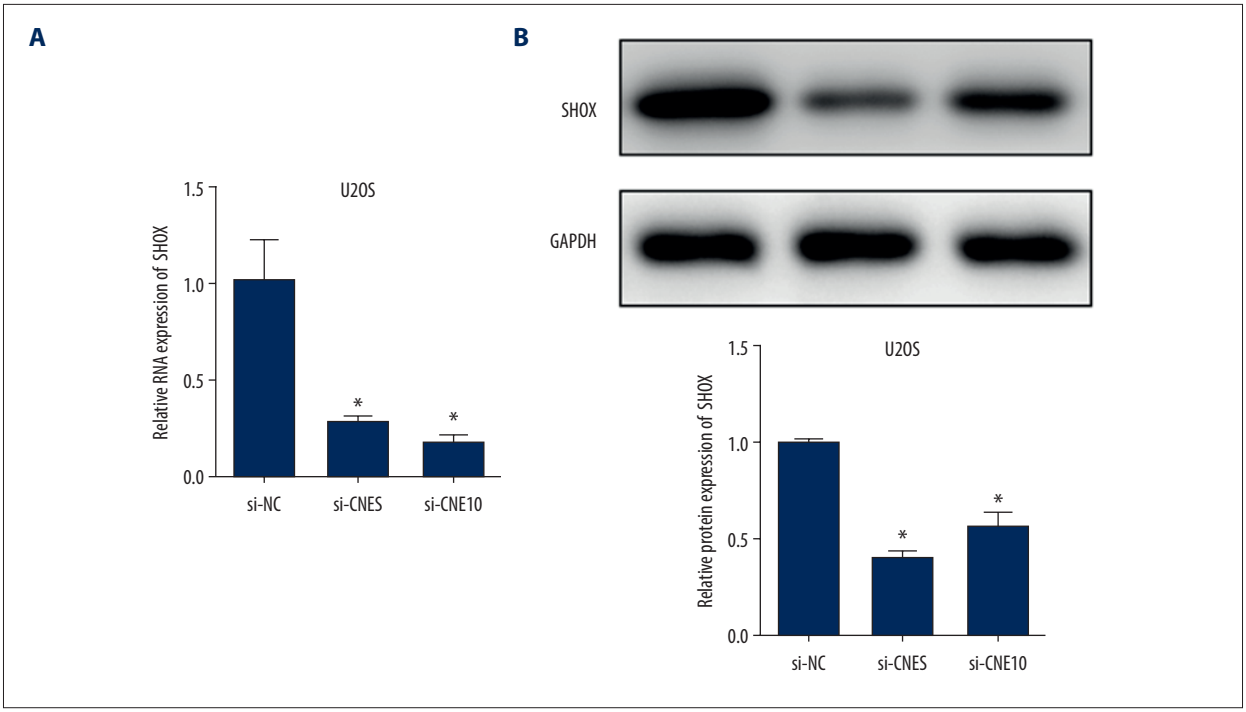

Figure 1. Knockdown of CNE9 and CNE10 inhibited SHOX. (**A, B**) The effects of knockdown of CNE9 and CNE10 on SHOX mRNA and protein in osteosarcoma cell U2OS. * *P*<0.05 versus si-NC group.

