

Knockdown of MSI1 inhibited the cell proliferation of human osteosarcoma cells by targeting p21 and p27

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Abstract. Osteosarcoma is the most common type of primary bone cancer in children and adolescents, but its mechanism remains unclear. Musashi RNA-binding protein 1 (MSI1) is highly expressed in certain cancer types and functions as a putative progenitor/stem cell marker. In the present study, it was demonstrated that MSI1 expression in osteosarcoma tissue was higher compared with in the paraneoplastic tissue samples. Knockdown of MSI1 using shRNA in MG-63 and HOS cells inhibited cell proliferation *in vitro* and tumor formation *in vivo*, suggesting that MSI1 serves an essential role in osteosarcomagenesis. Further investigations demonstrated that the knockdown of MSI1 leads to the cell cycle arrest at G₀/G₁ phase, and the upregulation of p21 and p27 protein expression in osteosarcoma cells. Additionally, luciferase assays demonstrated that MSI1 can bind to the 3' untranslated regions of p21 and p27 mRNA. In conclusion, the results of the present study suggest that the knockdown of MSI1 can suppress cell proliferation of osteosarcoma by targeting p21 and p27 and subsequently inhibiting cell cycle progression.

Introduction

Osteosarcoma is the most common non-haematological primary malignant bone tumor that occurs in children and adolescents and its overall relapse-free survival rate over 5 years is 65-75% (1). Osteosarcoma is characterized by a highly malignant and metastatic potential, and the leading cause of death of osteosarcoma patients is distant metastases (2). However, at present, the pathogenesis of osteosarcoma remains unclear.

Increasing evidences have showed that tumor stem cells are considered to be responsible for the metastasis of

tumors (3), and some stem cell-related genes are involved in tumorigenesis (4,5). MSI1 is a RNA-binding protein of the Musashi family involved in early asymmetric divisions generating differentiated cells from neural stem cells or progenitor cells. MSI1 is highly enriched in the nervous system and has been found to be related with the grade of the malignancy in glioma (6). Additionally, MSI1 drives progenitor cell expansion along the luminal and myoepithelial lineages in mammary glands and regulates the proliferation and apoptosis of mesenchymal stem cells. Recently, high MSI1 expression has been found in various types tumors, including medulloblastoma (7), colon cancer (8), lung cancer (9), cervical cancer (10) and breast cancer (11), and appeared to be a maker of poor prognosis. Moreover, MSI1 has been found to activate the Notch and Wnt signaling pathways in several types of normal and cancerous cells (12,13). However, the role of MSI1 in osteosarcoma progression is currently unclear.

Here, we investigated the function of MSI1 and its mechanism in the progression of osteosarcoma. In this study, we found that MSI1 expression is increased in osteosarcoma tissue compared with paraneoplastic tissue. Knockdown of MSI1 resulted in the decreased cell proliferation and slow growth of the tumor xenografts. Furthermore, knockdown of MSI1 resulted in the arrest of cell cycle and up-regulation of p21 and p27 protein expression. These results supported that MSI1 functions as an oncogene in osteosarcoma.

Materials and methods

Tissue samples. A total of 30 Fresh frozen specimens of matched osteosarcoma tissues and paraneoplastic tissues were collected from Shandong Jining No. 1 People's Hospital (Jining, China) from 2011 to 2014. None of the patients had received chemotherapy, immunotherapy or radiotherapy before the specimen collection. The study was approved by Institutional Research Ethics Committee, and patients gave their informed consent before sample collection.

Cell lines and cell culture. Human osteosarcoma cell lines, MG-63 and HOS, were purchased from the American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine

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serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin. All cells were cultured at 37°C in an atmosphere 5% CO₂.

Vector construction. The small interfering RNA expression vector that expresses MSI1-specific short hairpin RNA (shRNA) was purchased from GenePharma Co., Ltd. (Shanghai, China). To construct reporter vector containing the 3'UTR of p21 and p27, the fragments of the 3'UTR of p21 and p27 mRNA were separately extracted from MG-63 cells and amplified from cDNA by PCR using primers listed below, then cloned into the pMIR-REPORT luciferase vector. The following primers were listed in Table I.

