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MNX1-AS1 accelerates the epithelial-mesenchymal transition in osteosarcoma cells by activating MNX1 as a functional oncogene

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Abstract. – OBJECTIVE: To investigate whether MNX1-AS1 can accelerate epithelial-mesenchymal transition (EMT) of osteosarcoma cells *via* activating MNX1.

PATIENTS AND METHODS: The expression pattern of MNX1-AS1 in osteosarcoma tissues and cell lines was examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Moreover, the cytoplasmic and nuclear levels of MNX1-AS1 in osteosarcoma cells were also determined. The regulatory effects of MNX1-AS1 on viability, clonality, migratory, and invasive abilities of the osteosarcoma cells were evaluated. The relative levels of MNX1 and EMT-related genes influenced by MNX1-AS1 were detected. The methylation ability in the promoter of the osteosarcoma cells transfected with si-MNX1-AS1 or MNX1-AS1 vector was determined by the whole genome bisulfite sequencing.

RESULTS: MNX1-AS1 was upregulated in osteosarcoma tissues and cell lines, which was mainly expressed in the nucleus. The knockdown of MNX1-AS1 markedly attenuated viability, clonality, migratory, and the invasive abilities of the osteosarcoma cells. Besides, the transfection of si-MNX1-AS1 in U2OS and MG63 cells downregulated MNX1 and Snail, and upregulated E-cadherin. The methylation ability increased after the knockdown of MNX1-AS1, while the overexpression of MNX1-AS1 obtained the opposite trends.

CONCLUSIONS: MNX1-AS1 mediates EMT of the osteosarcoma cells *via* activating MNX1, thereafter accelerating the progression of the osteosarcoma.

Key Words:
Osteosarcoma, EMT, MNX1-AS1, MNX1.

Introduction

Osteosarcoma is the most common primary bone malignancy. Osteosarcoma cells originate from mesenchymal tissues, presenting strong proliferative, invasive, and angiogenesis abilities[1]. The incidence of osteosarcoma ranks second place in primary bone malignancies, accounting for 34%, and it is the fifth tumor prevalent in adolescents and young adults[2]. Currently, the therapeutic efficacy of osteosarcoma is far away from satisfying. The genetic therapy of osteosarcoma has been well concerned[3]. The epithelial-mesenchymal transition (EMT) is the process that transforms the adherent epithelial cells to migratory mesenchymal cells under certain specific factors. As a result, EMT induces in the epithelial cells the characteristics of the mesenchymal cells. In malignant tumors, EMT is highly correlated with the invasive and metastatic performances[4,5]. Yu et al[6] considered that EMT is related to the metastatic epithelial degeneration, but its role in interstitial cancers, such as osteosarcoma is still unknown. Therefore, it is of clinical significance to explore the mechanism of EMT and metastasis in osteosarcoma.

Long non-coding RNA (lncRNA) is a non-coding RNA with 200 nucleotides long[7]. It participates in the regulation of biological processes, such as embryonic development, tissue differentiation, and cell proliferation[8]. Plenty of lncRNAs are closely related to the occurrence and development of osteosarcoma. Zheng et al[9] showed that lncRNA HOTAIR is upregulated in osteosarco-

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ma cells, and it accelerates the proliferative rate and suppresses the apoptosis of MG63 cells. A current study has identified that the knockdown of MNX1-AS1 suppresses the ovarian cancer cells to proliferate and migrate[10]. Nevertheless, the potential function of MNX1-AS1 in osteosarcoma remains unclear.

MNX1 is a homologous structural gene, initially discovered in pancreatic and lymphoid tissues[11]. There have been over 69 cytogenetics or gene mutations reported at the MNX1 locus[12,13]. The association between MNX1 gene mutation and Currarino syndrome has been confirmed[14]. As a functional oncogene, MNX1-AS1 induces the tumorigenesis of breast cancer *via* activating MNX1[15]. In this study, we investigated whether MNX1-AS1 could accelerate EMT in osteosarcoma by activating MNX1, so as to provide novel directions in the clinical treatments of osteosarcoma.

