

## Original article

# LncRNA ZFAS1 promotes growth and metastasis by regulating BMI1 and ZEB2 in osteosarcoma

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**Abstract:** Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents. LncRNAs have recently gained widespread attention and have been shown to have crucial roles in various biological regulatory processes. ZFAS1, a newly identified lncRNA, was shown to be dysregulated in several cancers. However, little is known about the alteration and functional significance of ZFAS1 in OS. In the present study, for the first time, we revealed a functional role of ZFAS1 on OS growth and metastasis. The expression of ZFAS1 was significantly overexpressed in OS samples and cell lines, and upregulation of ZFAS1 is significantly associated with unfavorable prognosis of OS patients. Functional assays also demonstrated that ZFAS1 enhanced the growth and metastatic ability of OS cells *in vitro* and *in vivo*. Mechanistically, we found that ZFAS1 positively regulated malignant phenotypes by competitively binding the miR-200b and miR-200c and upregulating BMI1. ZFAS1 also interacted with ZEB2 and regulated ZEB2 protein stability. Furthermore, we demonstrated that SP1 functions as an upstream activated factor of ZFAS1. ZFAS1 may be a potential therapeutic target for OS tumorigenesis and progression.

**Keywords:** ZFAS1, miR-200b, miR-200c, SP-1, BMI1, ZEB2

## Introduction

Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents, which is characterized by highly aggressive and early systemic metastasis [1]. Most osteosarcoma patients are diagnosed under the age of 25 years, and the disease occurs more often in males than in females. Chemotherapy and/or radiation therapy are usually used before or after surgery to prevent tumors from spreading throughout the body. The prognosis of patients with distant metastases still unfavorable, as the 5-year survival rate in these patients is approximately 20% [2, 3]. Thus, understand the underlying molecular mechanism of osteosarcoma carcinogenesis and progression is very necessary for developing comprehensive and effective treatments for osteosarcoma.

Since the invention of DNA sequencing methods and the completion of the draft human genome sequence, researchers have found that more than 90% of the human genome DNA

is thought to be transcribed, while less than 3% of the human genome constitutes protein coding genes. Long noncoding RNAs (lncRNA) are new large and regulatory class of non-protein-coding transcripts greater than 200 nucleotides [4, 5]. LncRNAs have recently gained widespread attention and have been shown to have crucial roles in various biological regulatory processes. LncRNAs function through a variety of mechanisms including recruiters regulating gene expression *in cis* or *in trans*, scaffolds that assemble chromatin remodeling machinery, decoys that titrate away and prevent the action of effector molecules, mediators of alternative splicing and precursors for small ncRNAs. LncRNA expression is frequently dysregulated in cancer, and specific lncRNAs are correlated with cancer recurrence, metastasis, and poor prognosis in Osteosarcoma. For example, significantly higher TUG1 levels were detected by in OS tissues and inhibition of TUG1 expression distinctly impaired proliferation of OS cells [6]. Additionally, upregulated ZEB1-AS1 has connection with tumor metastasis in OS by

increasing ZEB1 expression [7]. ZFAS1, a newly identified lncRNA, was shown to be dysregulated in colonic cancer, breast cancer and hepatocellular carcinoma [8-10]. It has been reported that ZFAS1 promotes invasion and metastasis through upregulation of ZEB1 [10], and also associated with epithelial-mesenchymal transition (EMT) [11]. However, little is known about the alteration and functional significance of ZFAS1 in OS. It is also necessary to reveal the underlying molecular mechanisms by which ZFAS1 is involved in OS tumorigenesis and progression. In the current study, we investigated the expression pattern, biological function and underlying mechanism of ZFAS1 in OS metastasis.

## Materials and method

### *Cell culture and tissue samples*

OS cell lines (KHOS, 143b, LM7, U2OS, and MG-63) and a normal osteoblast cell line (Nhost) were obtained from the Chinese Academy of Sciences Cell Bank. These cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS, Gibco) at 37°C in 5% CO<sub>2</sub>. The tumor tissues obtained from Cangzhou Central Hospital of Hebei Medical University. All patients provided written informed consent and ethical consent was granted from the Committees for Ethical Review of Research involving Human Subjects of Cangzhou Central Hospital of Hebei Medical University.