Western blot analysis. Cells and clinical tissues were lysed on ice in lysis buffer containing freshly added protease inhibitor cocktail (Roche Diagnostics, Branchburg, NJ, USA). The protein extracts (15 µg) were separated using SDS-PAGE and transferred into polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The appropriate primary antibodies were used after the membranes were blocked in 5% fat-free milk. The primary antibodies included the following: anti-MSI1 (1:1,000, cat. no. sc-98845; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-p21 (1:500, cat. no. sc-397; Santa Cruz Biotechnology, Inc.), anti-p27 (1:500, cat. no. sc-397; Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:500, cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). Blots were incubated with a secondary antibody coupled to horseradish peroxidase (Thermo Fisher Scientific Inc.), and visualized on X-ray film. Relative quantitation was measured using the AlphaView system (Cell Biosciences, Santa Clara, CA, USA).

RT-qPCR analysis. Total RNA was extracted from cells with TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcription reactions were performed using RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). Relative mRNA levels were evaluated by quantitative PCR using SYBR-Green Mastermix (Takara Biotechnology Co., Ltd.). The primer sequences were listed in Table II. The results were analyzed according to the Cq (ΔΔCq) method using GAPDH as the normalizing gene.

MTT assays. Cell viability was assessed every other day using 3-(4,5-dimethylthiazole-yl) 2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) dye according to standard protocol. Approximately 2x10³ cells/well were seeded in a 96-well plate and incubated for 7 days. 20 µl of MTT solution was added to 200 µl of culture media and incubated for 4 h, and then dissolved in 100 µl of dimethylsulphoxide (DMSO; Sigma-Aldrich). Cell proliferation was determined by measuring the absorbance at 490 nm.

Flow cytometry analysis. Cells (2x10⁶) were harvested and fixed with 70% cold ethanol at 4°C overnight. After washed twice in PBS, the cells were suspended in PBS with 50 µg/ml propidium iodide and 10 µg/ml RNaseA, and then incubated at room temperature for 30 min in the dark. Then, the cells were measured by FACSCalibur flow cytometry (BD Biosciences,

Franklin Lakes, NJ, USA), and the cell cycle distributions were analyzed.

In addition, the cells were harvested, washed in PBS and stained in duplicate with APC annexin V and propidium iodide (BD Pharmingen, Franklin Lakes, NJ, USA) for 10 min in the dark to characterize cell apoptosis using flow cytometry.

Animal and tumor xenograft assay. Tumor cells collected from stable transfectants were bilaterally injected into subcutis on the dorsum of 6- to 7-week-old Balb/c nude mice. Tumor size was measured with calipers once every week, and volumes (cm³) were calculated according to the standard formula: V=length x width²/2. At the end of the experiment, tumors were dissected out, and the net weight was measured. All animals received humane treatment in accordance with institutional policies, and all experimental protocols were approved by the Animal Care and Use Committee of Jining First people's Hospital.

Luciferase reporter assay. In brief, plasmids containing firefly luciferase reporters were cotransfected into cells using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Then, the cells were lysed in 100 µl of passive lysis buffer (Promega Corporation, Madison, WI, USA) at 48 h after transfection and determined with a dual-luciferase assay according to the manufacturer's instructions. Luciferase activities were detected in a luminometer (Promega Corporation) and expressed at the ratio of Firefly to Renilla luciferase activity.

Statistical analysis. All results were confirmed in three independent experiments, and data were expressed as the mean ± standard deviation. Student's t-test or one-way ANOVA test were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

The expression of MSI1 was upregulated in osteosarcoma. To determine the expression of MSI1 in osteosarcoma, Western blot analysis was conducted. We found the expression of MSI1 in osteosarcoma tissues was markedly higher than that in the paraneoplastic tissues (P<0.01, Fig. 1A). Additionally, the expression of MSI1 in osteosarcoma cell lines was also measured by western blot, and the high expression of MSI1 in U-2OS, HOS, Hs-188T and MG-63 cell lines were observed (Fig. 1B). These data suggested MSI1 may contribute to the progression of osteosarcoma.

The effect of knockdown of MSI1 on the proliferation and apoptosis of osteosarcoma cells. To assess the effect of MSI1 on the proliferation and apoptosis of osteosarcoma cells, we established MG-63 and HOS osteosarcoma cells with MSI1 knockdown by shRNA, respectively (Fig. 2A and B). MTT assay revealed that MSI1 knockdown MG-63 cells had much lower proliferation ability than the corresponding control cells (Fig. 2C), and similar results were observed in MSI1 knockdown HOS cells (Fig. 2D). These findings suggested that

Table I. The primer sequence of p21 and p27.

