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Original Article

LncRNA GAS5-AS1 inhibits osteosarcoma cell growth and invasion by regulating MMP2

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Abstract: Human osteosarcoma is usually highly metastatic and leads to poor outcomes. However, the underlying mechanism is still largely unknown. In the present study, we investigated the role of lncRNA GAS5-AS1 (GAS5-AS1) on the proliferation, migration and invasion of osteosarcoma *in vitro* and *in vivo*. Our results indicated that GAS5-AS1 was commonly lowly expressed in osteosarcoma, particularly in patients with metastasis. Furthermore, we demonstrated that knockdown of GAS5-AS1 could notably promote cellular proliferation, invasion and increase the secretion of MMP2 in osteosarcoma cells. *In vivo* nude mouse assays were used to investigate the oncogenic role of GAS5-AS1. Collectively, our results suggest that the ectopic expression of GAS5-AS1 down-regulates the expression level of MMP2. Taken together, GAS5-AS1 might be a potent therapeutic target for osteosarcoma.

Keywords: LncRNA, GAS5-AS1, osteosarcoma, MMP2

Introduction

Osteosarcoma is the most common primary malignancy that originates from bone tissue [1] in the 15- to 19-year-old age group (0.8-11/100000 per year) [2]. Osteosarcoma has a poor prognosis due to a high tendency of metastatic spread. More than 20% of osteosarcoma patients had lung metastases at the initial diagnosis, and less than 30% of patients survive for 5 years when lung metastasis is present [3, 4]. Thus, exploring the detailed mechanisms and identifying novel agents to improve the treatment and prognosis of metastatic osteosarcoma is urgently required.

Long non-coding RNAs (lncRNAs) are a group of eukaryote RNAs longer than 200 nucleotides in length that do not encode proteins [5, 6]. Their function has not been well characterized compared to microRNA (miRNA). Recently, lncRNAs have been demonstrated to act as oncogenes or tumor suppressors in various cancers and are closely correlated with human malignancies [7-9]. LncRNAs play crucial roles in the progression and metastasis of osteosarcoma [10-17]. Thus, studying the roles of tumor-associated lncRNAs benefits our understanding of the molecular mechanism of osteosarcoma initiation and progression. Nonetheless, GAS5-AS1's expression and its biological role in osteosarcoma development and progression remain unknown.

In this study, we aimed to investigate the biological functions of GAS5-AS1 in osteosarcoma and its underlying mechanisms. Here, we discovered that the expression of GAS5-AS1 in osteosarcoma samples was lower than that in adjacent normal tissues, which was negatively correlated with tumor size. The lower expression of GAS5-AS1 is especially significant in patients with metastasis. Previous reports have shown that matrix metalloproteinases (MMPs) are correlated with various tumor metastases [18-20], including osteosarcoma [21]. Thus, we focused on the effects of GAS5-AS1 on the migration and invasion of osteosarcoma cells and its possible correlation with MMP2. According to our results, we found that lncRNA GAS5-AS1 inhibits osteosarcoma cell growth and invasion by regulating MMP2.

Materials and methods

Clinical specimens

Twenty-five patients with osteosarcoma were recruited from the Second Xiangya Hospital, which approved the study. Informed consent was signed by the participants.

Cell culture

The human normal osteoblast cell line NHOst and human osteosarcoma cell lines (HOS, Saos2, MG63 and U2-OS) were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and maintained in a humidified incubator at 37°C with a 5% CO_2 atmosphere.

RNA extraction and quantitative real-time polymerase chain reaction

For qRT-PCR analysis, total RNA was extracted using the Trizol reagent (Takara, Dalian, China) from cells and tumor samples, and reverse transcribed to cDNA using a miScript II RT Kit (Qiagen, Hilden, Germany). All of the steps were performed according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the standard SYBR-Green PCR kit on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA) with primers specific for the target genes and controls (Qiagen, Hilden, Germany). All data are shown as the mean ± SD of three independent experiments. The following forward and reverse primers were used, respectively: GAS5-AS1: 5'-TCC CAG CCT CAG ACT CAA CA-3' and 5'- GTT TCA TAG GCC CCT GTG CT-3' [22], GAPDH: 5'-CCA GCC GAG CCA CATCGC TC-3' and 5'-ATG AGC CCC AGC CTT CTC CAT-3'.

