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Long non-coding RNA MNX1-AS1 promoted osteosarcoma proliferation and invasion *via* inhibiting KISS1

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Abstract. – OBJECTIVE: Recent studies revealed that long non-coding RNAs (lncRNAs) participate in the progression and development of many human diseases. In this work, we are committed to uncovering the association between lncRNA MNX1-AS1 and the development of osteosarcoma.

PATIENTS AND METHODS: MNX1-AS1 expression of osteosarcoma cells and tissue samples was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). Besides, we conducted functional assays including cell proliferation assay, colony formation and transwell assays. Furthermore, the negative correlation was found between the expression of KISS1 and MNX1-AS1 in osteosarcoma tissues.

RESULTS: MNX1-AS1 was higher expressed in osteosarcoma samples than in tissues. The abilities of proliferation and invasion were suppressed after MNX1-AS1 was knocked down *in vitro*. Moreover, KISS1 expression was upregulated at mRNA and protein level via silence of MNX1-AS1. Furthermore, the negative correlation was found between the expression levels of KISS1 and MNX1-AS1 in osteosarcoma tissues.

CONCLUSIONS: Our study demonstrated that MNX1-AS1 could enhance osteosarcoma cell proliferation and invasion by inhibiting KISS1, which may contribute to the therapy for osteosarcoma.

Keywords:
Long non-coding RNA, MNX1-AS1, Osteosarcoma

Introduction

Osteosarcoma is one of the most common primary malignant bone tumors and presents a highly malignant tendency to damage the surrounding tissues. Because of the poor prognosis and high rate of disability in children and adolescents, osteosarcoma remains a threat to the patients and brings a huge economic burden to society. About 4 million new cases are diagnosed with osteosarcoma annually in the world[2,3]. The main interventions include surgery, chemotherapy and radiotherapy. Despite the advances have been made in the development of therapeutic strategies, most of the patients finally developed recurrent metastasis after surgery, with a 5-year survival rate of 50-60%[4,5]. Therefore, it is urgent to realize the underlying molecular mechanism of osteosarcoma tumorigenesis and find out new therapeutic targets for the patients.

Moreover, long non-coding RNAs (lncRNAs) have the potential to regulate the gene expression which is frequently sequence homology-dependent and the particular mechanism of regulation can be associated with homology to different regions of the regulated gene. Recently, a number of regulatory promoters associated lncRNAs have been characterized. Conversely, transcription of some promoter lncRNAs induces an open chromatin formation that facilitates activator binding and transcription of the associated protein-coding gene. For example, lncRNA linc00261 functions as a tumor suppressor in gastric cancer by depressing the stability of Slug proteins and inhibiting epithelial-mesenchymal transition[6]. LncRNA NR_036575.1 acts as an oncogene in papillary thyroid cancer by contributing to enhancing cell proliferation and cell migration, which suggests lncRNA NR_036575.1 could be applied as a potential biomarker and therapeutic target[7]. In addition, lncRNA AF147447 represses cell prolif-

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eration and cell invasion in gastric cancer infected with *Helicobacter pylori* by targeting MUC2 through regulation of miR-34c expression[8]. However, a few studies have explored the function of MNX1-AS1 and biological mechanism in the progression of osteosarcoma.

Patients and Methods

Tissue Specimens

Totally, we collected 44 paired osteosarcoma samples - and corresponding non-tumor samples from patients who had undergone surgeries at Jining No. 1 People's Hospital. Before surgery, none of the patients had received radiotherapy or chemotherapy treatment. All tissue samples collected in the surgeries were snap-frozen in liquid nitrogen immediately and then stored under -80ºC until extracting the total RNA. Signed informed consents were obtained from all participants before the study. This study was approved by the Ethics Committee of Jining No. 1 People's Hospital which was performed in compliance with the Declaration of Helsinki Principles.