Gene	Orientation	Primer sequence
WT p21	Forward	5'-ATTGAGCTCTAATCCGCCACAGGAAG-3'
	Reverse	5'-CTCAAGCTTACAAGTAAAGTCACTAAG-3'
MU p21	Forward	5'-TGGGAACGACTGTCTTTCCTGGCACTAACGTT-3'
	Reverse	5'-AGACAGTCGTTCCAGCCCCATATGAGCCAC-3'
WT p27	Forward	F: 5'-CGCGAGCTCGAATTAAGAATATGTTTC-3'
	Reverse	5'-TTGACGCGTATGCAACCTTTTAAAGCATAGC-3'
MU p27	Forward	5'-CCGCTCGAGTGATCTGCCTCTAAAAGCGT-3'
	Reverse	5'-CGGGATCCATTCTTAACATTCAAACACTCCC-3'

WT, wild-type; MU, mutant.

Table II. The primer sequence of p21 and p27.

Genes	Orientation	Primer sequence
p21	Forward	5'-GAGCAGTGCCCGAGTTAAGG-3'
	Reverse	5'-TGGAACAGGTCGGACATCAC-3'
p27	Forward	5'-GGTGCCTTCAATTGGGTCTC-3'
	Reverse	5'-GCTTCCTCATCCCTGGACAC-3'

knockdown of MSII suppressed the proliferation of osteosarcoma cells.

However, the apoptosis analysis of MG-63 and HOS cells using flow cytometry showed that there was no significant difference in the proportion of apoptotic cells between MSII knockdown cells and the control (Fig. 2E and F), which suggested that MSII had no significant influence on apoptosis in the osteosarcoma cells.

Knockdown of MSII inhibited the tumor formation of osteosarcoma cells. To further assess the effect of MSII on the tumor formation, nude mice were injected subcutaneously with MSII knockdown MG-63 and HOS cells, and the growth of tumors was monitored in terms of tumor volume every three days. At the end of the experiment, the mice were sacrificed, and the tumors were excised and weighed. The tumors formed by the MSII knockdown MG-63 and HOS cells grew much slower than those formed by the Control (Fig. 3A and B, $P < 0.01$). In addition, the weights of the tumors formed by the MSII knockdown cells were significantly reduced compared to the Control (Fig. 3C and D, $P < 0.01$). All these data suggested that knockdown of MSII inhibited the tumor formation ability of osteosarcoma cells *in vivo*.

Knockdown of MSII led to the cell cycle arrest of osteosarcoma cells. We all know that cell proliferation is usually associated with the modulation of the cell cycle, then, we characterized cell cycles by flow cytometry analysis. As shown in Fig. 4A and B, the proportion of MSII knockdown MG-63 cells in G0/G1 phase was markedly increased, while proportion in S phase was significantly decreased compared to the

control ($P < 0.05$), and a similar result was observed in MSII knockdown HOS cells (Fig. 4C and D). These data indicated that the knockdown of MSII resulted in the cell cycle arrest.

Knockdown of MSII increased the expression of p21 and p27 protein. Previous research reported that MSII regulated cell cycle by negative regulation of p21 expression in bladder carcinoma (14) and breast cancer (15). To explore the molecular mechanism of MSII on the regulation of cell cycle of osteosarcoma cells, the expression of p21 and p27 were examined by Western blot (Fig. 5A-D). A significant increase in the levels of p21 and p27 protein expression were observed in the MSII knockdown MG-63 and HOS cells compared to their control cells, respectively ($P < 0.01$), suggesting that MSII may reduce the levels of p21 and p27 protein expression. However, the examination using RT-qPCR assay showed that no significant differences in the expression of p21 and p27 mRNA were observed in the MSII knockdown MG-63 and HOS cells and their control (Fig. 5E and F). Therefore, these data indicated that MSII could influence the expression of p21 and p27 at protein level, not mRNA level in MG-63 and HOS cells.

It has been reported that p21 and p27 were the MSII target genes in which there is a binding sequence region in their 3'UTR (16,17). The MSII binding sites in p21 and p27 protein are shown in Fig. 6A, respectively. To test the direct interaction between MSII and its putative target site, the wild-type and mutant MSII binding sequence was inserted downstream of the luciferase vectors (Fig. 6B). After transfected by p21 wild-type vector, the relative luciferase activity of MSII knockdown MG-63 and HOS cells was significantly increased compared to the control ($P < 0.01$), however, no significant difference in the luciferase activities between the MSII knockdown MG-63 and HOS cells and their control cells transfected with the mutant vector was observed (Fig. 6C and D). Similarly, the luciferase activity levels in MSII knockdown MG-63 and HOS cells were found to be higher than their control cells after being transfected with the p27 1&2 wild-type vector ($P < 0.01$), while, the differences were not significant between the MSII knockdown MG-63 and HOS cells and their control cells after being transfected by p27 1&2 mutant vector. These data suggested that MSII