Cell proliferation assay

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) following the manufacturer's instructions. Briefly, a total of 3.0×10^3 treated cells were seeded in each well of 96-well plates with complete medium for 12, 24, 48, and 72 hrs. The absorbance was observed at 570 nm (Bio-Tek Instruments, Winooski, VT, USA) at each time point to plot the cell proliferation curves. All of the experiments were performed in triplicate.

Cell invasion assay

A cell invasion assay was performed using transwell chambers. MG63 or U2OS cells were seeded in the upper compartment with serum-free medium. The lower compartment was filled with medium supplemented with 10% fetal bovine serum. After 48 hrs, the cells that migrated to the bottom surface of the filters were fixed with methanol and stained with crystal violet. Cell numbers were counted under the microscope in five representative fields.

Colony formation assay

For the colony formation assay, the treated cells were seeded into 6-well plates at the same densities. After 7 days, the cells were stained with crystal violet. The total number of colonies in each plate was counted to evaluate the formation ability.

Western blotting

A western blot assay was used to determine the protein expression level as previously described [23-25]. In brief, the treated osteosarcoma cells were homogenized in RIPA buffer (Beyotime, Shanghai, China) containing a protease inhibitor cocktail (Roche Diagnostics). The protein concentrations were determined using a BCA Protein Assay kit (Thermo Fisher Scientific., Rockford, IL, USA). Equal amounts of total protein were separated by 10% SDS-PAGE gels and transferred onto a PVDF membrane (PerkinElmer, Boston, MA) and then incubated with a primary antibody overnight at 4°C. The blots were subsequently incubated with HRP-conjugated secondary antibodies for 1 h at room temperature and then detected using an ECL detection kit (Millipore Corp. Bedford, MA). The primary antibodies for MMP2 (CST, 4022) and GAPDH (CST, #5174) were from Cell Signaling Technology (Bioke, The Netherlands).

Transfection

For the transfection treatment, 2×10^5 MG-63, U2OS or HOS cells were seeded into 6-well plates with antibiotic-free complete medium. For GAS5-AS1 overexpression, the pCDNA-GAS5-AS1 or empty vector (GenePharma Co., Ltd., Shanghai, China) was transfected into MG-63 or U2OS cells. For knockdown of GAS5-AS1, HOS cells were transfected with GAS5-AS1 siRNA recombinant plasmid or control siRNAs. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidance.

Tumor formation assay in nude mice

Five-week-old male nude mice were divided into two groups for subcutaneous injection of 2×10^6 U2OS cells with or without the GAS5-AS1 overexpression vector. The formula tumor vol-