Cell Culture

Supplemented with 10% fetal bovine se and 2 mmol/L l-glutamine, human osteosar ma cell lines (Saos-2, MG-63 OSP-960 and osteoblastic cell line hF e main- tained in Dulbecco's Mod Eagle Medium (DMEM; Gibco, Grand d, NY humidified atmospher

Cell Transfecti

GenePharma us lentiv xpress- ing short-hai RN NA) against MNX1-AS1 (MN AS1/shRNA lentivirus against MNX1- (MNX1-AS1). X1-AS1/shRNA was into he pGPH1/Neo vector (Gene- Pha Shan China), which was then used for of Sao cells. MNX1-AS1 lentivirus oned the pGPH1/Neo vector Phar ha i, China), which was then us r tran of SOSP-9607 cells. Lipofect ne 2000 (Invitrogen, Carlsbad, CA, USA) w y out the transfection of cells.

NA Extraction and Real Quantitative Polymerase Ch Reaction (RT-qPCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from tissues and cells according to the manufacturer's instructions. The Reverse Transcription System Kit (TaKaRa, Otsu, Shiga, Japan) was utilized to generate first strand complementary d nucleic acid (cDNA). Quantitative Re e-Po merase Chain Reaction (RT-qPCR alyses utilized SYBR Green I (TaKaRa, Shiga, Japan) and were performed in triplic Primers were as follows: MNX1- primer ard 5′-GTGACTTCGCCTGT GGA-3′, 5′-GGCCTCTATCTG CTTT CC-3′; p tin primers forward ATG AATC TCA- GAGGCT-3' and rs CACT TG- GAAATGC-3' $2^{-\Delta\Delta C}$ od used to calculate the ive fold ch xpression. The therm s as follows sec at 95°C, 5 sec for cycles C, 35 sec at 60°C.

Ce ting Kit-8 (8) Assay

10^3 cells were plated in 96-well plates. Counting K 8 (CCK-8; KeyGEN biotech, ing, China) s used to evaluate cell pro- li on in acc nce with the manufacturer's inst bsorbance values were used to plot ce eration curves at a different time oint. All experiments were repeated three times.

Formation Assay

For the colony formation assay, specific numbers of transfected cells were placed into each well of a six-well plate. All the cells were cultured in medium for 15 days which contained 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and was replaced every 5 days. Methanol was used to fix the colonies and 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) in Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) was used to stain the colonies for 15 min. Colony formation was detected by counting the number of stained colonies. Wells were counted in triplicate for each treatment group.

Transwell Assay

4×10^4 cells in 200 µL of serum-free DMEM were added to top chamber of an insert (8 µm pore size; Corning, Corning, NY, USA) coated with 50 µg of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). DMEM and FBS were added to the lower chamber. 24 h later, the top surface of chambers was wiped by cotton swab and immersed for 10 min with precooling methanol. Then, they were stained in crystal violet for 30 min. Five fields were used to count the data.

Western Blot Analysis

Cells were lysed in cell lysis buffer containing Tris-HCl and Triton X-100 to extract total protein. An equal amount of denatured protein was decentralized on sodium dodecyl sulphate (SDS) polyacrylamide gel transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in Tris-Buffered Saline and Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA). Specific primary antibody was added to the membrane and incubated at 4°C overnight. Then, the samples were incubated with horseradish peroxidase-conjugated second antibody at room temperature for 30 min. Protein bands were visualized using enhanced chemiluminescence (ECL) reagents (Pierce, Waltham, MA, USA) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Statistical Analysis

In this work, Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA) was chosen for statistical analysis. Data analysis was conducted by two-tailed Student's *t*-test. It was considered of statistically significance [illegible] $p<0.05$.