Patients and Methods

Clinical Samples

Osteosarcoma tissues were surgically resected from osteosarcoma patients treated in First People's Hospital of Fuyang District from March 2017 to October 2018. They did not receive preoperative anti-tumor therapy and were pathologically diagnosed. During the same period, normal bone tissues were harvested from patients undergoing joint replacement. This study was approved by the Ethics Committee of the First People's Hospital of Fuyang District. Patients with other malignancies or osteoporosis were excluded. All subjects volunteered to participate in the study and signed written informed consent.

Cell Culture and Transfection

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 37°C, 5% CO_2 incubator. For transfection, the cells were pre-seeded in a 6-well plate and grown to 80% of confluence. The transfection of si-MNX1-AS1 and MNX1-AS1 vector or the negative control was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 6 h. Sequences of si-MNX1-AS1 were 5′-GCUACGUGAGUCUUGCAAATT-3′ (sense) and 5′-UUUGCAAGACUCACGUAGCTT-3′ (antisense).

Chromatin Fractionation

Until cell growth to 1 × 106 cells, 200 μL of Lysis Buffer J was added to fully lyse the cells. After centrifugation, the supernatant contained cytoplasmic RNA, and the remaining precipitant contained nuclear RNA. The supernatant was transferred to a new tube. Subsequently, Buffer SK and absolute ethanol were added to cytoplasmic RNA and nucleus RNA, respectively, followed by extraction with column centrifugation.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and quantified by a UV spectrophotometer. The extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the 5×-AU-In-One RT MasterMix at 25°C for 10 min, 42°C for 15 min, and 85°C for 5 min. QRT-PCR system was prepared, including 0.4 μL of cDNA template, 5 μL of EvaGreen 2× qPCR MasterMix, 0.3 μL of forward primer, 0.3 μL of reverse primer and ddH2O was supplied to a total of 10 μL. Subsequently, qRT-PCR was conducted at 95°C for 10-min denaturation, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Each sample was performed in triplicate, and the relative level calculated by the $2^{-\Delta\Delta Ct}$ method was analyzed by iQ5 2.0 (Bio-Rad, Hercules, CA, USA). The primer sequences were as follows: MNX1-AS1: F: 5'-AAGGTAGCCACCAAACAC-3', R: 5'-AGACTCACGTAGCACTGT-3'; MNX1: F: 5'-CGTTGCTGTAGGGGAAATGGT-3', R: 5'-AGGAAGCGGAGAAACAGAAGG-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: 5'-CAGGGCTGCTTTTAACTCTGGTAA-3', R: 5'-GGGTGGAATCATATTGGAACATGT-3'; U6: F: 5'-CGCTTCGGCAGCACATATACTAAAATTGGAAC-3', R: 5'-GCTTCACGAATTTGCGTGTCATCCTTGC-3'.

Cell Counting Kit-8 (CCK-8)

The cells were seeded in the 96-well plate with 2×10^4 cells per well. The absorbance (A) at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

Colony Formation Assay

The cells were seeded in the 6-well plate with 500 cells per well and cultured for 2 weeks. Subsequently, the cells were subjected to 15-min fixation in 4% paraformaldehyde and 30-min

staining in 0.1% violet crystal. After removing the staining solution, the colonies were aired dried and observed under a microscope.

Transwell

The transwell chamber was pre-coated with Matrigel (diluted in DMEM at a ratio of 1:8) overnight at 4°C. The cells were digested, washed once with Phosphate-Buffered Saline (PBS) and serum-free medium, and suspended in serum-free medium. The cell density was adjusted to 1×105/mL. 500 μL of medium containing 10% FBS was added in the bottom chamber of the 24-well plate. 200 μL of cell suspension was seeded in the upper chamber. At 24 h, the cell fixation with methanol for 30 min and staining with 0.1% crystal violet for another 30 min were performed. The invasive cells were observed and photographed using an inverted microscope. A migration assay was conducted in the same procedures except for Matrigel pre-coating.