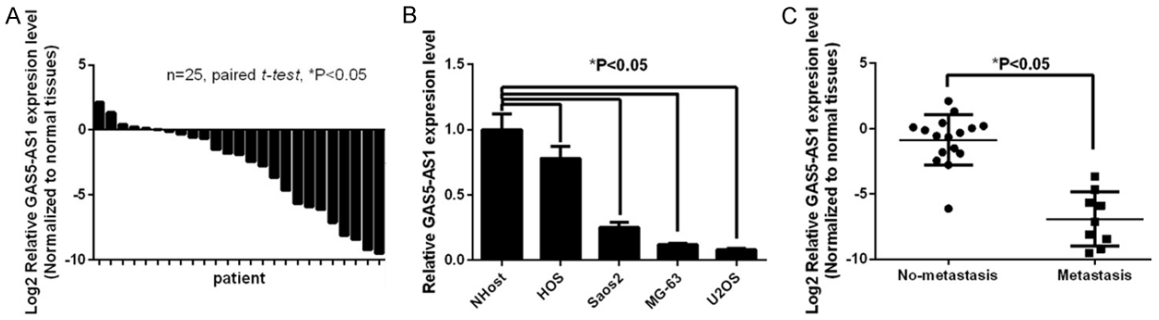

Figure 1. LncRNA GAS5-AS1 was down-regulated in osteosarcoma. A. The mRNA expression level of GAS5-AS1 was detected by qRT-PCR in 25 paired osteosarcoma and matched normal tissues. The Y-axis displays a ratio of GAS5-AS1 expression in osteosarcoma normalized to the matched normal tissues. Each bar is the log2 value of the ratio of GAS5-AS1 expression between osteosarcoma and matched normal tissues from the same patient. Bar value > 1 represents > 2-fold increases, and bar value < -1, represents > 2-fold decreases (*P < 0.05). B. The mRNA expression level of GAS5-AS1 was significantly lower in four osteosarcoma cell lines (HOS, Saos2, MG-63 and U2OS) compared with the normal human osteoblast (NHOst) cells (*P < 0.05). C. The mRNA expression level of GAS5-AS1 was significantly decreased in metastatic patients compared with the non-metastatic patients (*P < 0.05).

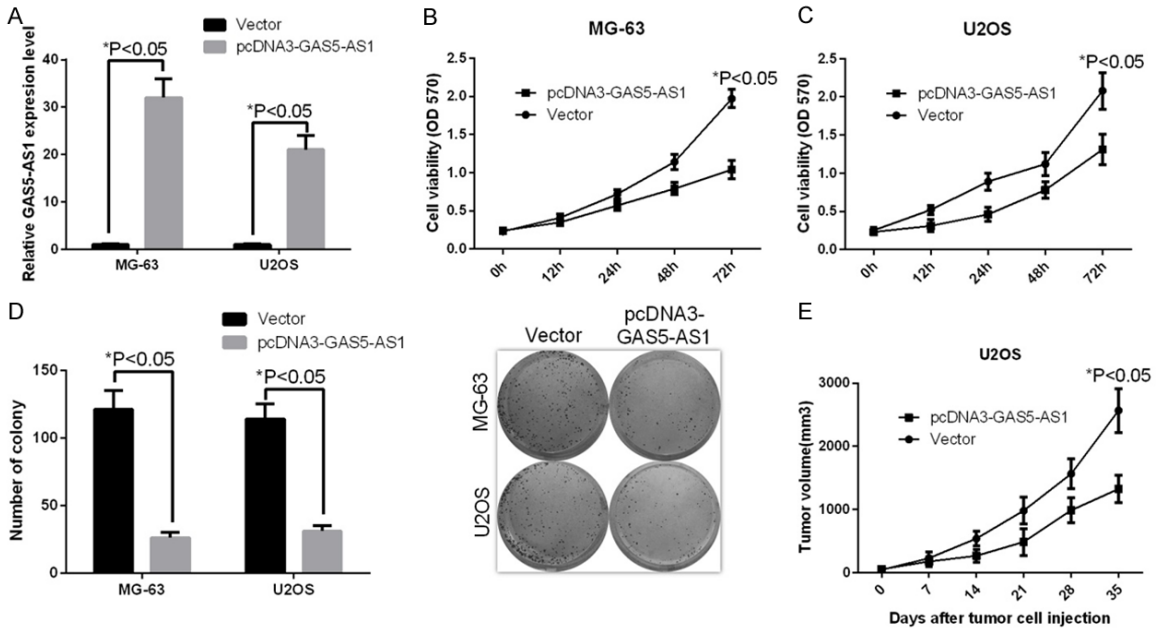

Figure 2. GAS5-AS1 inhibited the proliferation ability of osteosarcoma cells. MG-63 and U2OS cells were transfected with a control vector or pcDNA3-GAS5-AS1. A. The relative expression level of GAS5-AS1 was increased in MG-63 and U2OS cells transfected with pcDNA3-GAS5-AS1 compared with the control group (*P < 0.05). B. MTT assays were performed to detect the effects of GAS5-AS1 on the cell viability of MG-63 (*P < 0.05). C. MTT assays were performed to detect the effects of GAS5-AS1 on the cell viability of U2OS (*P < 0.05). D. Colony formation assays were used to measure the long term proliferation capacities of MG-63 and U2OS cells transfected with the control vector or pcDNA3-GAS5-AS1 (*P < 0.05). E. The dissected tumor volumes were measured at different days after injection (*P < 0.05).

ume = (length × width^2)/2 was used to calculate the tumor volume once a week. The animals were sacrificed, and the tumors were collected for further analysis at day 35. All animal experiments were performed in accordance with the institutional guidelines.

