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Original Article

MSX2 inhibits the growth and migration of osteosarcoma cells by repressing SOX2

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Abstract: SRY (sex determining region Y)-box 2 (SOX2) plays a key role in the maintenance of stemness and resistance to drugs, whereas tumor necrosis factor (TNF)-α is essential for maintaining cancer cell proliferation and metastasis. Accumulation of muscle segment homeobox 2 (MSX2) leads to downregulation of SOX2 expression. Here, we explored the MSX2-SOX2-TNF-α signaling axis and its function in the tumor phenotypes of osteosarcoma cells. Colony formation assay, cell counting kit (CCK)-8 assay, and Flow cytometry were used to examine cell growth, viability, and death, respectively. Wound healing and Transwell invasive assay were employed to examine cell migratory and invasive activities, respectively. Western blotting and RT-qPCR were used to determine the protein and mRNA expressions of MSX2, SOX2, TNF-α, Bax, and matrix metalloproteinase-2 (MMP-2). Osteosarcoma clinical samples and cells showed lower levels of MSX2 than normal healthy control samples. Overexpression of MSX2 led to a reduced activity of SOX2 and TNF-α, whereas MSX2 depletion did not contribute to upregulated SOX2 levels. A gain-of-function experiment showed that osteosarcoma cell viability and growth were reduced, cell death was increased, and migration and invasion were inhibited in the MSX2 overexpression group compared with those in the non-transfected group. Furthermore, co-overexpression of MSX2 and SOX2 counteracted the inhibitory effects of MSX2 on the abovementioned tumor phenotypes of osteosarcoma cells. An *in vivo* tumor growth assay showed that MSX2 overexpression slowed the growth rate of osteosarcoma xenograft tumors. Thus, MSX2 loss plays a crucial role in the osteosarcoma phenotype by elevating SOX2 and TNF-α levels.

Keywords: Osteosarcoma, metastasis, MSX2, SOX2, TNF-α

Introduction

Osteosarcoma is one of the most common orthopedic malignancies, mainly affecting children and adolescents. It constitutes approximately 5% of all pediatric cancers, and the mortality rate is approximately 8.9% [1]. The most commonly affected sites include the distal femur and proximal humerus [2]. Although surgery and chemotherapy have been acknowledged as the standard treatment for osteosarcoma, the 5-year survival rate remains at 65-70% [3], thus necessitating the identification of novel therapeutic strategies.

SRY (sex determining region Y)-box 2 (SOX2), a pluripotent stem cell factor, plays a crucial role in cell fate and maintenance of stem cell properties, thereby regulating developmental processes [4]. SOX2 is aberrantly expressed in many cancers, including lung, breast, colon, ovary, and prostate cancer [5]. Importantly, SOX2 expression is positively correlated with cancer cell stemness and poor patient outcomes, suggesting its important role in cancer stem cell generation and biology [6, 7]. In osteosarcoma, Sox2 maintains the survival of tumor-initiating cells and allows these cells to exhibit higher expression of stem cell markers, higher colony-formation ability, higher proliferation rate, and poorer osteoblastic differentiation [8]. These characteristics are absent in non-tumorigenic cancer cells and can easily be diverted to osteoblastic differentiation [9-11]. Knockdown (KD) of Sox2 activity can abrogate tumorigenicity [10]. Moreover, conditional kno-

ckout (CKO) of Sox2 may lead to decreased tumorigenicity [12].

A recent report revealed that SOX2 is transcriptionally downregulated by the muscle segment homeobox 2 (MSX2) transcription repressor [13], suggesting a novel approach to target SOX2. MSX2, a widely expressed transcription factor, belongs to the MSH family [14-16]. Although MSX2 is generally recognized as a transcriptional repressor, it can also activate some downstream genes [17, 18]. Our *in vivo* experiments demonstrated the vital role of MSX2 in organogenesis, and the depletion of this gene results in serious developmental defects [16, 19]. Consistent with the defects observed in mice, MSX2 mutations increase the risk of Boston-type parietal foramina and craniosynostosis [20, 21]. The role of MSX2 in embryonal development lies in its ability to regulate the epithelial-to-mesenchymal transition [22, 23]. However, the mechanism by which MSX2 regulates osteosarcoma remains to be elucidated. Our research, therefore, aimed to investigate the impact of MSX2 on SOX2-facilitated migration and growth in osteosarcoma cells.

Material and methods

Sample collection

Ten patients with primary osteosarcoma treated at The First Affiliated Hospital of South China University, were enrolled in this study between September 2014 and July 2018. Written informed consent for core-needle biopsy and for the scientific analysis of clinical specimens was obtained from all patients. Our procedures followed a protocol approved by the Ethics Committee of The First Affiliated Hospital of South China University (Approval number: ChiCTR1900023977).

Cell culture

KHOS and U2OS cells (human osteosarcoma cell lines) were obtained from the Cell Bank of the China Science Academy, Shanghai. Both cell lines were cultured at 37°C in Roswell Park Memorial Institute medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco Laboratories) in an environment of 5% CO_2 and 95% air.

Quantitative real-time PCR (qPCR)

Total RNA from osteosarcoma cell lines and clinical osteosarcoma tissues (100 mg) was extracted using TRIzol (Invitrogen, USA). RNA concentrations were quantified using a NanoDrop2000 system. mRNA was reverse-transcribed into cDNA using the TaqMan Reverse Transcription Kit (Ambion). The amplification reactions were performed on a Stratagene MX3005P machine using the TaqMan Universal Master Mix (Ambion). These cDNAs were subjected to cycles at 95°C for 10 min (40 cycles in total, each lasting for 15 s) and annealing/extension at 60°C for 40 s. The relative expression levels of each gene were calculated by normalizing them to GAPDH (an endogenous control) using the $2^{-\Delta\Delta CT}$ method. Sequences of primers used in the present study are the following: MSX2: F 5'-CAT AAA AGC ATC CCC CTC CC-3', R 5'-AGG AGC AGA GTT GGC ACC AC-3'; SOX2: F 5'-CAC CTA CAG CAT GTC CTA CTC G-3', R 5'-GGT TTT CTC CAT GCT GTT TCT T-3'; Bax: F 5'-AGC TGA GCG AGT GTC TCA AG-3', R 5'-GTC CAA TGT CCA GCC CAT GA-3'; MMP-2: F 5'-AGC GAG TGG ATG CCG CCT TTA A-3', R 5'-CAT TCC AGG CAT CTG CGA TGA G-3'; TNF-α: F 5'-CTC TTC TGC CTG CTG CAC TTT G-3', R 5'-ATG GGC TAC AGG CTT GTC ACT C-3'; GAPDH: F 5'-TGT TCG TCA TGG GTG TGA AC-30, R 5'-ATG GCA TGG ACT GTG GTC AT-3'. All experiments were performed in triplicate.