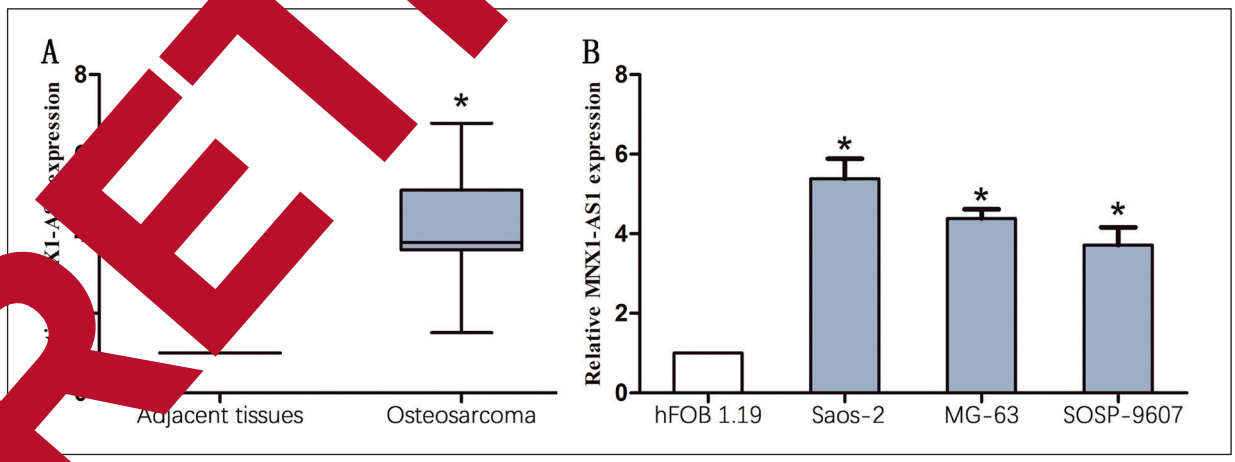
Results

The Expression Level of [illegible] in Osteosarcoma Tissues [illegible] Cells

First, RT-qPCR was [illegible]cted f[illegible] MNX1-AS1 expressio[illegible] in [illegible] tients' tissues and 3 osteosarcoma cell lines. As a result, MNX1-AS1 was significantly upregulated in osteosarcoma tumor tissues compared with adjacent non-tumor tissues (Fi[illegible] The MNX1-AS1 expression level o[illegible]osarc[illegible] ma cells was markedly higher tha[illegible]t of hFOB 1.19 (osteoblastic cell line), (Fig[illegible])

MNX1-AS1 Promoted [illegible]l Proliferati[illegible] in Osteosarcoma C[illegible]

In our work, we [illegible]e S[illegible]2 oste[illegible]arcoma cell lines fo[illegible]he [illegible]own o[illegible]NX1-AS1. Then, RT[illegible]CR was [illegible]ed f[illegible]tecting transfection [illegible]ency (Figu[illegible] Moreover, CCK-8 as[illegible]led that af[illegible] MNX1-AS1 was kno[illegible]d do[illegible]e cell viability of osteosarcoma cells wa[illegible]arkably decreased in Sa[illegible]osarcoma ce[illegible]igure 2B). Colony f[illegible]ation assay revealed that, after MNX1-AS1 [illegible] knocked de[illegible] the number of colonies was [illegible]ced in Sao[illegible]osteosarcoma cells (Figure 2[illegible]Ve then [illegible]e SOSP-9607 osteosarcoma cell[illegible] overexpression of MNX1-AS1. RT-qP[illegible] utilized for detecting transfection efficiency (Figure 2D). Moreover, CCK-8 [illegible]vealed that after MNX1-AS1 was over[illegible]d, the cell viability of osteosarcoma cells was significantly increased in SOSP-9607 osteosarcoma cells (Figure 2E). Colony formation assay revealed that after MNX1-AS1 was overexpressed, the number of colonies was increased in SOSP-9607 osteosarcoma cells (Figure 2F).