Statistical analysis

Statistical analysis was performed using the SPSS version 22 software (SPSS, Chicago, IL, USA). Student's t-test or one-way ANOVA were used to analyze the data. All results were sum-

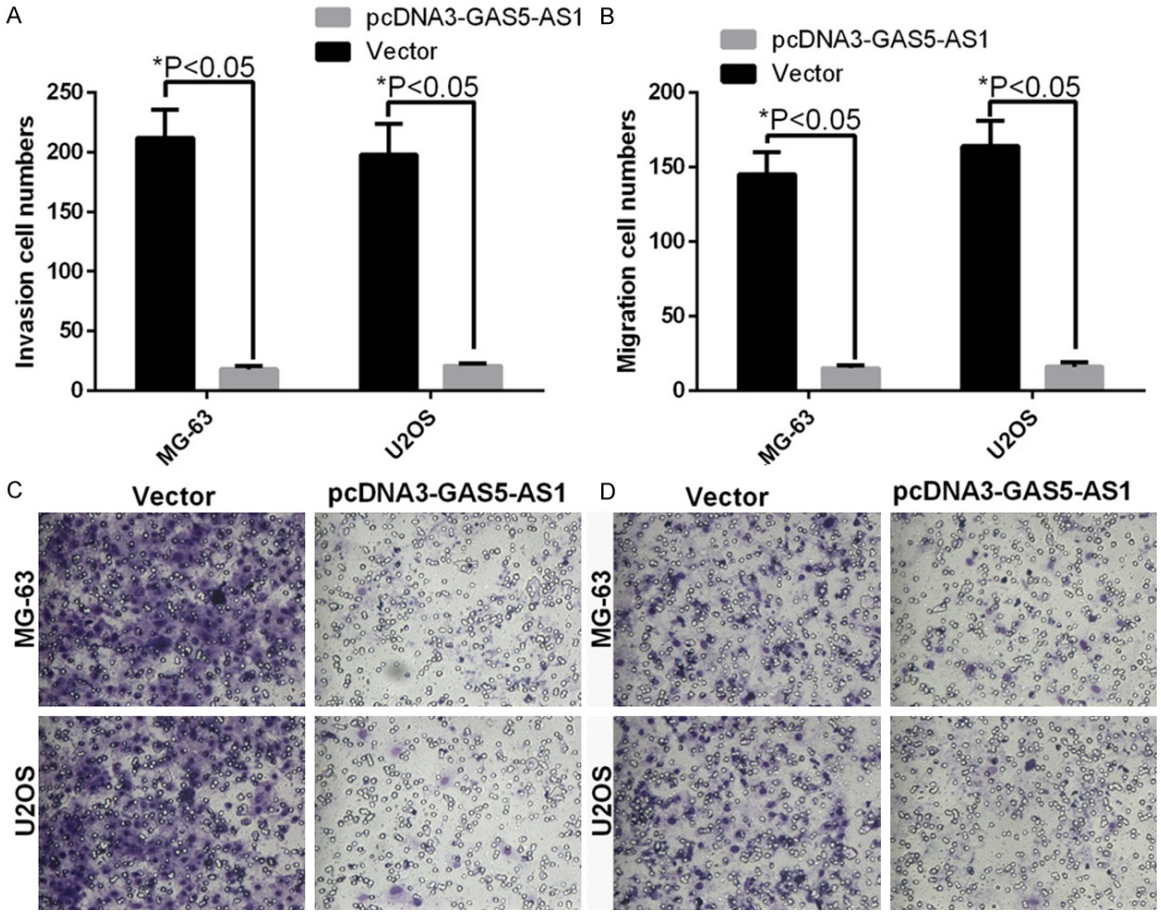

Figure 3. GAS5-AS1 suppressed the migration and invasion abilities of osteosarcoma cells. A. The effect of GAS5-AS1 overexpression on the invasion abilities of MG-63 or U2OS cells *in vitro*. The invasion levels were expressed as number of invading cells. Error bars represent standard deviation of triplicate experiments (*$P < 0.05$). B. The migration abilities of MG-63 and U2OS cells transfected with vector or pcDNA3-GAS5-AS1 *in vitro*. The migration abilities were expressed as number of migratory cells. Error bars represent standard deviation of triplicate experiments (*$P < 0.05$). C. The invasive cells were shown in MG-63 and U2OS cells transfected with a control vector or pcDNA3-GAS5-AS1 (magnification, × 100). D. The migratory cells were indicated in MG-63 and U2OS cells transfected with the control vector or pcDNA3-GAS5-AS1 (magnification, × 100).

marized and presented as the means ± SD. $P < 0.05$ was considered statistically significant.