Lentivirus production, infection, and plasmid transfection

A lentiviral vector encoding MSX2 (pCCL-MSX2) was constructed using GenScript (Shanghai, China). To produce sufficient lentivirus, 10 mg of pCCL-MSX2 was added to 15 mg of packaging plasmids CMVDR8.91, and Lipofectamine 3000 (Invitrogen, USA) was used to transfect HEK293T cells according to the manufacturer's instructions. Forty-eight hours post-transfection, we collected the viral supernatant, which was separated using a 0.45 mm syringe filter (Millipore, USA). The viral supernatant was subsequently concentrated and stored at -80°C. For transfection, KHOS and U2OS cells were cultured in 6-well plates in culture medium containing 5 mL of viral concentrate and polybrene (5 mg/mL, Sigma-Aldrich, USA).

For the overexpression of SOX2, 25 μg of pcDNA3.1 vector carrying the SOX2 coding

sequence was transfected into KHOS and U2OS cells (5 × 10^6 cells/mL). After 48 h, the cells were lysed for further examination.

Cell proliferation assay

The proliferation of osteosarcoma cells was examined using a colony formation assay. Cells were seeded into 35-mm Petri dishes at a density of 1000 cells/well, after which they were cultured for 24 h at 37°C. Following various treatments for 48 h, the culture medium was renewed, and the cells were cultured for another 2 weeks. Finally, colonies were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet solution. The number of colonies in each dish was then counted under a microscope.

CCK-8 assay

Cell viability was examined using the Cell Counting Kit-8 (CCK-8). Briefly, cells were plated onto 96-well plates, and 10 μL of CCK-8 solution was added to each sample, followed by incubation for 2 h at 37°C. Optical density (OD) values were measured at 450 nm using an auto-microplate reader (Infinite M200, Switzerland).

Flow cytometry

The percentage of apoptotic osteosarcoma cells was assessed using flow cytometry. In brief, KHOS and U2OS cells were separated using trypsin and washed with cold phosphate-buffered saline (PBS) three times. Annexin fluorescein isothiocyanate (Beyotechology, China) and propidium iodide (Beyotime, China) were added to the samples and incubated in the dark for 15 min. Finally, the percentage of apoptotic cells was determined using a FACSCalibur flow cytometer (BD Biosciences, USA).

Western blotting (WB)

Cellular lysates were obtained by lysing osteosarcoma cells or tissues with RIPA buffer. Protein concentration was determined, and lysates were subsequently separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel. Polyvinylidene fluoride (PVDF) membranes (Millipore, USA) were used to transfer the immunoblots. After successful protein blot transfer, the immunoblots were blocked with 5% bovine serum albumin at 25°C for 1 h. Protein blots were incubated with primary antibodies against.

MSX2 (1:1000, ab223692; Abcam, UK), β-actin (1:10000, ab8226; Abcam, UK), SOX2 (1:1000, ab97959; Abcam, UK), TNF-α (1:2000, ab6671; Abcam, UK), Bax (1:2000, ab53154; Abcam, UK), and MMP-2 (1:1000, ab86607; Abcam, UK) at 4°C overnight, and then with the corresponding secondary antibodies at 25°C for 1 h, following which the blots were washed. The reactivity of immunoblots was evaluated using the Super Signal West Femto Maximum Sensitivity Substrate Kit (Invitrogen, USA). Pictures were taken for further analysis of relative protein expression.

Transwell migration assay

The Transwell migration assay was used to examine cell migration. Twenty-four hours post-transfection, osteosarcoma cells were dispersed using trypsin and then gently washed with D-Hank's solution. Matrigel inserts (pore size: 8 μm) were utilized in 24-well culture plates to divide each well into a top compartment and a bottom chamber. In the bottom chamber, 400 μL of F-12 medium mixed with 20 ng/mL HGF and 10% fetal bovine serum (FBS) was placed; osteosarcoma cells were plated in the upper chamber. After incubation for 24 h, Matrigel inserts were removed and washed gently, and the cells were fixed with ethanol. Finally, cells that had migrated through the pores in the Matrigel inserts were stained with crystal violet solution. The cells were observed and imaged under a microscope.

Scratch wound healing assay

To evaluate cell migration, a wound-healing assay was performed. First, cells were cultured in 6-well plates at a density of 1 × 10^6 cells/well for 24 h in Dulbecco's Modified Eagle's medium containing 10% FBS. Subsequently, we utilized a sterile pipette tip to create a straight scratch on the cells at the surface of each well. The width of the scratch was observed under an Olympus IX71 microscope (Olympus Corporation, Japan) and imaged.

In vivo tumor formation assay

Athymic nude mice (6-week-old, female) were purchased from Vitalriver Laboratory Animal

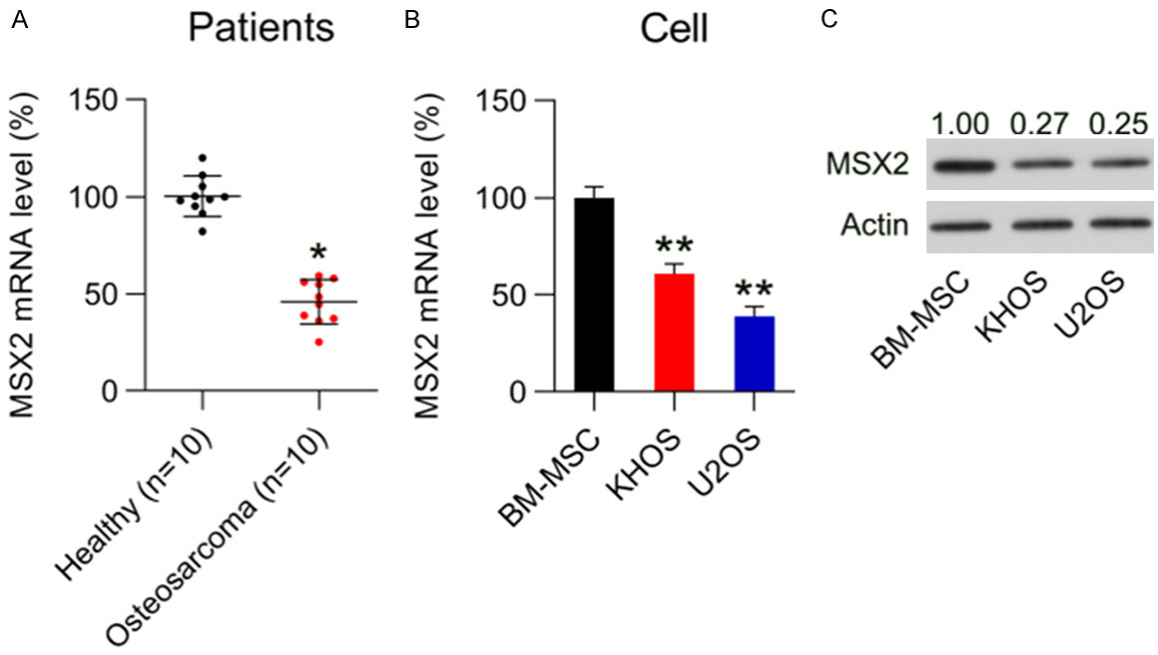

Figure 1. MSX2 levels in tumor specimens of osteosarcoma patients and cell lines. A. The mRNA expression of MSX2 in osteosarcoma specimens from patients (n = 10) and healthy volunteers (n = 10) was examined by qPCR. B, C. MSX2 mRNA and protein expression in osteosarcoma cell lines and BM-MSCs was analyzed using qPCR and WB analyses. *P < 0.05, **P < 0.01 vs. indicated group.