### *Lentiviral knockdown and overexpression of ZFAS1*

ShRNAs were used to silence ZFAS1 expression. Two different pairs of shRNAs were inserted to pLKO.1 lentiviral plasmid (Addgene). The target sequence of ZFAS1 was as follow: sh1: CAGGAAGCCATTCGTTCTT, sh2: GTGGTACTTCTCCTAGTTG. ZFAS1 was cloned into pLV lentiviral plasmid (Addgene). The above lentiviral expressing vectors and packaging vectors were transfected into the 293T cells (ATCC) using the Lipofectamine 3000 (Invitrogen). The lentivirus was collected and centrifuged, and stored at -80°C. Cells were infected with indicated lentivirus. The stable cells were selected by 1 µg/ml puromycin for 1 week.

### *siRNA transfection*

siRNAs against BMI1 were purchased from RiboBio company. The target sequence against BMI1 is as follow: AGCTAAATCCCCACCTGAT.

### *RNA isolation and quantitative real-time PCR (qPCR)*

Total RNAs from cells or tissues were extracted by using TRIzol (Invitrogen) reagent according to the standard protocol. First-strand cDNA was synthesized by using the Reverse Transcriptase Kit (Transgene). qRT-PCR was performed using SYBR Green Mixture (Takara) in the ABI StepOne-Plus System. Data were normalized to control group. Primers sequences as follows: ZFAS1-F: 5'-CTATTGTCCTGCCCCGTTAGAG CTATGTCCTGCCCCGTTAGAG-3', ZFAS1-R: 5'-GTCAGGAGATCGAAGGTTGTAG-3'; BMI1-F: 5'-CCAGAGAGATGGACTGACAAAT-3', BMI1-R: 5'-CAAGAAGAGGTGGAGGGAATAC-3'. Comparative quantification was determined using the 2<sup>-ΔΔCt</sup> method.

### *Isolation of cytoplasmic and nuclear*

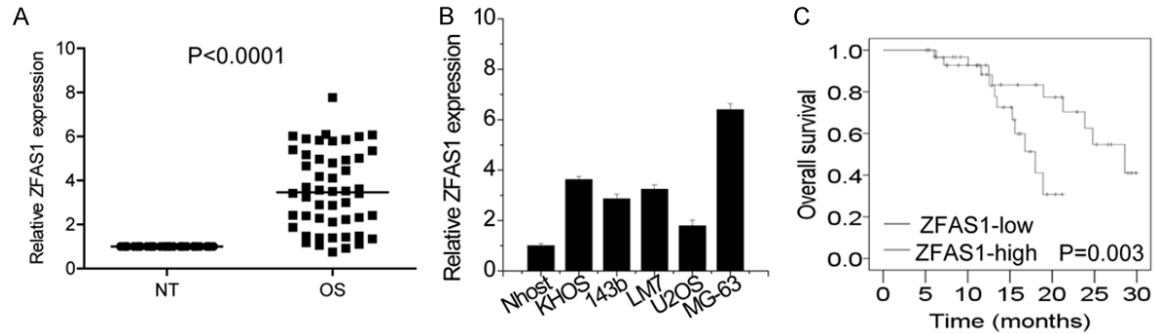
Cytoplasmic and nuclear RNA were isolated and purified using the Cytoplasmic & Nuclear RNA Purification Kit (Norgen, Belmont, CA) according to the manufacturer's instructions.

### *Western blot*

Western blotting was performed as previously described [12]. In briefly, cell lysates were subjected to SDS-PAGE and transferred to a PVDF membrane. The PVDF membrane was incubated with 1:200 ZEB2 (Santa Cruz), 1:1000 anti-BMI1 (Millipore) and 1:10000 anti-GAPDH (Proteintech) overnight. After wash, the membrane was incubated with 1:10000 anti-mouse IgG-HRP conjugate (Jackson) or anti-rabbit IgG-HRP conjugate (Jackson). Blots were detected by ECL system (Millipore) and analyzed on an imager (GE Healthcare).