Western Blot

The total protein was extracted from cells using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and quantified by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) method. The protein sample was loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), the protein sample was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and with secondary antibodies for 2 h. The bands were exposed by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Whole Genome Bisulfite Sequencing

After bisulfite treatment, all cytosines that have not been methylated were converted to uracil, whereas the methylated cytosine remained unchanged. The target products were subjected to purification and TA cloning, and those positive colonies were harvested for sequencing. Finally, the measured sequences were aligned with the original sequences. The methylation sites and numbers were counted for calculating the degree of methylation.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. The data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

Upregulated MNX1-AS1 in Osteosarcoma

U2OS, MG63, and HOS cells were subjected to chromatin fractionation. QRT-PCR data revealed that MNX1-AS1 was mainly distributed in the nucleus of the osteosarcoma cells (Figure 1A). Besides, MNX1-AS1 was upregulated in the osteosarcoma cells compared to normal osteoblasts (Figure 1B). Osteosarcoma tissues presented a higher abundance of MNX1-AS1 compared to normal tissues (Figure 1C). It is believed that MNX1-AS1 was involved in the occurrence and progression of osteosarcoma.

Knockdown of MNX1-AS1 Suppressed Proliferative, Migratory and Invasive Abilities of Osteosarcoma Cells

The transfection of si-MNX1-AS1 effectively downregulated the MNX1-AS1 level in U2OS and MG63 cells (Figure 2A). Subsequently, the CCK-8 assay revealed the inhibited viability in osteosarcoma cells transfected with si-MNX1-AS1 (Figure 2B). The colony formation assay showed fewer colonies in U2OS and MG63 cells transfected with si-MNX1-AS1, indicating an inhibited clonality (Figure 2C). Moreover, the migratory and invasive cells were reduced after the knockdown of MNX1-AS1 (Figure 2D).

Overexpression of MNX1-AS1 Accelerated Proliferative, Migratory and Invasive Abilities of Osteosarcoma Cells

To further explore the biological function of MNX1-AS1 in osteosarcoma, the MNX1-AS1 vector was constructed. The transfection of MNX1-AS1 vector markedly upregulated MNX1-AS1 level in osteosarcoma cells (Figure 3A). The overexpression of MNX1-AS1 enhanced the viability of U2OS and MG63 cells (Figure 3B). More colonies were observed in osteosarcoma cells overexpressing MNX1-AS1 (Figure 3C). Furthermore, the transwell assay illustrated more migratory and invasive cells after transfection of MNX1-AS1 vector (Figure 3D).