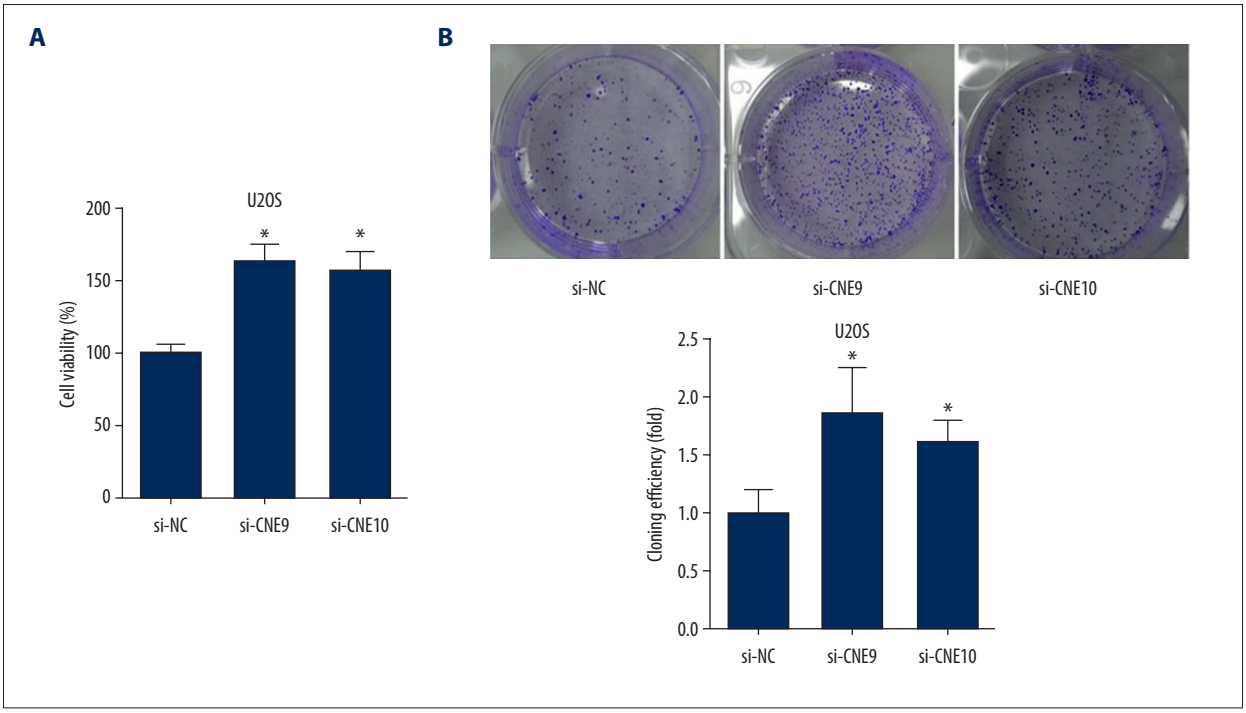

Figure 2. Knockdown of CNE9 and CNE10 promoted cell growth and proliferation. (**A, B**) The effects of knockdown of CNE9 and CNE10 on cell viability and proliferation of U2OS cells.* *P*<0.05 versus si-NC group.

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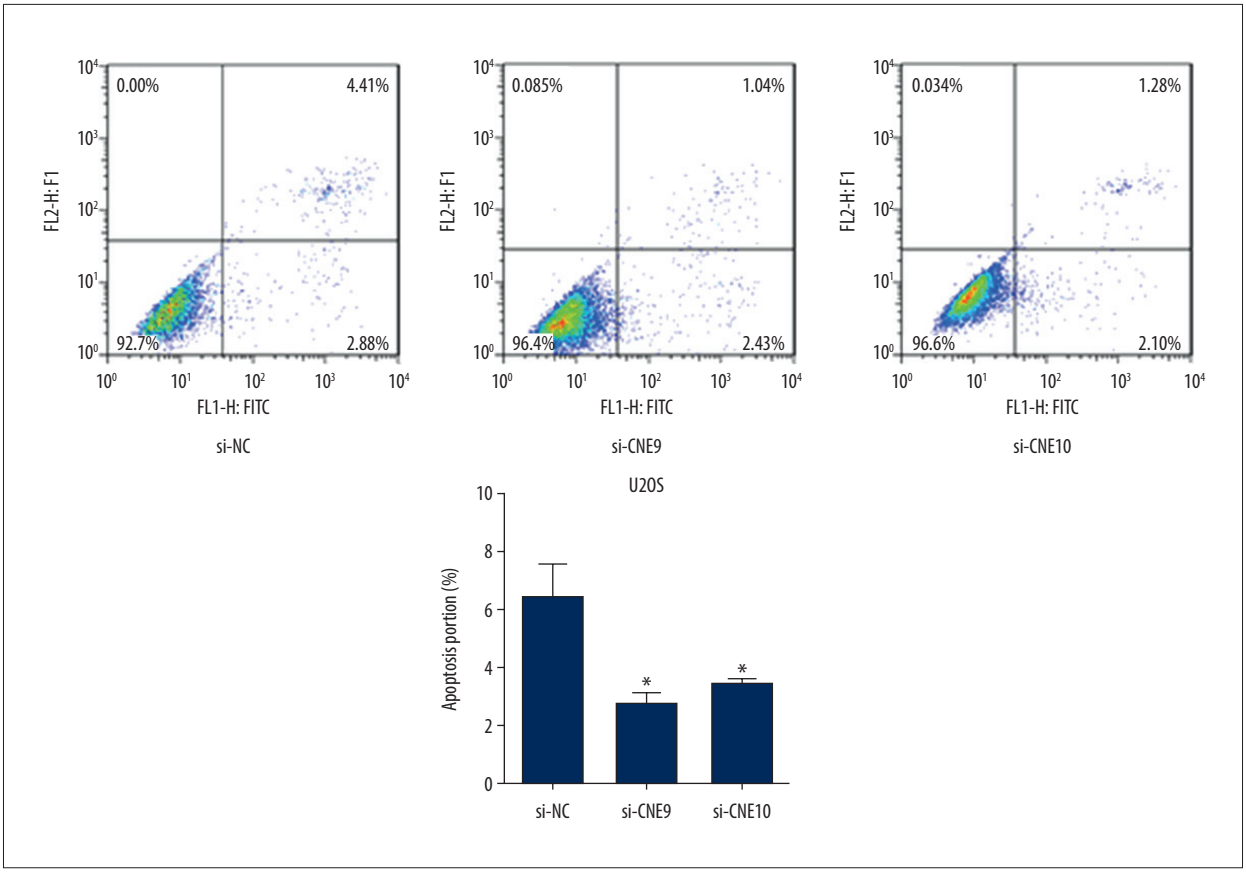