Results

GAS5-AS1 expression was down-regulated in osteosarcoma tissues and osteosarcoma cells

We randomly selected 25 pairs of osteosarcoma tissues and adjacent normal tissues and detected the mRNA expression level of LGAS5-AS1 using a qRT-PCR assay. We found that GAS5-AS1 was significantly decreased in osteosarcoma tissues normalized to the relatively normal areas. Analysis of the mRNA expression level of 25 samples showed a significant (> 2-fold decreased) downregulation of GAS5-AS1 in 60% (15/25) of patients, whereas 8% (2/25) of patients showed a significant (> 2-fold increased) upregulation of GAS5-AS1 (**Figure 1A**). The mRNA expression level of GAS5-AS1 was significantly lower in four osteosarcoma cell lines (HOS, Saos2, MG-63 and U2OS) compared with NHOst cells. (*$P < 0.05$*) (**Figure 1B**), and low expression of GAS5-AS1 was observed in metastatic patients compared with the non-metastatic patients (*$P < 0.05$*) (**Figure 1C**).

GAS5-AS1 acted as a cancer suppressor gene in osteosarcoma

The transfection efficiency was determined by qRT-PCR. The result showed that the relative GAS5-AS1 expression level was significantly

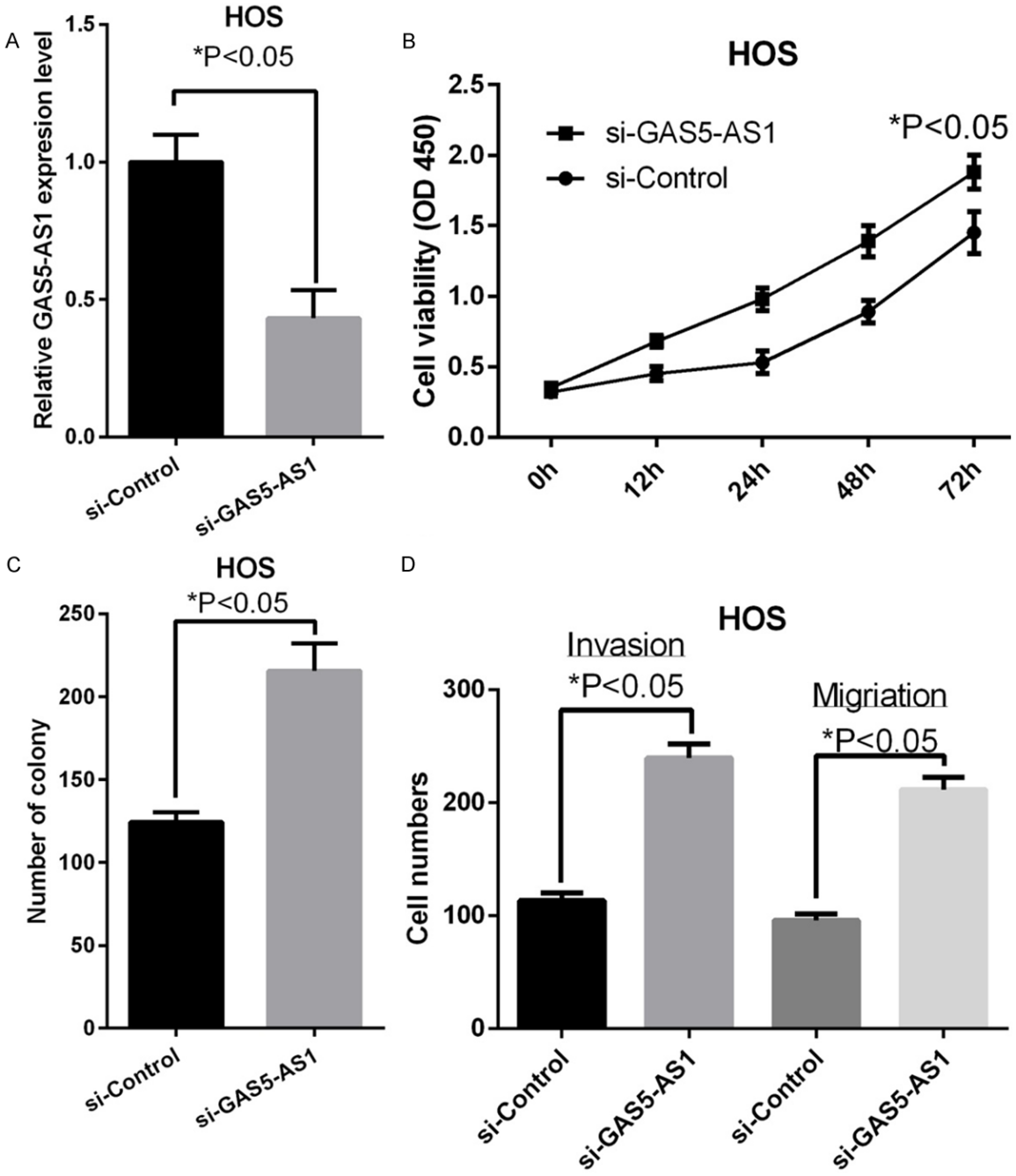

Figure 4. Silencing GAS5-AS1 by siRNAs promoted the proliferation, colony formation, migration and invasion abilities of osteosarcoma cells. A. The mRNA expression level of GAS5-AS1 was assessed by qRT-PCR in HOS cells transfected with GAS5-AS1 siRNAs or control siRNAs. As expected, the mRNA expression level of GAS5-AS1 was inhibited by GAS5-AS1 siRNAs. *P < 0.05 compared with the control group. B. A cell viability assay was performed in HOS cells transfected with equal amounts of GAS5-AS1 siRNAs and control siRNAs. The cell viability was measured at 0, 12, 24, 48 and 72 hrs after transfection (*P < 0.05). C. A colony formation assay was used to detect the proliferation capabilities of HOS cells in soft agar. Silencing GAS5-AS1 by siRNAs significantly increased the proliferation capabilities of HOS cells (*P < 0.05). D. The invasion and migration cell numbers were detected by transwell assays in HOS cells transfected with equal amounts of GAS5-AS1 siRNAs and control siRNAs (*P < 0.05).

higher in MG-63 and U2OS cells transfected with pcDNA3-GAS5-AS1 compared with the control group ($P < 0.05$) (**Figure 2A**). A CCK-8 assay and colony formation assay demonstrat-