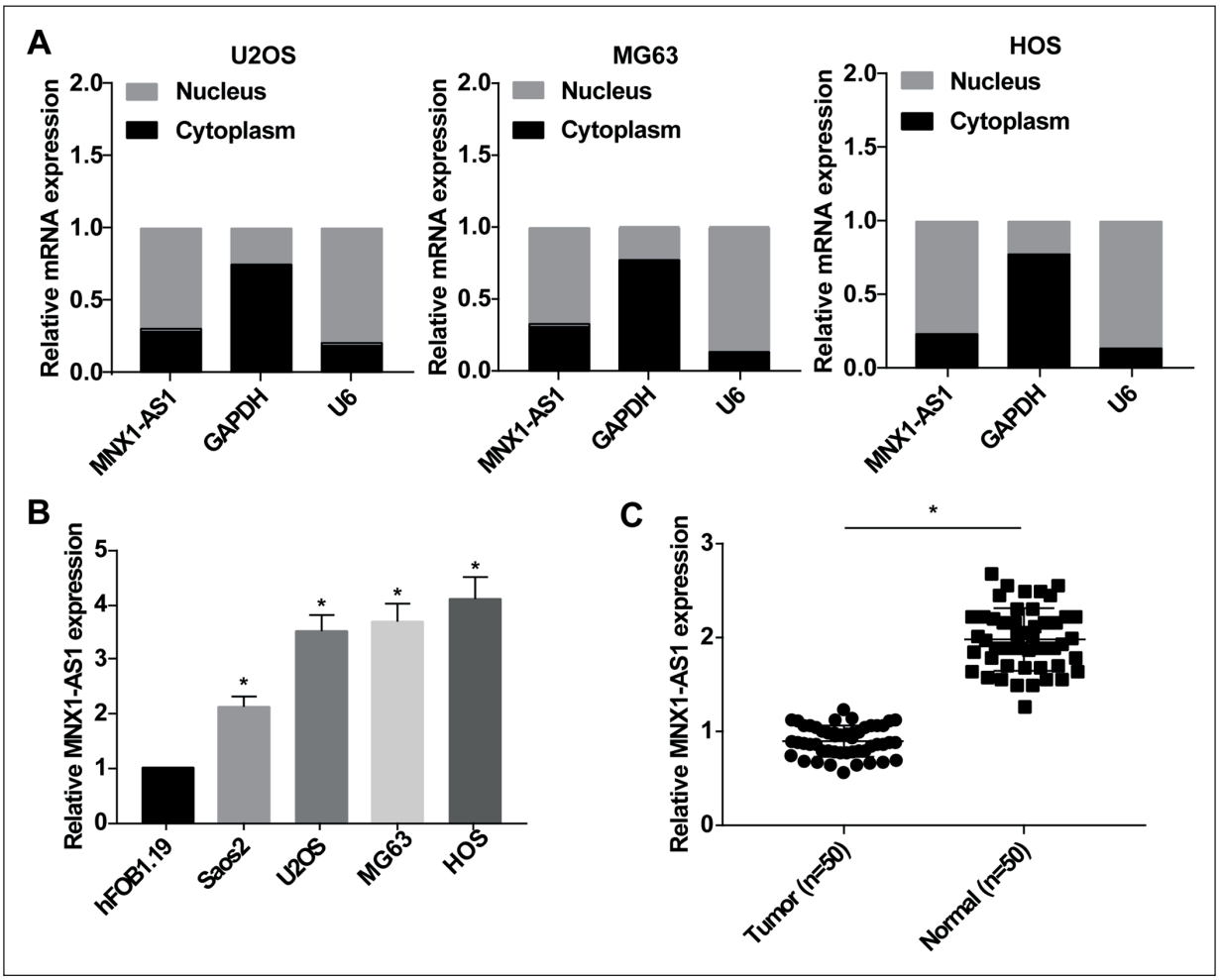

Figure 1. Upregulated MNX1-AS1 in osteosarcoma. **A,** Cytoplasmic and nuclear levels of MNX1-AS1 in U2OS, MG63, and HOS cells. GAPDH and U6 were used as cytoplasmic or nuclear internal references, respectively. **B,** Relative level of MNX1-AS1 in osteoblasts (hFOB1.19) and osteosarcoma cell lines (Saos2, U2OS, MG63, and HOS). **C,** Relative level of MNX1-AS1 in osteosarcoma tissues and normal tissues (n=50).

MNX1-AS1 Mediated EMT in Osteosarcoma by Regulating MNX1 Level

We next explored the role of MNX1-AS1 and MNX1 in the EMT of osteosarcoma. It is found that the MNX1 level was downregulated after transfection of si-MNX1-AS1 in U2OS and MG63 cells (Figure 4A). Conversely, the overexpression of MNX1-AS1 markedly upregulated MNX1 level (Figure 4C). The protein levels of MNX1 and Snail were downregulated, whereas E-cadherin was upregulated after the knockdown of MNX1-AS1 (Figure 4B). The overexpression of MNX1-AS1 has shown opposite trends in the protein levels of MNX1, E-cadherin, and Snail (Figure 4D). Notably, the knockdown of MNX1-AS1 enhanced the methylation ability in the promoter of U2OS and MG63 cells, and the overexpression of MNX1-AS1 achieved the opposite results (Figure 4E).