Fig[illegible]1. Expression levels of MNX1-AS1 were increased in osteosarcoma tissues and cell lines. ***A,*** MNX1-AS1 expression was [illegible]nificantly increased in the osteosarcoma tissues compared with adjacent tissues. ***B,*** Expression levels of MNX1-AS1 were detected in the human osteosarcoma cell lines and hFOB 1.19 (osteoblastic cell line) by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

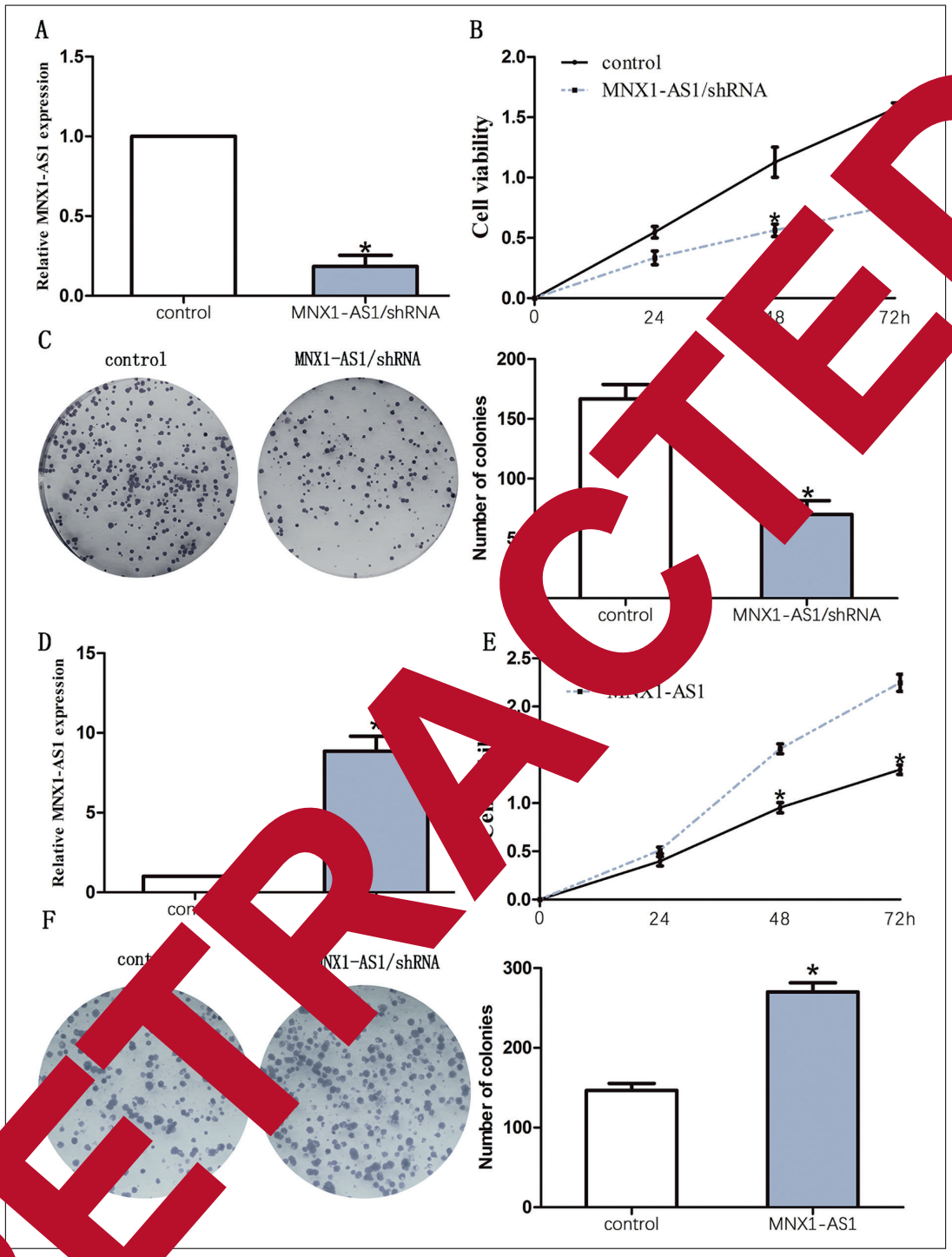


Fig. 2. MNX1-AS1 promoted osteosarcoma cell proliferation. ***A,*** MNX1-AS1 expression in Saos-2 osteosarcoma cells transduced with MNX1-AS1 shRNA (MNX1-AS1/shRNA) and the control vector was detected by RT-qPCR. β-actin was used as an internal control. ***B,*** Cell proliferation assay showed that cell viability was significantly inhibited in Saos-2 osteosarcoma cells after knockdown of MNX1-AS1. ***C,*** Colony formation assay showed that number of colonies was markedly reduced after knockdown of MNX1-AS1 in Saos-2 osteosarcoma cells (magnification: 10×). ***D,*** MNX1-AS1 expression in SOSP-9607 