Co. Ltd., China. The animals were raised according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of South China University. U2OS cells were transfected with MSX2-overexpression lentivirus or a negative control. The cells (1×10^6 U2OS cells) were suspended in 100 μL of Matrigel (BD Biosciences), and the cell suspension was then diluted at a ratio of 1:1. Next, the cell suspension was intradermally administered into the left flank of mice (n = 8 per group). The size of the tumors was measured every 5 days, and the tumor volume was determined based on the following formula: Volume = (Length × Width^2)/2. All experimental protocols were approved by the Animal Experimentation Ethics Committee of The First Affiliated Hospital of South China University (Approval number: ChiCTR1900023977) and all efforts were made to minimize animal suffering and reduce the number of animals used.

Statistical analysis

Data are presented as mean ± standard deviation. The statistical analysis was performed using the GraphPad Prism software (GraphPad Software, Inc., USA) and SPSS predictive analytics software (version 18.0; SPSS, Inc., USA). Normally distributed data were compared using one-way analysis of variance, whereas the Mann-Whitney U test was employed to compare the non-parametric variables with a post-hoc test used for multiple comparisons between groups. Two-tailed P < 0.05 was considered as the statistical cutoff value for a significant difference.

Results

MSX2 expression is decreased in osteosarcoma clinical samples and cell lines

To evaluate the function of MSX2 in the development of osteosarcoma, MSX2 expression in osteosarcoma samples and normal healthy controls was analyzed by qPCR. MSX2 mRNA levels in osteosarcoma tissues were remarkably decreased compared to those in normal controls (**Figure 1A**). Next, we measured MSX2 expression levels in BM-MSCs and osteosarcoma cells (KHOS and U2OS cells). It was found that mRNA and protein expression levels of MSX2 in the two osteosarcoma cell lines were lower than those in BM-MSCs (**Figure 1B**, **1C**). These data imply that MSX2 might be involved in the progression of osteosarcoma.

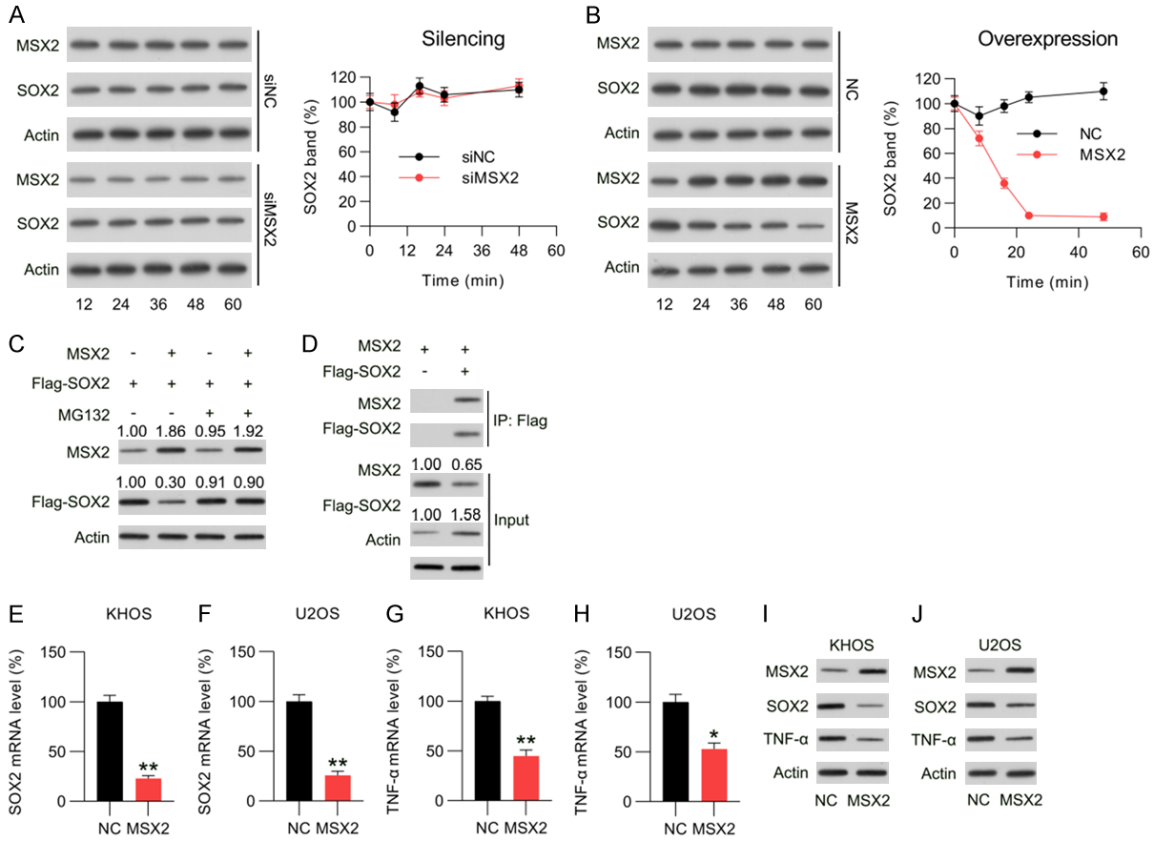

Figure 2. MSX2 repressed the expression of SOX2 transcriptionally and translationally. A, B. U2OS cells were transfected with Lentiviral-MSX2 siRNA (siMSX2) or Lentiviral-negative control siRNA (siNC), or Lentiviral-MSX2 or Lentiviral-NC, as indicated. WB analysis were performed to examine the expression of SOX2 protein. The band intensities of SOX2 protein were quantified by densitometry and normalized to GAPDH levels. C. Whole-cell lysates from U2OS cells were infected with Lentiviral-NC or MSX2, and then transfected with SOX2 expression vector, followed by treatment with 30 μM MG132 for 4 h. WB was used to probe for MSX2 and SOX2. D. U2OS cells were infected with Lentiviral-MSX2, and subsequently transfected with Flag-SOX2 plasmids. Lysates were IP by anti-Flag antibody and WB by the indicated antibodies. E-J. KHOS and U2OS cells were infected with Lentiviral-NC or MSX2; qPCR and WB were carried out to examine the mRNA and protein level of SOX2 and TNF-α, respectively. *P < 0.05, **P < 0.01 vs. indicated group.