Discussion

Osteosarcoma is a relatively rare malignant tumor in children[16]. The occurrence and development of osteosarcoma are related to cell cycle and apoptosis. The expressions of the osteosarcoma-related genes in distant metastasis organs, the invasiveness of the osteosarcoma cells, and the metastatic triggers are responsible for the metastasis of osteosarcoma[17]. Although the combination of traditional chemotherapy with surgery significantly improves the 5-year survival of

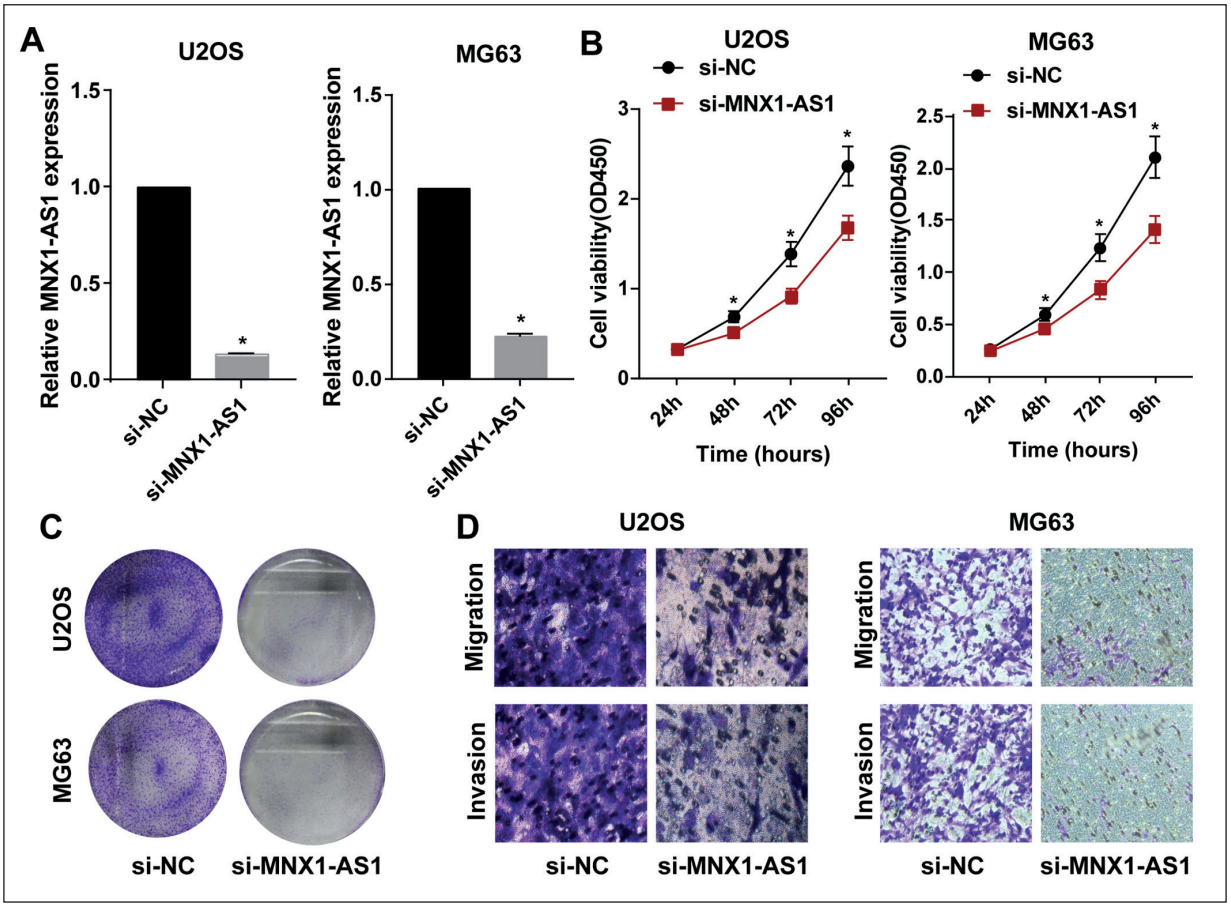

Figure 2. Knockdown of MNX1-AS1 suppressed proliferative, migratory, and the invasive abilities of the osteosarcoma cells. **A,** Relative level of MNX1-AS1 in U2OS and MG63 cells transfected with si-NC or si-MNX1-AS1. **B,** CCK-8 assay revealed the viability in U2OS and MG63 cells transfected with si-NC or si-MNX1-AS1. **C,** The colony formation assay revealed the clonality in U2OS and MG63 cells transfected with si-NC or si-MNX1-AS1. **D,** The transwell assay revealed the migration and invasion in U2OS and MG63 cells transfected with si-NC or si-MNX1-AS1 (magnification: 40×).