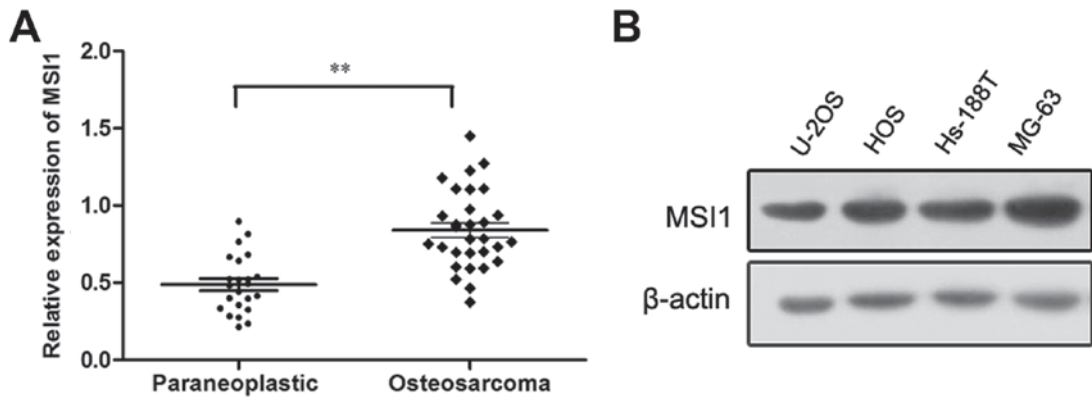


Figure 1. The expression of Musashi RNA-binding protein 1 (MSI1) in osteosarcoma tissues, paraneoplastic tissues and osteosarcoma cell lines. (A) MSI1 protein expression in osteosarcoma tissues was higher than that of the paraneoplastic tissues (** $P < 0.01$). (B) MSI1 expression in human osteosarcoma cell lines was shown by western blot analysis.