### *Cell proliferation assay*

Cell proliferation was determined by the Cell Counting Kit-8 (CCK-8) assay. 2 × 10<sup>3</sup> cells were seeded in 96-well plates. Each well was incubated with WST-8 solution (Dojindo) for 3 hours, and the absorbance was measured at 450 nm using a spectrophotometer.



**Figure 1.** Increased ZFAS1 expression predicts poor prognosis of osteosarcoma patients. A. The relative expression of ZFAS1 in osteosarcoma tissues and corresponding noncancerous tissues from 50 patients. NT, noncancerous tissues; OS, osteosarcoma tissues. B. The expression level of ZFAS1 in OS cell lines (KHOS, 143b, LM7, U2OS, and MG-63) and a normal osteoblast cell line Nhost. The expression of Nhost was taken as control reference. C. Kaplan-Meier survival curve and log-rank test were used to evaluate the association of ZFAS1 expression level with overall survival rate. Patients were segregated into ZFAS1-high group and ZFAS1-low according to the median of ZFAS1 expression in OS tissues.

#### Apoptosis and cell cycle analysis

Apoptosis and cell cycle was detected as previously describe [13]. Cells were then analyzed with flow cytometer, and the data were studied using Flow Jo software (Tree Star).

#### Transwell assays

$1 \times 10^5$  cells were counted and seeded in serum-free DMEM in the upper chamber of a 24-well transwell or Matrigel-coated transwell insert (Corning). The lower chamber was filled with DMEM with 10% FBS. After 24 hours of incubation, the cells that had traversed the membrane were fixed and stained, and the cells in the upper chamber were removed.

#### RNA immunoprecipitation (RIP)

RIP assays were performed as previously described [14]. In specific, wild-type ZFAS1 or ZFAS1-mut (mutant in miR-200b/c binding site) was inserted into pcDNA3.1-MS2 (Addgene). Cells were co-transfected with pcDNA3.1-MS2 or pcDNA3.1-ZFAS1-MS2 or pcDNA3.1-ZFAS1-mut-MS2 and pMS2-GFP (Addgene). Cells were used to perform RIP experiments by using Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to its instructions. In brief, 5  $\mu$ g of GFP antibody (Abcam) or negative control IgG (Abcam) and magnetic beads were added to the cell lysate and incubated for overnight at 4°C with gentle rotation. The purified co-precipitated RNAs

were reverse transcribed and detected by RT-PCR. For anti-AGO2 RIP, cells were transfected with negative control miRNA (miR-NC) or miR-200b or miR-200c mimic. Cells were used to perform RIP assay by using an anti-AGO2 antibody (Millipore) as described above.