osteosarcoma, the long-term prognosis of osteosarcoma has stagnated in the past 30 years. The adverse effects of traditional chemotherapy for osteosarcoma have been extensively identified[18]. It is urgent to develop more effective strategies to treat osteosarcoma.

During the process of EMT, the epithelial cells lose polarity and obtain the migratory ability of mesenchymal cells. EMT is proved to be crucial in tumor invasion and metastasis[19]. Multiple cytokines are changed after the occurrence of EMT, such as the upregulated N-cadherin, etc. Meanwhile, EMT induces the remodeling of the cytoskeleton, increases migratory capacity and affinity with other interstitial tissues, which in turn lead to the spread of tumor cells[20]. It is reported that EMT is involved in the invasion and metastasis of osteosarcoma[21]. Ishikawa et al[22] believed that EMT enhances the malignant level of tumor cells. Relevant transcriptional factors in EMT are important in triggering the progression of malignant epithelial tumors. Further explorations on the relation between osteosarcoma and EMT are required to improve the prognosis of the affected patients.

LncRNA was previously considered as transcriptional noise. With the deepening of researches, lncRNA is found to be crucial in biological processes and disease progression[23]. Notably, abnormally expressed lncRNAs serve as oncogenes or tumor suppressors in tumor diseases[24]. The search for osteosarcoma-related lncRNAs contributes to clarify the pathogenesis of osteosarcoma and develop novel therapeutic targets. Li et al[25] demonstrated that UCA1 is highly expressed