Negative correlation between MSX2 and SOX2 expression in osteosarcoma cells

To understand how MSX2 regulates SOX2 expression in osteosarcoma cells, U2OS cells were infected with Lentiviral-siRNA-MSX2 or Lentiviral-MSX2 to silence or overexpress MSX2, respectively. Data clearly showed that silencing of MSX2 in U2OS cells did not cause any changes in SOX2 expression. In contrast, with MSX2 overexpression, SOX2 protein levels decreased significantly faster in U2OS cells than in cells with Lentiviral-NC infection (**Figure 2A**, **2B**). Furthermore, MSX2-induced downregulation of SOX2 protein was abolished after treatment with the proteasome inhibitor MG132 (**Figure 2C**). The Co-IP assay results revealed a direct link between MSX2 and SOX2 (**Figure 2D**). Lysates from KHOS and U2OS cells co-expressed with MSX2 and Flag-SOX2 plasmids or vector controls were used to immunoprecipitate SOX2 with the Flag antibody. WB analysis showed that MSX2 coimmunoprecipitated with SOX2, but not with the empty vector control (**Figure 2D**). These data suggest that MSX2 expression contributes to the proteasomal degradation of SOX2.

Furthermore, qPCR data indicated that mRNA levels of SOX2 and its upstream TNF-α were downregulated in osteosarcoma cells in the MSX2 overexpression group compared with those in the NC group (**Figure 2E-H**). WB data confirmed that, following MSX2 overexpres-

sion, the protein expression of SOX2 and TNF-α was downregulated (**Figure 2I**, **2J**). Collectively, these data suggest that MSX2 transcriptionally and translationally represses SOX2 expression in osteosarcoma cells.

Influence of MSX2 overexpression on cell migration and proliferation in osteosarcoma

The influence of MSX2 on cell viability and growth was investigated. The results of colony formation and CCK-8 assays demonstrated that MSX2 overexpression significantly decreased the number of colonies compared to that in the control groups (**Figure 3A**, **3B**). Cell viability was notably reduced as a consequence of MSX2 overexpression, when compared with that in the NC group (**Figure 3C**, **3D**). These data suggest an inhibitory role of MSX2 in osteosarcoma cell viability.

Next, we evaluated the effect of MSX2 on apoptosis in osteosarcoma cells. Bax, a well-recognized apoptotic marker, was examined by qPCR and WB. MSX2 overexpression resulted in a significant increase in Bax mRNA and protein levels in both KHOS and U2OS cells (**Figure 3E-H**). The results of flow cytometry showed significantly increased apoptosis in osteosarcoma cells with Lentiviral-MSX2 infection compared to that in the NC group (**Figure 3I**, **3J**).

We subsequently examined whether cell invasion and migration were affected by MSX2 overexpression using Transwell and wound-healing assays. We found that MSX2 overexpression suppressed the invasion ability of these cells (**Figure 4A**, **4B**). In the wound-healing assay, MSX2 overexpression significantly impaired the cell migration rate in the two osteosarcoma cell lines (**Figure 4C**, **4D**). In addition, the activity of MMP-2 in both osteosarcoma cell lines was also suppressed by MSX2 upregulation, as revealed by qPCR and western blotting (**Figure 4E-H**). These data suggest that MSX2 inhibits the migration and growth of osteosarcoma cells.

Overexpression of SOX2 abrogates the effects of MSX2 on growth and migration of osteosarcoma cells

To better understand whether and how SOX2 was involved in the effects of MSX2 on the growth and migration of osteosarcoma cells, both U2OS and KHOS cells were transfected with pcDNA3.1-empty or pcDNA3.1-SOX2 to overexpress SOX2. Transfection with pcDNA3.1-SOX2 led to an increased SOX2 activity in osteosarcoma cells already overexpressing MSX2 (**Figure 5A-D**).

We subsequently performed colony formation and CCK-8 assays to evaluate cell proliferation and viability after overexpressing SOX2 in osteosarcoma cells overexpressing MSX2. The number of colonies generated was significantly restored owing to SOX2 upregulation in osteosarcoma cells overexpressing MSX2 (**Figure 6A**, **6B**). Results from the CCK-8 assay indicated that SOX2 overexpression also resulted in the recovery of cell viability (**Figure 6C**, **6D**).

Furthermore, qPCR and WB results showed that SOX2 activity was increased in the SOX2-overexpressed group, in contrast to that in the empty vector transfection group (**Figure 6E-H**). Flow cytometry data suggested that SOX2 upregulation contributed to downregulation of the apoptotic cell proportion in KHOS and U2OS cells overexpressing MSX2 (**Figure 6I**, **6J**).

The Transwell invasion assay revealed that SOX2 upregulation notably increased the number of invasive KHOS and U2OS cells (**Figure 7A**, **7B**). Meanwhile, SOX2 restoration remarkably increased the number of migrated KHOS and U2OS cells with MSX2 overexpression, as determined by the wound healing assay (**Figure 7C**, **7D**). Data from both qPCR and WB indicated that MMP-2 expression was elevated after SOX2 overexpression in osteosarcoma cells compared to that in the empty vector transfection group (**Figure 7E-H**). These data suggest that SOX2 participates in the MSX2-repressed cell growth and migration of KHOS and U2OS cells.

MSX2 overexpression inhibits in vivo osteosarcoma tumor growth

The abovementioned results suggest that MSX2 serves as a tumor suppressor in osteosarcoma. Here, to probe the impact of MSX2 on *in vivo* tumorigenesis of U2OS cells, tumor growth was further observed by conducting a xenograft tumor growth assay with administration of U2OS cells with or without MSX2 overexpression. The tumor growth curve clearly indicated that the growth rate in the MSX2-