osteosarcoma cells transduced with MNX1-AS1 lentivirus (MNX1-AS1) and the control vector was detected by RT-qPCR. β-actin was used as an internal control. ***E,*** Cell proliferation assay showed that cell viability was remarkably promoted in SOSP-9607 osteosarcoma cells after overexpression of MNX1-AS1. ***F,*** Colony formation assay showed that number of colonies was increased *via* overexpression of MNX1-AS1 in SOSP-9607 osteosarcoma cells (magnification: 10×). The results represent the average of three independent experiments (mean ± standard error of the mean). **p*<0.05.

MNX1-AS1 Promoted Cell Invasion in Osteosarcoma Cells

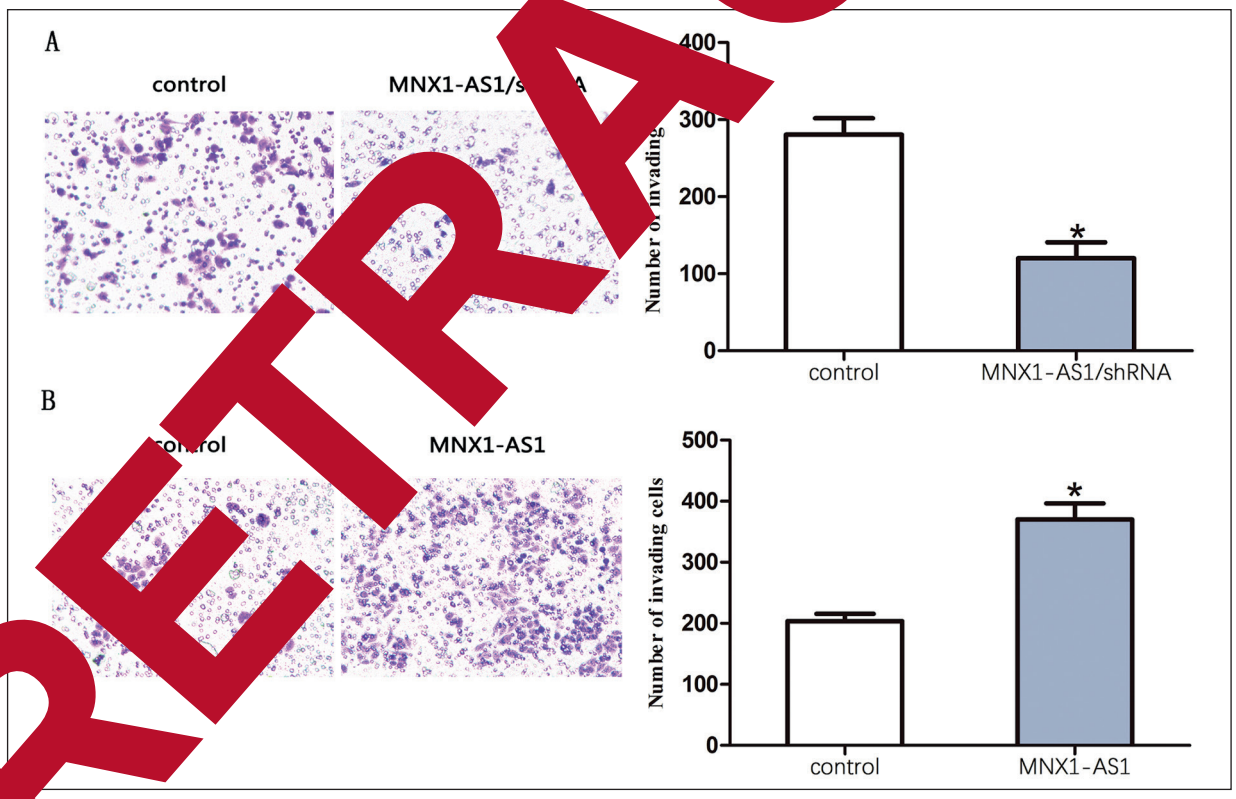
To identify the function of MNX1-AS1 in the metastasis of osteosarcoma *in vitro*, we performed transwell assay and found that after MNX1-AS1 was knocked down in Saos-2 osteosarcoma cells, the number of invaded cells was markedly reduced (Figure 3A). After MNX1-AS1 was overexpressed in SOSP-9607 osteosarcoma cells, the number of invaded cells was significantly increased (Figure 3B).