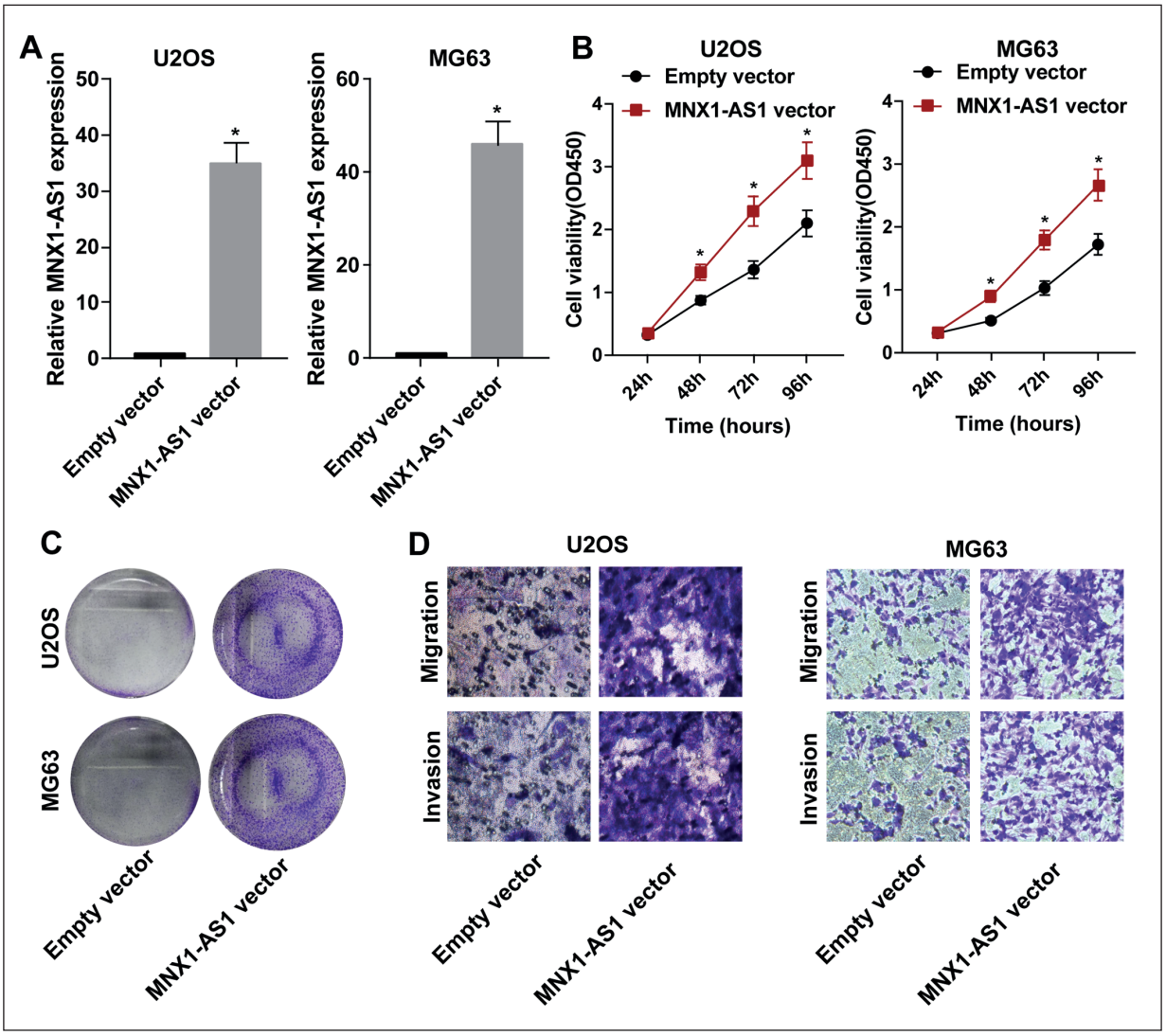

Figure 3. Overexpression of MNX1-AS1 accelerated the proliferative, migratory, and invasive abilities of the osteosarcoma cells. **A,** The relative level of MNX1-AS1 in U2OS and MG63 cells transfected with empty vector or MNX1-AS1 vector. **B,** CCK-8 assay revealed the viability in U2OS and MG63 cells transfected with empty vector or MNX1-AS1 vector. **C,** The colony formation assay revealed the clonality in U2OS and MG63 cells transfected with empty vector or MNX1-AS1 vector. **D,** The transwell assay revealed the migration and invasion in U2OS and MG63 cells transfected with empty vector or MNX1-AS1 vector (magnification: 40×).

in osteosarcoma, which is able to enhance proliferative and invasive abilities and suppresses apoptosis. The knockdown of UCA1 achieved the opposite trends. In this study, the knockdown of MNX1-AS1 attenuated invasive and migratory abilities of osteosarcoma cells, and the overexpression of MNX1-AS1 obtained the opposite results. It is verified that MNX1-AS1 accelerated the progression of the osteosarcoma.

The regulatory pattern of lncRNA is complex and can be mediated by specific signaling pathways. Methylation modification is one of the reasons that influence the relative gene expressions in osteosarcoma[26]. Here, it is demonstrated that MNX1-AS1 could mediate EMT-related gene expressions and decrease methylation ability by activating MNX1.

Conclusions

We found that MNX1-AS1 can activate MNX1 and promote EMT in osteosarcoma cells. We clarified the emerging role of MNX1-AS1 in the