#### RNA pull-down and mass spectrometry assay

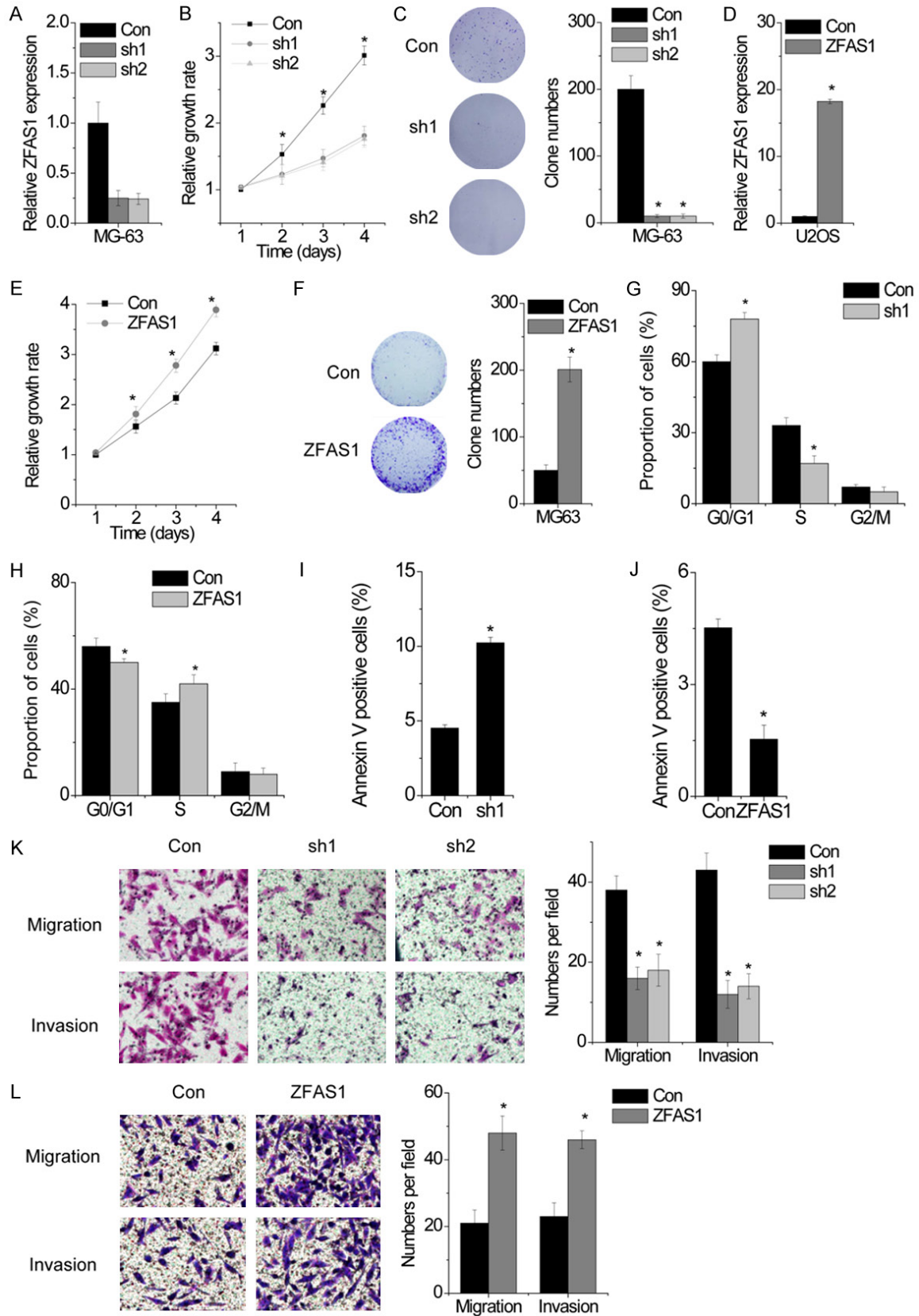
RNA pull-down was performed as previously described [15]. *In vitro* biotin-labeled RNAs (ZFAS1 and antisense ZFAS1) were transcribed with the biotin RNA labeling mix (Roche) and T7 RNA polymerase (Roche) treated with RNase-free DNase I (Promega) and purified with RNeasy Mini Kit (QIAGEN). Biotinylated RNA was incubated with nuclear extracts of breast cancer cells, and pull-down proteins were run on SDS-PAGE gels. Mass spectrometry followed.

#### Dual-luciferase reporter assay

ZFAS1 or ZFAS1-mut was inserted into pmirGLO reporter vector, respectively. The pmirGLO containing nothing, ZFAS1 or ZFAS1-mut was transfected with miR-200b or miR-200c mimic or miR-NC into cells by Lipo 3000 (Invitrogen). 48 hours after transfection, the luciferase activity was detected. The relative luciferase activity was normalized to Renilla luciferase activity.

#### Statistical analysis

All experiments were performed in triplicate. All statistical analyses were analyzed using 19.0