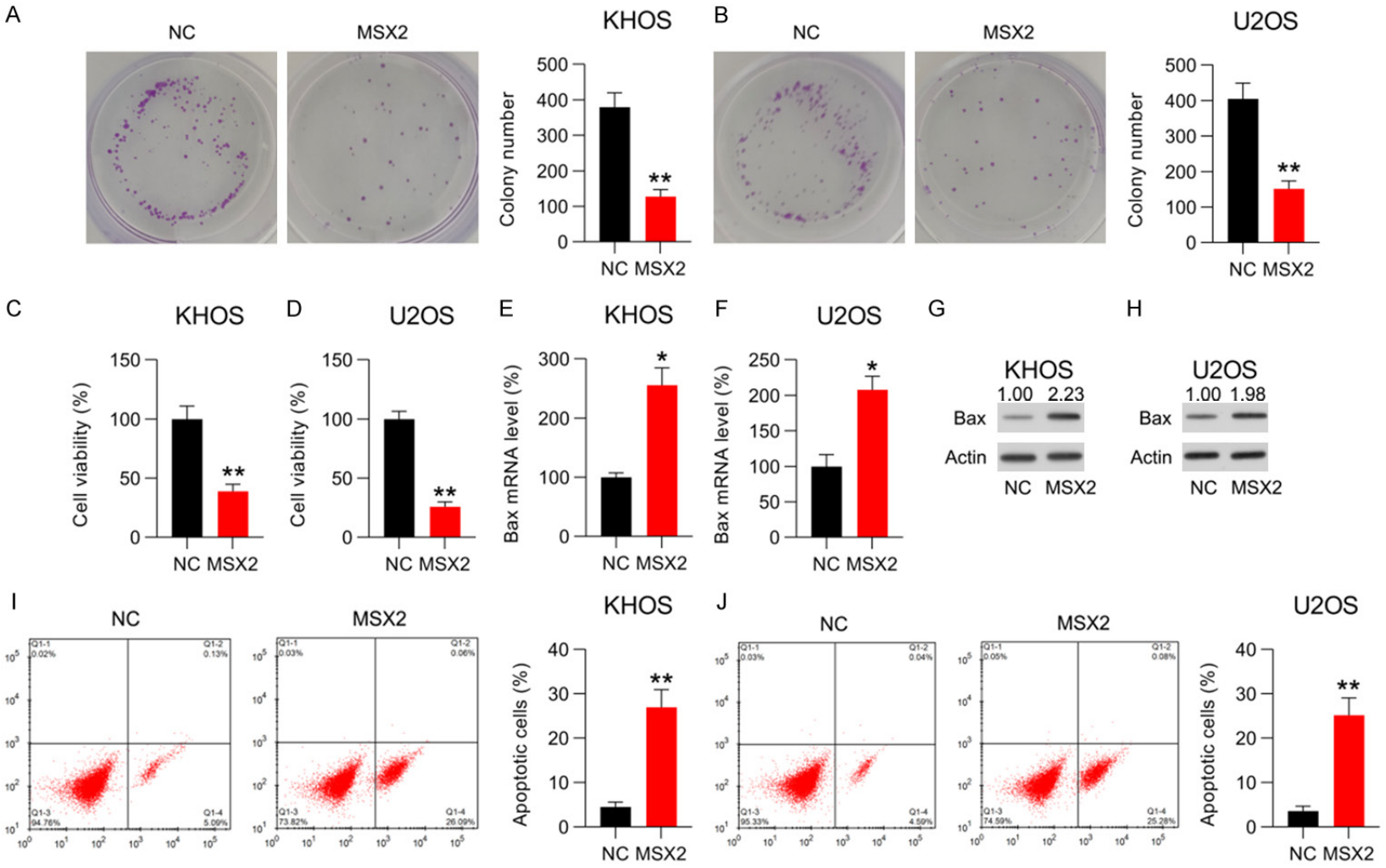

Figure 3. Impact of MSX2 overexpression on the growth of osteosarcoma cells. Osteosarcoma cells were infected with Lentiviral-NC or Lentiviral-MSX2 for 48 h. A, B. Soft agar colony formation assay was performed. C, D. Cell viability was measured 72 h post-transfection using the CCK-8 assay. E-H. The mRNA and protein levels of Bax in KHOS and U2OS cells. I, J. Flow cytometry to assess the number of apoptotic cells in KHOS and U2OS cells. *P < 0.05, **P < 0.01 vs. indicated group.

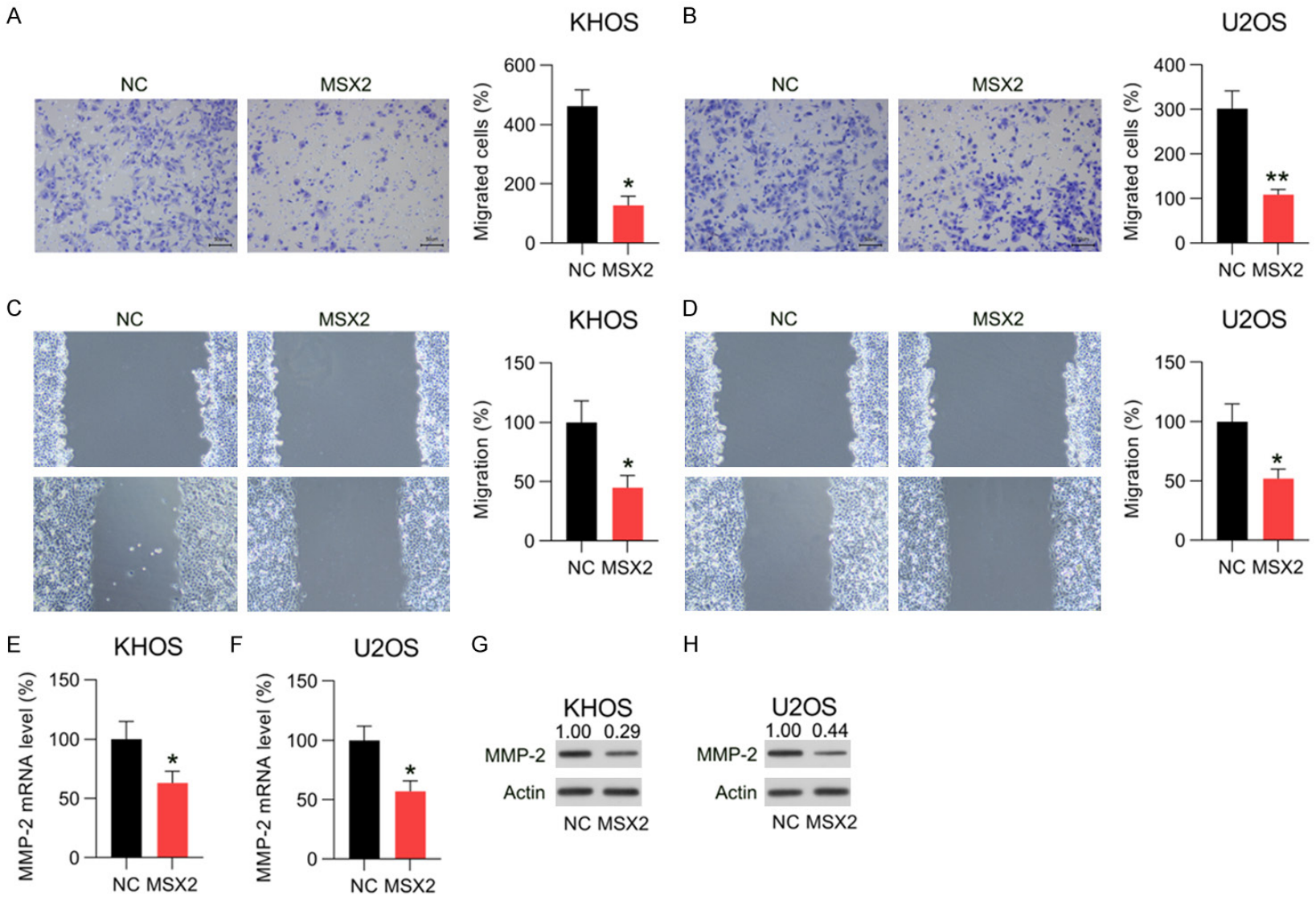

Figure 4. Effect of MSX2 overexpression on the migration and invasion of osteosarcoma cells. KHOS and U2OS cells were infected with Lentiviral-MSX2 for 48 h. A, B. Wound healing assay was performed to detect the migration rate of KHOS and U2OS cells. C, D. The Transwell invasion assay was used to detect the invasion ability of KHOS and U2OS cells. E-H. The mRNA and protein levels of MMP-2 in KHOS and U2OS cells. *P < 0.05, **P < 0.01 vs. indicated group.