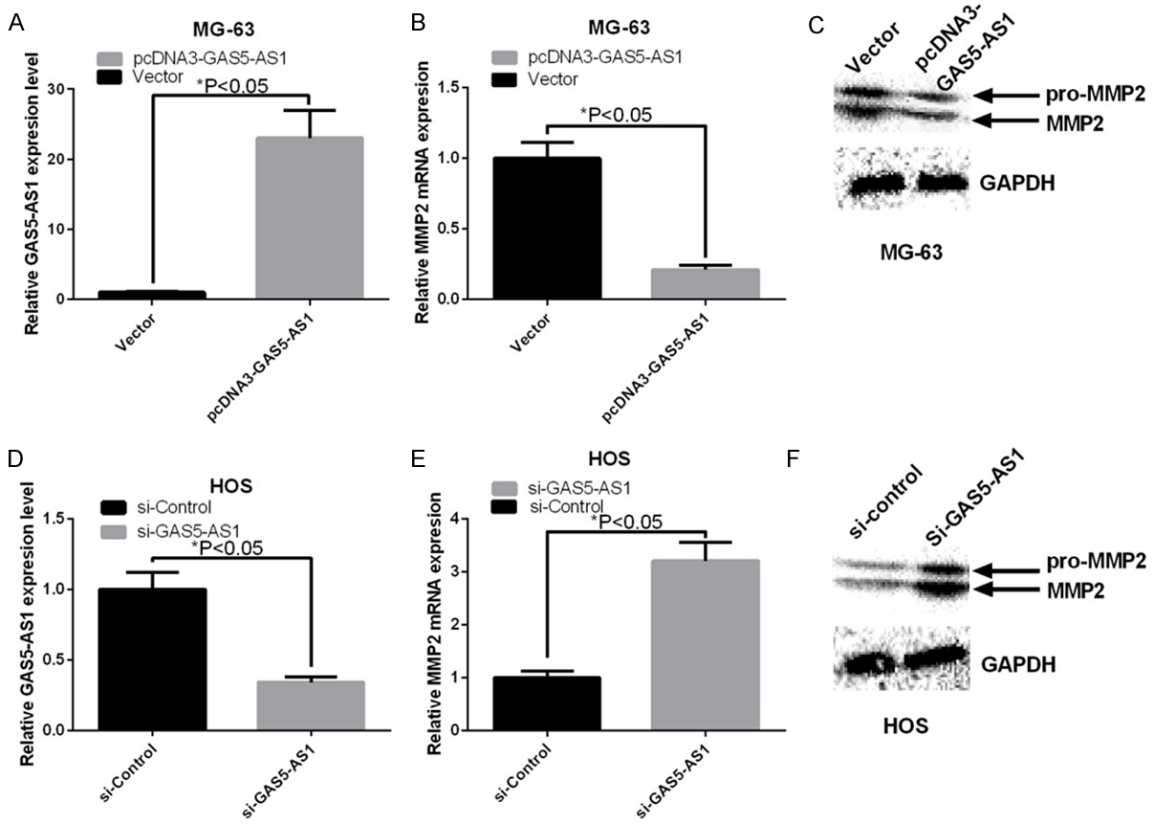

Figure 5. MMP2 is a target gene of GAS5-AS1 in osteosarcoma cells. MG-63 cells were transfected with pcDNA3-GAS5-AS1 or a control vector, qRT-PCR was used to detect the mRNA expression levels of GAS5-AS1 (A) and MMP2 (B), and western blotting was used to detect the protein level of MMP2 (C). GAPDH was used as a control. Then, HOS cells were transfected with GAS5-AS1 siRNAs and a negative control, qRT-PCR was performed to measure the mRNA expression levels of GAS5-AS1 (D) and MMP2 (E), and western blotting was used to detect the protein levels of MMP2 (F). GAPDH was used as a control. All of the error bars indicate the means ± SDs. Experiments were performed in triplicate. *$P < 0.05$ compared with the control group.

ed that GAS5-AS1 significantly decreased the cell viability and colony formation of MG-63 and U2OS cells compared with the control group ($P < 0.05$) (**Figure 2B-D**). To further assess the cell growth effect of GAS5-AS1, we examined the tumorigenesis of the treated osteosarcoma cells in nude mice. The result indicated that the overexpression of GAS5-AS1 in U2OS cells could decrease the tumor growth rate in the nude mouse model (**Figure 2E**). Finally, we suggest that GAS5-AS1 acted as a cancer suppressor gene in osteosarcoma.

GAS5-AS1 suppressed the migration and invasion of MG-63 and U2OS cells

To measure the effect of GAS5-AS1 on tumor cell migration and invasion, we performed a transwell apparatus assay (**Figure 3**). We found that GAS5-AS1 significantly inhibited the migratory and invasive abilities of MG-63 and U2OS cells. These results indicated that GAS5-AS1 reduced the migration and invasion abilities of osteosarcoma cells.

Silencing GAS5-AS1 by siRNAs promoted the proliferation, migration and invasion abilities of osteosarcoma cells

We further analyzed if GAS5-AS1 affected the proliferation, colony formation, migration and invasion abilities of osteosarcoma cells. Based on the lower expression level of GAS5-AS1 in HOS cells (**Figure 1B**), we chose the HOS cells for knockdown study. HOS cells were transfected with GAS5-AS1 siRNAs and control siRNAs, and we found that the mRNA expression level of GAS5-AS1 was decreased in HOS cells transfected with GAS5-AS1 siRNAs compared with the control group ($P < 0.05$) (**Figure 4A**). The

silencing of GAS5-AS1 by siRNAs accelerated the proliferation ($P < 0.05$) (**Figure 4B**), colony formation ($P < 0.05$) (**Figure 4C**), invasion ($P < 0.05$) and migration ($P < 0.05$) (**Figure 4D**) abilities of HOS cells. In this study, we confirmed that silencing GAS5-AS1 by siRNAs promoted osteosarcoma cell proliferation, colony formation, migration and invasion.