**Figure 2.** ZFAS1 positively regulates cell proliferation, migration and invasion in OS. A. ZFAS1 expression was silenced in MG-63 cells by two shRNAs. B. The proliferation of control and ZFAS1-silenced MG-63 cells was detected by CCK-8 assay. C. The clone formation in control and ZFAS1-silenced MG-63 cells. D. The ZFAS1 expression in control and ZFAS1-overexpressed U2OS cells was detected by qPCR. E. The relative proliferation rate in control and ZFAS1-overexpressed U2OS cells was determined by CCK-8 assay. F. The clone formation in control and ZFAS1-overexpressed U2OS cells. G. The cell cycle distribution was analyzed by FACS in control and ZFAS1-silenced MG-63 cells. H. The cell cycle distribution was analyzed by FACS in control and ZFAS1-overexpressed U2OS cells. I. The cell apoptosis was analyzed by FACS in control and ZFAS1-silenced MG-63 cells. J. The cell apoptosis was analyzed by FACS in control and ZFAS1-overexpressed U2OS cells. K. The cell migration and invasion in control and ZFAS1-silenced MG-63 cells. L. The cell migration and invasion in ZFAS1-overexpressed U2OS cells. Data are shown as mean  $\pm$  s.d. (n = 3) and are representative of three independent experiments. \*P<0.05.

SPSS software. *P* value less than 0.05 was taken as statistically significant.

## Results

### *Increased ZFAS1 expression predicts poor prognosis of OS patients*

At first, we performed qPCR to determine the differential expression of ZFAS1 in OS tissues and corresponding noncancerous tissues from 50 patients. The expression of ZFAS1 was significantly increased in osteosarcoma tissues compared with corresponding noncancerous tissues (**Figure 1A**). Moreover, we found that the mRNA expression level of ZFAS1 was upregulated in OS cell lines (KHOS, 143b, LM7, U2OS, and MG-63) compared with a normal osteoblast cell line Nhost (**Figure 1B**). The results also indicated that ZFAS1 reduced the survival rate of osteosarcoma patients (**Figure 1C**). These results indicated that ZFAS1 may play an oncogene in OS pathogenesis.

### *ZFAS1 positively regulates cell proliferation, migration and invasion in OS*

Tumor growth and metastasis are critical steps in tumor progression. To determine the role of ZFAS1 in growth and metastasis, we constructed stable MG-63 cells with ZFAS1 knockdown by two different shRNA-expressing lentiviruses. The qPCR results indicated that both sh1 and sh2 effectively knocked down ZFAS1 expression (**Figure 2A**). We found that the cell proliferation of MG-63 cells with ZFAS1 knockdown were significantly decreased compared with the control cells by CCK-8 assays (**Figure 2B**). In clone formation assays, the ZFAS1-knockdown MG-63 cells displayed less clones (**Figure 2C**). In contrast, we constructed stable U2OS cells with ZFAS1 overexpression (**Figure 2D**). We found that overexpression of ZFAS1 significantly promoted cell proliferation and colony formation ability (**Figure 2E** and **2F**).

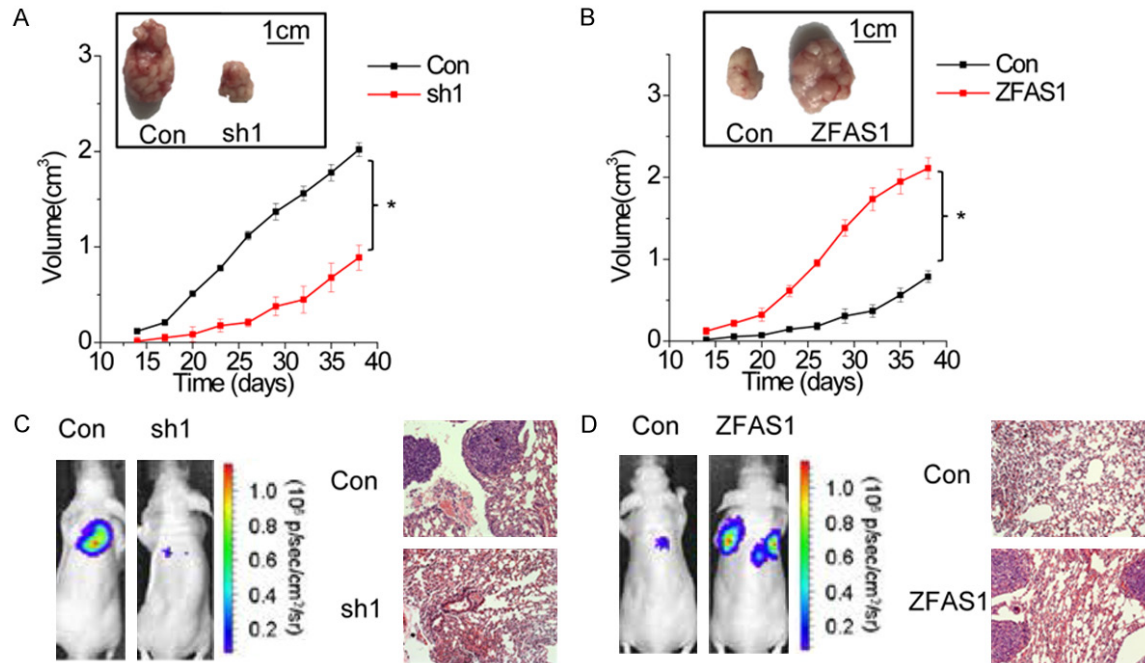
To gain insight into the mechanism by which ZFAS1 regulates cell proliferation, we performed flow cytometry to analyze the effect of ZFAS1 on cell cycle and apoptosis. We found that significant G1/S arrest was observed in ZFAS1-knockdown MG-63 cells, whereas ZFAS1 knockdown suppressed progression beyond the G1/S transition in MG-63 cells (**Figure 2G**), whereas ZFAS1 overexpression showed the opposite effect (**Figure 2H**). In addition, the results showed that ZFAS1-knockdown MG-63 had a significantly higher percentage of Annexin V-positive cells compared with control cells (**Figure 2I**), while protective effects were observed in ZFAS1-overexpressing U2OS cells (**Figure 2J**).