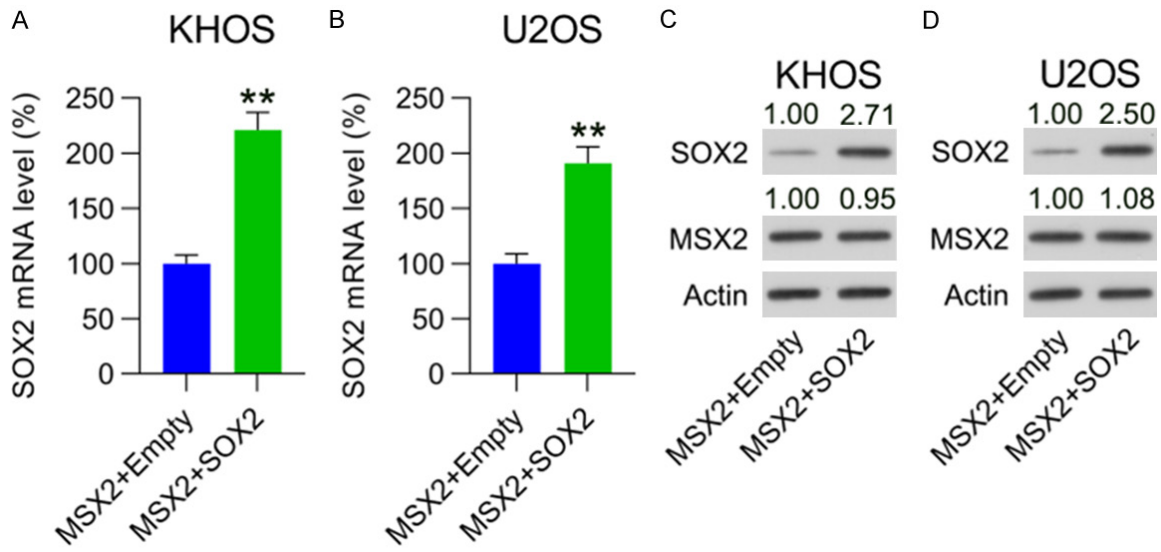

Figure 5. Overexpression of SOX2 in osteosarcoma cells with MSX2 overexpression. Osteosarcoma cells were infected with Lentiviral-MSX2 for 24 h and then transfected with Flag-SOX2 plasmid or empty plasmid for 36 h. A-D. qPCR and WB were carried out to determine the mRNA and protein levels of SOX2 in KHOS and U2OS cells, respectively. **P < 0.01 vs. indicated group.

overexpression group was considerably lower than that in the control group on day 25 (**Figure 8A**). The tumor weight in the MSX2-overexpression group was also lower than that in the control group (**Figure 8B**, **8C**). The expression of MSX2, SOX2, and TNF-α in tumor tissues was determined by qPCR and WB. The results indicated increased expression of MSX2 and decreased expression levels of SOX2 and TNF-α in tumor tissue in the MSX2-overexpression group compared to those in the NC group (**Figure 8D-G**). These results provide further evidence that MSX2 serves as a tumor suppressor in osteosarcoma by mediating SOX2 expression.

Discussion

This study unveils a novel mechanism by which loss of MSX2 maintains osteosarcoma tumor phenotypes through the degradation and transcriptional suppression of SOX2. Lower MSX2 expression in osteosarcoma clinical tissues and cells than that in normal healthy controls or BM-MSCs has been observed. A previous study showed that overexpression of MSX2 promoted SOX2 degradation by the proteasome, while MSX2 also led to downregulation of SOX2 transcription. Furthermore, we found that in vitro overexpression of MSX2 inhibited osteosarcoma cell viability and growth, induced apoptotic death, and repressed migration and invasion. In contrast, overexpression of SOX2 could counteract the effects of MSX2 overexpression. In addition, MSX2 showed an in vivo inhibitory effect on tumor growth and induction of apoptosis. Our data demonstrated that MSX2 might serve as a tumor suppressor to decrease the proliferation, migration, and invasion of osteosarcoma cells via transcriptional and translational mediation of SOX2 expression.

MSX2 has been reported to be essential for mesendoderm differentiation in human pluripotent stem cells (hPSCs) [13]. In the BMP signaling of hPSCs, MSX2 is considered a direct target of this signaling pathway. During mesendoderm differentiation, MSX2 can be synergistically activated by LEF1 via Wnt signaling. Typically, MSX2 can bind to the promoter of SOX2 and suppress the transcription of SOX2, thus making the circuitry of pluripotency unstable [13]. Therefore, according to a previous study, MSX2 may transcriptionally repress SOX2 expression [13]. Here, our data indicated that MSX2 not only affected SOX2 expression transcriptionally but also caused SOX2 degradation via proteasomal patterns in KHOS and U2OS cells. Previous studies have demonstrated that MSX2 is a BMP-associated apoptotic inducer at the transcriptional level [24, 25]. In