The Interaction Between KISS1 and MNX1-AS1 in Osteosarcoma

RT-qPCR results showed that the expression level of KISS1 in osteosarcoma cells was higher in MNX1-AS1/shRNA group compared with the KISS1 level in the control group (Figure 4A). The expression level of KISS1 in osteosarcoma cells was lower in MNX1-AS1 lentivirus group compared with the KISS1 level in the control group (Figure 4B). Western blot assay also showed that after MNX1-AS1 was knocked down, KISS1 could be upregulated (Figure 4C). Western blot assay also showed that after MNX1-AS1 was over-expressed, KISS1 could be downregulat (Figure 4D). We further detected KISS1 e ssion human tissues and found that it remarkably lower-expressed in osteosarcoma t compared with that in adjacent non-tumor ti (Figure 4E). Besides, the KISS1 ex ion leve eg atively correlated to M -AS1 expres osteosarcoma tissues (e 4F).

Discuss

Osteosa he most co n malignant bone tum affect adults with the median age is 20 years in a s[1]. The survival rate is patients wi tastasis than those w nonmetastatic disease. Developing effec- and targete herapies for osteosarcoma is ndent on ga g an improved understand-



e 3. MNX1-AS1 promoted osteosarcoma cell invasion. ***A,*** The transwell assay showed that the number of invading c remarkably reduced *via* downregulation of MNX1-AS1 in Saos-2 osteosarcoma cells (magnification: 40×). ***B,*** The trans ell assay showed that number of invading cells was remarkably increased *via* upregulation of MNX1-AS1 in SOSP-9607 osteosarcoma cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). **p*<0.05.