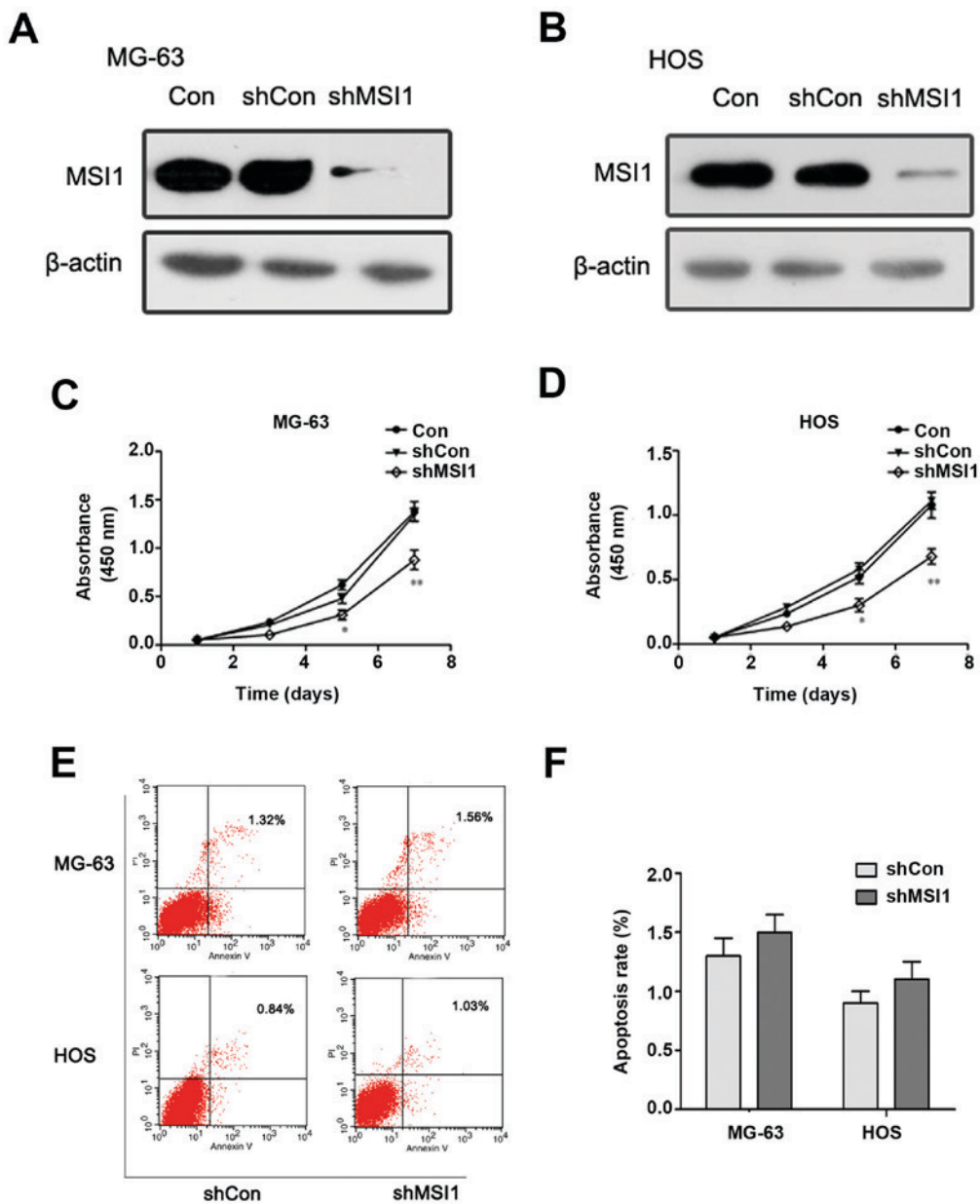


Figure 2. The effect of knockdown of Musashi RNA-binding protein 1 (MSI1) on the proliferation and apoptosis of osteosarcoma cells. Western blot analysis of the expression of MSI1 in (A) MG-63 and (B) HOS cells with MSI1 knockdown by shRNA. The cell proliferation of MSI1 knockdown (C) MG-63 and (D) HOS cells were evaluated by MTT assay. (E) Apoptosis assay for MG-63 and HOS was performed and (F) quantitative analysis. ** $P < 0.01$ compared to the shCon.