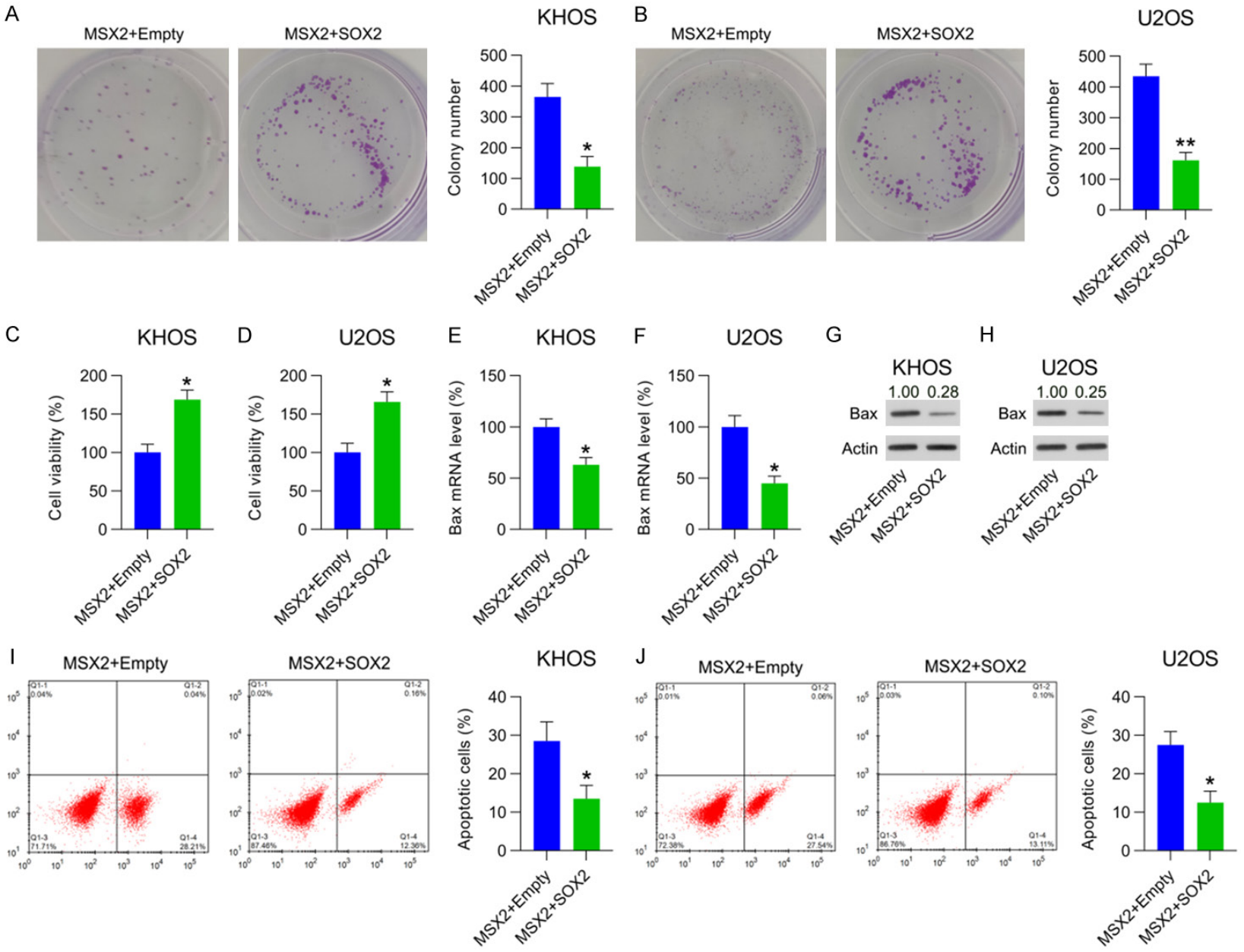
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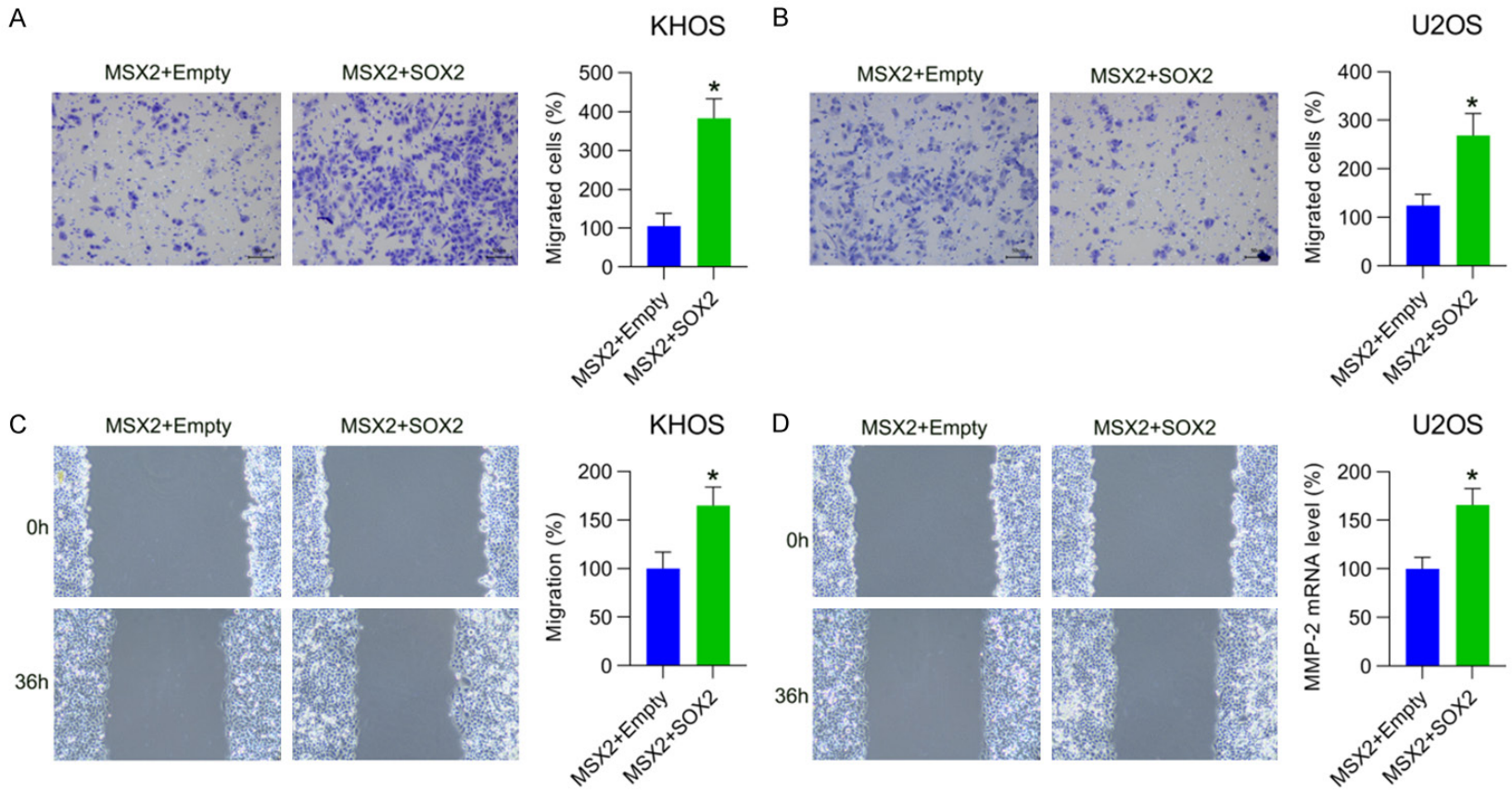
Figure 6. Effects of SOX2 overexpression on the growth of osteosarcoma cells with MSX2 overexpression. Osteosarcoma cells were infected with Lentiviral-MSX2 for 24 h and then transfected with Flag-SOX2 plasmid or empty plasmid for 36 h. A, B. Cell growth of KHOS and U2OS cells were examined by colony formation assay. C, D. Viability of KHOS and U2OS cells was measured at 72 h post-transfection using the CCK-8 assay. E-H. qPCR and WB were carried out to determine the mRNA and protein levels of Bax in KHOS and U2OS cells, respectively. I, J. Flow cytometry was used to assess the apoptotic percentage of KHOS and U2OS cells. *P < 0.05, **P < 0.01 vs. indicated group.