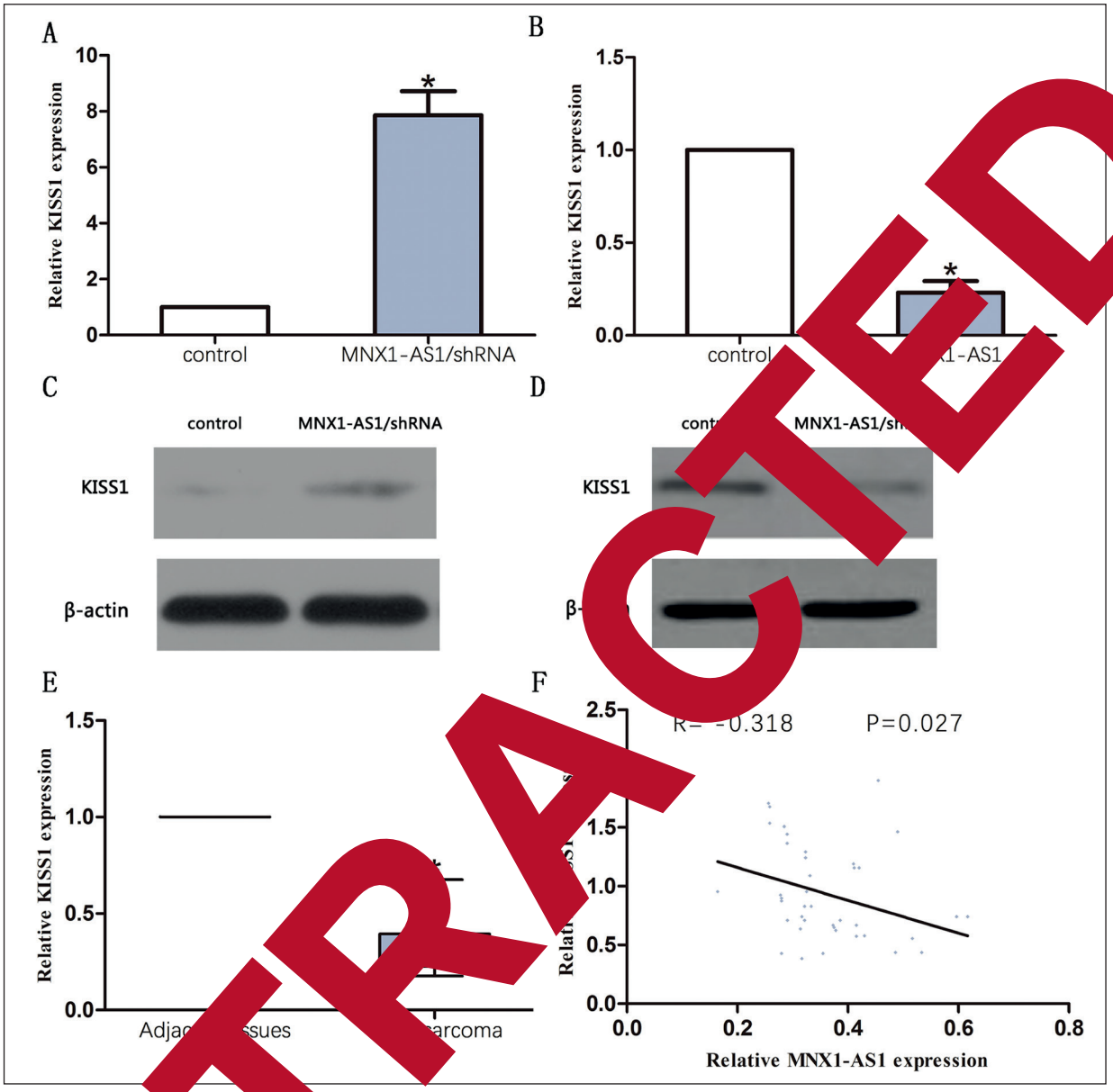


Figure 4. interaction be KISS1 and MNX1-AS1 in osteosarcoma. ***A,*** RT-qPCR results showed that KISS1 expressio higher in MNX1-A RNA group compared with the control vector (control). ***B,*** RT-qPCR results showed that K expressi was lower in NX1-AS1 lentivirus (MNX1-AS1) group compared with the control vector (control). ***C,*** W n blot a evealed that KISS1 protein expression was increased in MNX1-AS1/shRNA group compared with the cont or ol). ***D,*** W tern blot assay revealed that KISS1 protein expression was decreased in MNX1-AS1 lentivirus (MNX up comp with the control vector (control). ***E,*** KISS1 was significantly downregulated in osteosarcoma s co with ent tissues. ***F,*** The expression level of KISS1 was negatively associated with MNX1-AS1 in coma results represent the average of three independent experiments. Data are presented as the mean ± sta error o n. *$p<0.05$.

of the molecular mechanisms underlying os- sarcoma-genesis, proliferation, invasion and tasis.