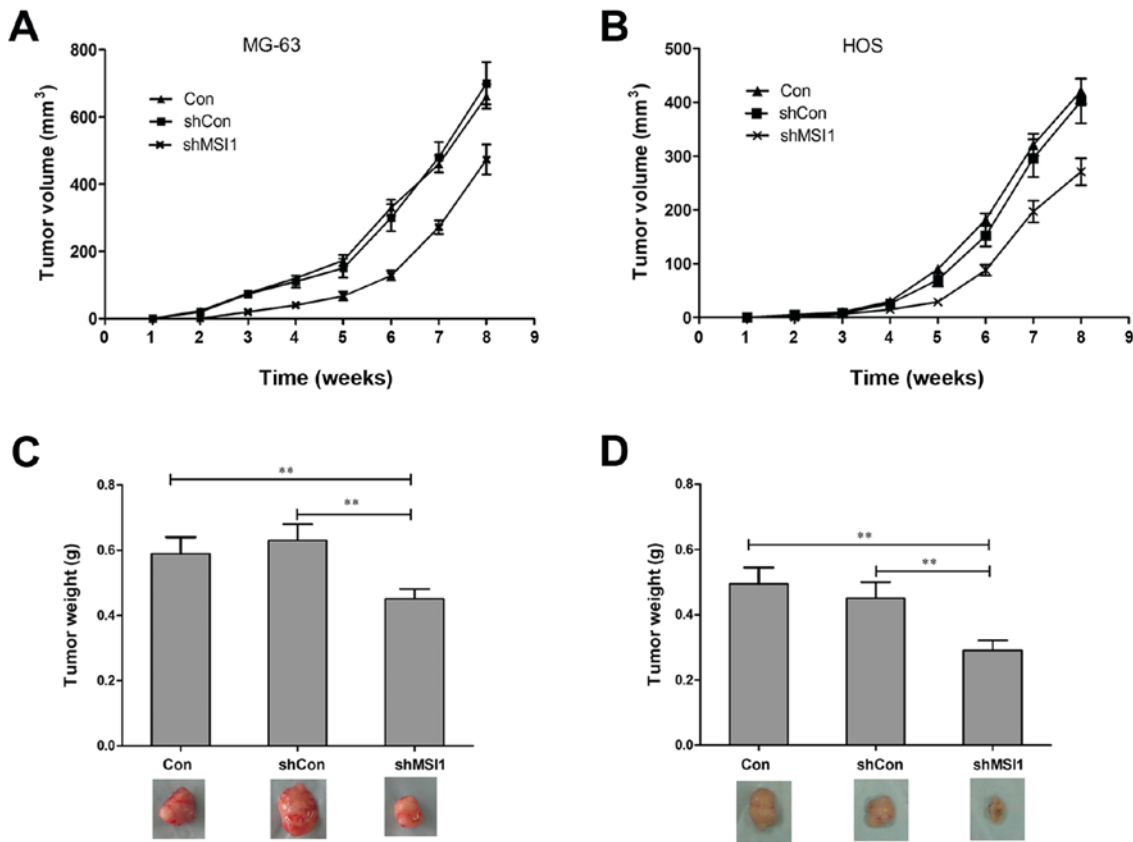


Figure 3. Knockdown of Musashi RNA-binding protein 1 (MSI1) inhibited the growth of xenograft tumors *in vivo*. The growth of xenograft tumors formed by MSI1 knockdown (A) MG-63 and (B) HOS cells were monitored longitudinally for 8 weeks. (C and D) At end of the experiment, the tumors were dissected out, and the tumor wet weights were measured. **P<0.01 compared to the shCon.

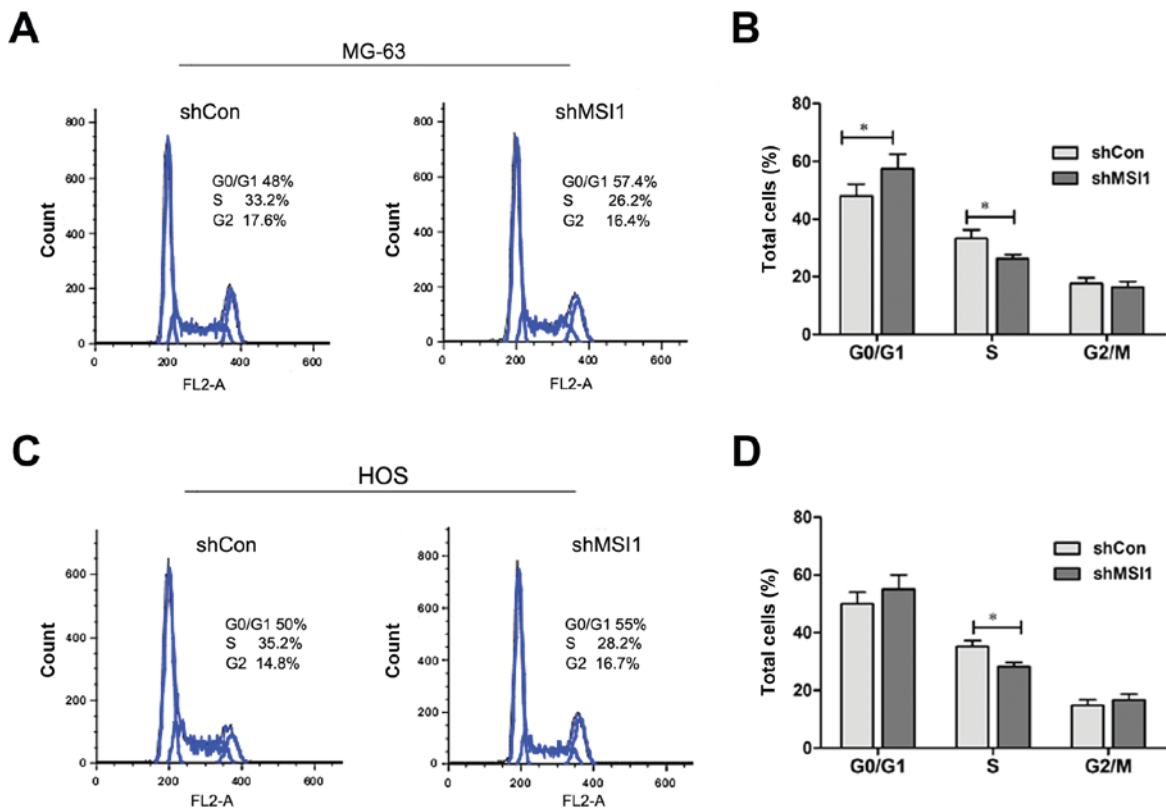


Figure 4. The effect of Knockdown of Musashi RNA-binding protein 1 (MSI1) on cell cycle and apoptosis of MG-63 and HOS cells. Cell cycle analysis was performed by flow cytometry, and the representative cell cycle histograms of MSI1 knockdown (A) MG-63 and (C) HOS cells were shown. Quantitative analysis was shown for the cell cycle distribution of (B) MG-63 and (D) HOS. *P<0.05 compared to the shCon.