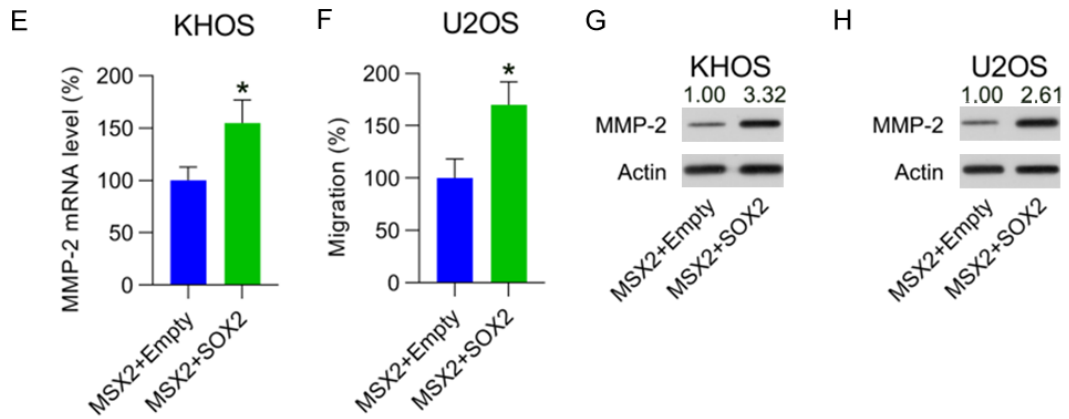


Figure 7. Effects of SOX2 overexpression on the migration and invasion ability of osteosarcoma cells with MSX2 overexpression. Osteosarcoma cells were infected with Lentiviral-MSX2 for 24 h and then transfected with Flag-SOX2 plasmid or empty plasmid for 36 h. A, B. Wound healing assay was performed to measure the migration rate in KHOS and U2OS cells. C, D. Transwell assay was employed to detect the invasion ability of KHOS and U2OS cells. E-H. qPCR and WB were performed to determine MMP-2 mRNA and protein levels, respectively. *P < 0.05 vs. indicated group.

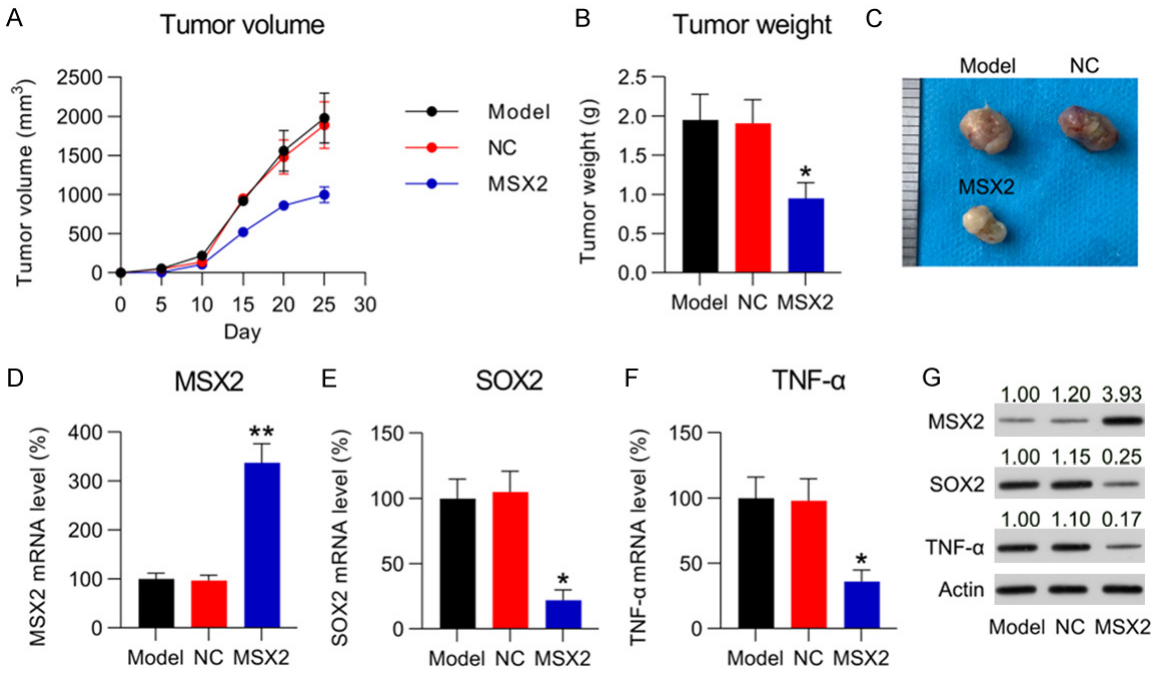


Figure 8. Influence of MSX2 on xenograft osteosarcoma tumorigenesis in mice. The U2OS cells with Lentiviral-NC or Lentiviral-MSX2 were intradermally administered to mice (n = 8 in each group). At day 26 post-inoculation, the animals were sacrificed, and tumors were weighed. A. The proliferation of tumors at 25 days post-inoculation was measured with an interval of 5 days. B. Tumor weight on day 26 after inoculation. C. Images of tumors from nude mice at 26 days post-inoculation. D-G. MSX2, SOX2, and TNF-α expression levels were measured in each group using qPCR and WB. *P < 0.05, **P < 0.01 vs. indicated group.

pancreatic cancer cells, MSX2 overexpression enhances the malignant phenotype, which is associated with Twist 1 expression [26, 27]. This current study implied that MSX2 overexpression in osteosarcoma cells resulted in the attenuation of cell viability and stimulation of cell apoptosis, which is consistent with previous studies showing that MSX2 acts as an apoptotic inducer [24, 25]. However, data from the Transwell invasion assay and wound healing assay revealed that MSX2 upregulation is harmful to the migration and invasion of KHOS and U2OS, which is contradictory to previously reported findings in pancreatic cancer cells [26, 27]. Thus, MSX2 may play a bidirectional role in metastasis in different cancer cells.

SOX2, which belongs to the SOX family, is a well-recognized transcription factor that is crucial to the development and maintenance of stemness [4, 28]. SOX2 forms a pluripotency regulatory network in NANOG. SOX2 promotes the malignant behavior of cancers by promoting cell invasion, migration, and proliferation [29]. It is noteworthy that SOX2 is essential for maintenance of osteosarcoma stem cell-like cells. Basu-Roy et al. reported that SOX2 is necessary for the self-renewal of osteosarcoma cells and that SOX2 antagonized their differentiation [10]. SOX2 is vital for the self-renewal of osteoblast precursors. Combined with these data, we further demonstrated that MSX2 repressed SOX2 to inhibit the malignant phenotypes, suggesting the importance of SOX2-targeted therapy for osteosarcoma.

In brief, our research sheds light on the regulatory mechanism of action of MSX2 and SOX2 in osteosarcoma and reveals that MSX2 is a transcriptional and translational inhibitor of SOX2 expression. Functional studies revealed that the overexpression of MSX2 inhibited osteosarcoma tumor phenotypes both *in vitro* and *in vivo*. In summary, this newly identified MSX2-SOX2 axis provides valuable insights into potential prognostic markers and therapeutic targets for osteosarcoma.

Disclosure of conflict of interest

None.

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