GAS5-AS1 inhibited cell migration and invasion by regulating the expression of MMP2 in osteosarcoma

MMP2 has been reported as a downstream effector in tumorigenesis. Thus, we assessed whether GAS5-AS1 changed the osteosarcoma cell phenotype by regulating MMP2. **Figure 5A** shows that there was a negative correlation between the expression levels of GAS5-AS1 and MMP2. Elevated expression of GAS5-AS1 in MG-63 cells clearly decreased the mRNA level of MMP2 (**Figure 5B**). Similarly, overexpression of GAS5-AS1 in MG-63 cells clearly decreased the protein level of MMP2 (**Figure 5C**). Conversely, silencing GAS5-AS1 by siRNAs significantly increased the mRNA expression level of MMP2 in HOS cells (**Figure 5E**) and significantly increased the protein expression level of MMP2 in HOS cells (**Figure 5F**).

Discussion

Osteosarcoma (OS) is a highly malignant tumor, and metastasis is the major cause of death in patients with osteosarcoma [26, 27]. Thus, developing comprehensive treatments targeting osteosarcoma metastasis may be a promising strategy. However, the molecular mechanisms underlying osteosarcoma invasion and metastasis remain unclear, and there are currently no effective therapies.

Numerous studies have shown that lncRNAs have important roles in tumorigenesis and contribute to diverse biological functions in various tumors [9, 28]. For example, Defeng K et al suggested that decreased expression of long noncoding RNA TUG1 inhibited ovarian cancer cell proliferation and metastasis [29]. She K et al revealed that lncRNA-SNHG7 promoted the proliferation, migration and invasion and inhibited apoptosis of lung cancer cells by enhancing FAIM2 expression [30]. Zhang et al suggested that upregulation of lncRNA MALAT1 correlated with tumor progression and poor prognosis in clear cell renal cell carcinoma [31]. Yan et al suggested that upregulation of the lncRNA HOTAIR predicted recurrence in stage Ta/T1 bladder cancer [32]. Ying Wu et al discovered that GAS5-AS1 was downregulated in NSCLC tumors and was significantly associated with larger tumors (> 3 cm), higher TNM stage, and lymph node metastasis [22]. In this study, we investigated the expression level of GAS5-AS1 in osteosarcoma tissues and discovered that GAS5-AS1 was downregulated in osteosarcoma tumors compared to the adjacent normal tissues. In addition, the expression of GAS5-AS1 was significantly lower in four osteosarcoma cell lines (HOS, Saos2, MG-63 and U2OS) compared with NHOst cells. We also indicated that GAS5-AS1 may function as a tumor suppressor in the modulation of osteosarcoma progression. Conversely, knockdown of GAS5-AS1 (GAS5-AS1 siRNAs), which lowered the expression levels of GAS5-AS1, significantly increased the proliferation, colony formation, and invasion and migration abilities of HOS cells. Furthermore, we also demonstrated that the overexpression of GAS5-AS1 in MG-63 and U2OS cells could decrease the tumor growth rate in a nude mouse model. Then, we explored the underlying molecular basis responsible for the inhibition of migration and invasion mediated by GAS5-AS1 in osteosarcoma, and we found that MMP2 served as a potential target of GAS5-AS1. Elevated expression of GAS5-AS1 in MG-63 cells clearly decreased the mRNA and protein levels of MMP2. Conversely, silencing GAS5-AS1 by siRNAs significantly increased the expression level of MMP2.

In summary, our results demonstrated that ectopic expression of GAS5-AS1 inhibited the proliferation, migration and invasion abilities of osteosarcoma cells through regulating the expression of MMP2. This result provides preclinical support for the therapeutic potential of GAS5-AS1 in osteosarcoma.

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Disclosure of conflict of interest

None.

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