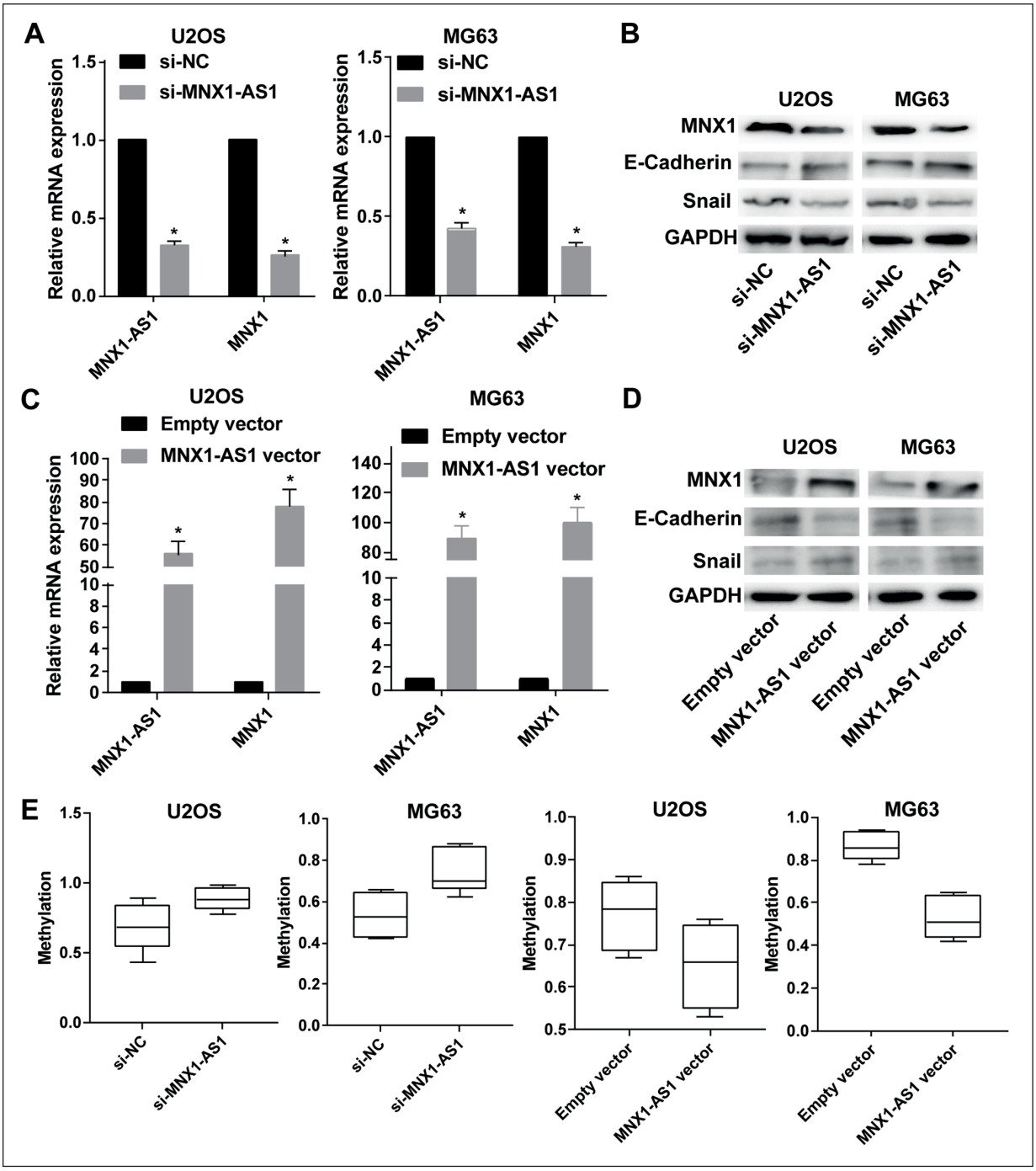

Figure 4. MNX1-AS1 mediated EMT in osteosarcoma by regulating MNX1 level. **A,** The relative levels of MNX1-AS1 and MNX1 in U2OS and MG63 cells transfected with si-NC or si-MNX1-AS1. **B,** Protein levels of MNX1, E-cadherin, and Snail in U2OS and MG63 cells transfected with si-NC or si-MNX1-AS1. **C,** Relative levels of MNX1-AS1 and MNX1 in U2OS and MG63 cells transfected with empty vector or MNX1-AS1 vector. **D,** The protein levels of MNX1, E-cadherin, and Snail in U2OS and MG63 cells transfected with the empty vector or MNX1-AS1 vector. **E,** The methylation in U2OS and MG63 cells transfected with si-MNX1-AS1 (si-NC) or MNX1-AS1 vector (empty vector).

progression of osteosarcoma, which provided a fundamental basis for developing effective therapeutic drugs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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