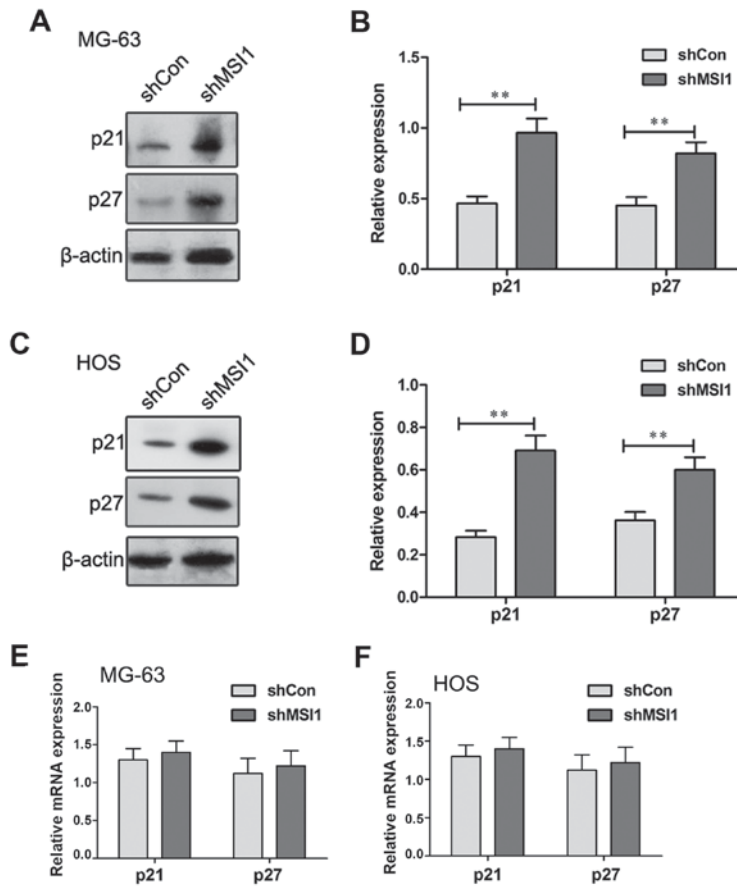


Figure 5. Musashi RNA-binding protein 1 (MSI1) regulated the expression of p21 and p27 at protein level. The expression of p21 and p27 proteins in MSI1 knockdown (A) MG-63 and (C) HOS cells were measured by western blot assay, and (B and D) the relative expression of p21 and p27 were calculated based on β -actin. The levels of p21 and p27 transcripts in MSI1 knockdown (E) MG-63 and (F) HOS cells were measured by qPCR. **P<0.01 compared to the shCon.