ent studies indicate that a substantial portion of transcribed sequences may be non-protein-coding, which represents a higher percentage of transcribed sequences than protein-coding transcripts. LncRNAs are a multifarious class of transcripts longer than 200 base pairs in length. LncRNAs have been implicated in a variety of regulatory roles, including the regulation of potential activity and splicing event *via* small RNA

regulatory pathways. Although many therapy methods were available for osteosarcoma in the past few decades, the prognosis of osteosarcoma patients remains poor. Plenty of lncRNAs have been revealed to play important roles in oncogenesis and progression of osteosarcoma. For example, lncRNA SNHG1 enhances tumorigenesis in osteosarcoma through regulation of NOB1 expression level by sponging miR-326[9]. LncRNA PVT1 promotes glucose metabolism, cell motility, cell proliferation and tumor progression in osteosarcoma by modulation of miR-497/HK2 axis[10]. In addition, lncRNA MEG3 inhibits cell proliferation and cell metastasis in osteosarcoma by depressing Notch and TGF-beta signaling pathway[11].

Recently, several reports have revealed that MNX1-AS1 is closely related to diverse cancers. MNX1-AS1 plays a vital role in tumor progression, including cell proliferation, migration and invasion. The knockdown of MNX1-AS1 suppresses the proliferation and migration of ovarian cancer cells[12]. By activating the MAPK pathway, MNX1-AS1 functions as an oncogene *via* promoting the progression of cervical cancer[13]. The overexpression of MNX1-AS1 facilitates cell proliferation and inhibits cell apoptosis in lung adenocarcinoma which may be a novel biomarker for predicting malignant progression and poor prognosis[14]. Through regulation of miR-218-5p/SEC61A1 axis, MNX1-AS1 influences the progression of colon adenocarcinoma. In this work, MNX1-AS1 was first identified as a novel oncogene in osteosarcoma. Our study showed that MNX1-AS1 was significantly upregulated in osteosarcoma tissues. Furthermore, after MNX1-AS1 was knocked down, osteosarcoma cell proliferation and invasion were markedly inhibited. Meanwhile, after MNX1-AS1 was overexpressed in osteosarcoma cells, cell proliferation and invasion were significantly promoted. In this work, MNX1-AS1 was first identified as a novel oncogene in osteosarcoma.

KISS1 is a member of the still-expanding family of metastasis suppressors, which are defined by their ability to block metastasis without preventing primary tumor development[16,17]. KISS1 is a tumor suppressor, defined by the ability to suppress metastasis without blocking primary tumor growth. Nascent KISS1 is a 145-amino acid secreted a protein that is cleaved by the proprotein convertase furin into polypeptides, termed kisspeptins[18]. KISS1 encodes a 145-amino acid protein that is processed into KISSpeptins (KP), including KP10, KP13, KP14 and KP54. Recent studies have reported that KISS1 exhibits anti-metastatic and anti-tumoral roles in a variety of cancers including osteosarcoma. For example, KISS1 expression level is reduced during the malignant transformation of the gastric mucosa and upregulation of KISS1 expression is associated with better prognosis in colorectal cancer[19]. Low-expression of KISS1 promotes cell proliferation and inhibits cell apoptosis in renal cell carcinoma. KISS1 functions as a tumor suppressor and inhibits lung cancer brain metastases, which helps to sensitize oncolytic virotherapy[21]. Our further studies revealed that KISS1 expression was upregulated *via* knockdown of MNX1-AS1 in osteosarcoma cells and KISS1 expression was downregulated *via* overexpression of MNX1-AS1 in osteosarcoma cells. We also showed the negative correlation between KISS1 expression and MNX1-AS1 expression in osteosarcoma tissues.

Conclusions

We found that MNX1-AS1 was remarkably elevated in osteosarcoma patients and could promote osteosarcoma cell proliferation and invasion by suppressing KISS1, which provided a novel therapeutic target for osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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