Figure 3. Knockdown of CNE9 and CNE10 inhibited cell growth and apoptosis. The effects of CNE9 and CNE10 knockdown on U2OS cell apoptosis. * $P<0.05$ versus si-NC group.

Knocking down CNE9 and CNE10 inhibited apoptosis

Knockdown of CNE9 and CNE10 significantly inhibited U2OS cell apoptosis (Figure 3). This suggested that downregulation of CNE9 and CNE10 inhibited osteosarcoma cell apoptosis.

Discussion

In this study, we successfully established CNE9 and CNE10 knockdown osteosarcoma cell line U2OS cells. It was found that knocking down CNE9 and CNE10 could decrease the expression levels of the SHOX gene and promote osteosarcoma cell proliferation and inhibit apoptosis.

Osteosarcoma is the most common primary malignancy in children and adolescents, and the 5-year survival rate for osteosarcoma patients receiving standard therapy is approximately 70%. Surveillance, Epidemiology, and End Results (SEER) data in children and adolescents show that the survival for patients age <25 years is 61.6% (females, 65.8%; males, 58.4%) [19,20]. Analysis of the pathogenesis of osteosarcoma lung metastasis is one of the means of discovering new approaches to osteosarcoma treatment. In humans, SHOX is mainly found in the bone marrow fibroblasts, skeletal muscles, and placenta and plays an important role in the formation of the upper and lower limbs of the fetus. A recent study showed that in U2OS osteosarcoma cells, SHOX expression causes oxidative stress and induces rupture of the lysosomal membrane, causing cell death [14]. A study also found that the loss of SHOX promotes the expression of osteogenic genes during osteoblast differentiation [12]. There are many conserved CNE-regulated transcription factors upstream and downstream of the SHOX gene [21,22]. To analyze the effects of CNE9/CNE10 on the expression level of SHOX, we constructed a CNE9/10 knockout U2OS cell line using the CRISPR/Cas9 system. The knockdown results were verified by sequencing techniques. The protein expression levels of SHOX in CNE9 and CNE10 knockout U2OS cells were decreased. This suggested that knockdown of CNE9/CNE10 could downregulate the level of SHOX transcription and translation and regulate the expression of the SHOX gene.

To further analyze the effects of knockdown of CNE9 and CNE10 on osteosarcoma cells, we examined the proliferation and apoptosis levels of cells. The results showed that after knocking down CNE9 and CNE10, cell viability was significantly

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promoted, while apoptosis was inhibited. Mutations in CNE affect the expression of SHOX, which may affect cellular function [17]. Bunyan et al. [23] also showed that SHOX expression is regulated by upstream and downstream regulatory elements, thereby participating in disease development. This indicates that knockdown of CNE9 and CNE10 could inhibit the expression of SHOX, thereby promoting the growth of osteosarcoma cells and inhibiting apoptosis.

Conclusions

We successfully constructed CNE9/CNE10 knockdown U2OS cells. Furthermore, we found that knockdown of CNE9 and CNE10 promotes osteosarcoma cell growth and inhibits apoptosis by inhibiting SHOX expression. This CNE9/CNE10 knockout U2OS cell model could provide a bridge for the research between SHOX and CNEs in osteosarcoma.

Acknowledgements

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Conflict of interests

None.

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