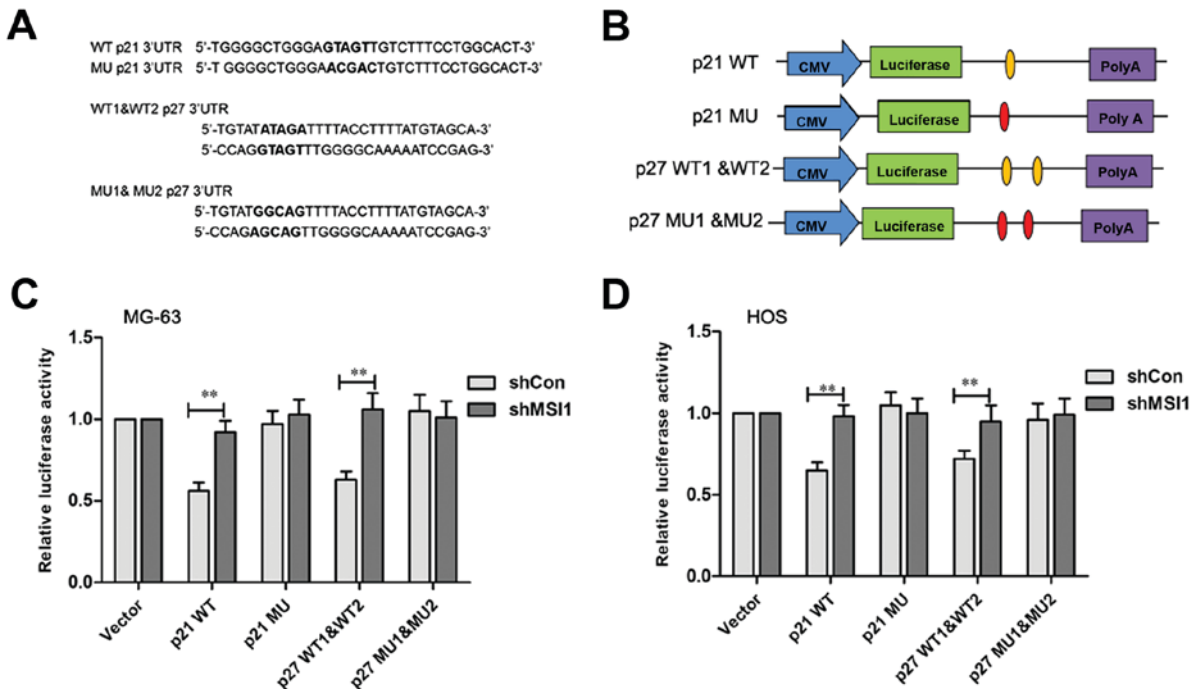


Figure 6. Musashi RNA-binding protein 1 (MSI1) binded to the 3'UTR of p21 and p27. (A) Schematic construction of MSI1 binding sites with wild-type (WT) and mutant (MU) 3'UTR of p21 and p27 as predicted. (B) Schematic representation of the reporter construct containing the firefly luciferase coding sequence fused to p21 and p27 3'UTR. The predicted binding site regions within the wild-type p21 and p27 3'UTR are noted in orange ovals, and the residues altered in the p21 3'UTR/p27 3'UTR mutants construct are marked on the red ovals. Luciferase reporter assays for p21 and p27 targeted by MSI1 in (C) MG-3 and (D) HOS cells, and the histogram shows the ratio of firefly to *Renilla* luciferase activity normalized to empty vector transfected cells. **P<0.01 compared to the shCon.

might inhibit p21 and p27 translation by specifically binding site.

Discussion

Increasing evidence showed that RNA binding proteins play an important role in the cell proliferation and apoptosis involved in tumorigenesis (18-20). MSI1, a multifunction RNA binding protein of MSI family, was initially identified as a marker of neuronal stem cell (21,22) and more recently identified as a putative marker of intestinal stem cell (23), was overexpressed in several types of cancers, such as glioma (13), medulloblastoma (24), and breast cancer (15).

In this study, we found that the expression of MSI1 protein in osteosarcoma tissues was significantly higher than that of the paraneoplastic tissues, suggesting MSI1 was likely to play an important role in osteosarcomagenesis. To further seek for the function of MSI1 involved in osteosarcomagenesis, the functional characterization was conducted by knockdown of MSI1 in osteosarcoma cell lines (MG-63 and HOS cells). We found that knockdown of MSI1 significantly inhibited the proliferation of MG-63 and HOS cells *in vitro*. Moreover, knockdown of MSI1 also significantly suppressed the tumor growth *in vivo*. These results suggested that MSI1 played an important role in the cell proliferation and tumor growth. In addition, the analysis of cell cycle and apoptosis showed that knockdown of MSI1 induced cell cycle arrest in the G0/G1 phase, but no markedly change of apoptosis, suggesting that MSI1 might have important impact on the cell cycle, not apoptosis. It is well known that cell proliferation is usually regulated by cell cycle progression. Therefore, our data suggested that the inhibitory effect of MSI1 knockdown on cell proliferation and tumor formation of osteosarcoma cells were probably associated with the regulation of cell cycle. Our finding was in agreement with previous findings in other types of tumors (25-27), suggesting that MSI1 related signaling may have a similar regulatory effect on the growth of different types of human malignancies.

It has been reported that increased expression of p21 and p27 leads to apoptosis and cell cycle arrest in various cancer cells (16,28,29). In this study, we found the expression of p21 and p27 protein levels were up-regulated in the MSI1 knockdown MG-63 and HOS cells compared to the control. As p21 and p27 is universal cyclin-dependent kinase (CDK) inhibitor that directly inhibits the activity of cyclin-CDK complexes (30,31), resulting in cell cycle arrest at G0/G1 phase, these results suggested that the cell cycle arrest caused by MSI1 knockdown in osteosarcoma cells might be associated with the overexpression of p21 and p27. Furthermore, luciferase assay revealed that MSI1 could directly bind to the consensus sequence of p21 and p27 3'UTR in osteosarcoma cells, indicating that knockdown of MSI1 could lead to the up-regulation of the expression of p21 and p27.

In summary, our study demonstrated that MSI1 was high expressed in osteosarcoma tissue, and knockdown of MSI1 inhibited the cell proliferation and tumor formation by the arrest of cell cycle involved the activation of p21 and p27. Our study provided a potential target for clinical therapy of osteosarcoma.

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