Next, we detected whether ZFAS1 influences cell migration and invasion in OS. Transwell assays and Matrigel-coated transwell assays demonstrated the positive effect of ZFAS1 on OS cell migration and invasion, with inhibited cell migration and invasion after ZFAS1 knockdown in MG-63 cells (**Figure 2K**). And ZFAS1 overexpression significantly increased the number of cells crossing the membrane (**Figure 2L**).

### *ZFAS1 also promotes OS growth and metastasis in vivo*

On the basis of the above findings that ZFAS1 promotes cell proliferation, migration and invasion, we next investigated the effects of ZFAS1 on cancer growth and metastatic ability *in vivo*. Xenograft tumors grown from ZFAS1-silenced MG-63 cells had smaller mean volumes than tumors grown from control cells (**Figure 3A**). Tumors grown from ZFAS1 overexpression were larger than controls (**Figure 3B**).

To establish a metastatic cancer model *in vivo*, MG-63 cells with stable knockdown of ZFAS1 were labeled with firefly luciferase and injected into tail vein of nude mice. A bioluminescent signal was assayed at 10 weeks after tail vein



**Figure 3.** ZFAS1 also promotes OS growth and metastasis *in vivo*. A. Subcutaneous tumor model of ZFAS1-silenced MG-63 cells ( $n = 7$  for both groups). Tumor growth curves in mice over time after subcutaneous injection with  $1 \times 10^6$  of the indicated MG-63 cells. B. Subcutaneous tumor model of ZFAS1-overexpressed U2OS cells ( $n = 7$  for both groups). Tumor growth curves in mice over time after subcutaneous injection with  $1 \times 10^6$  of the indicated U2OS cells. C. Left: Representative photographs of mice by imaging with the IVIS Imaging System and photographs of lung metastasis formed by control and ZFAS1-silenced MG-63 cells. Right: Visualization of the H&E-stained lung sections. D. Left: Representative photographs of mice by imaging with the IVIS Imaging System and photographs of lung metastasis formed by control and ZFAS1-overexpressed U2OS cells. Right: Visualization of the H&E-stained lung sections. Data are shown as mean  $\pm$  s.d. \* $P < 0.05$ .

injection. We found that the incidence of lung metastasis in the ZFAS1-silenced group is significantly decreased compared with the control group (**Figure 3C**). In contrast, ZFAS1 overexpression in U2OS cells inhibits lung metastasis (**Figure 3D**). Taken together, these data demonstrated that ZFAS1 promotes both growth and metastasis of OS tumors *in vivo*.

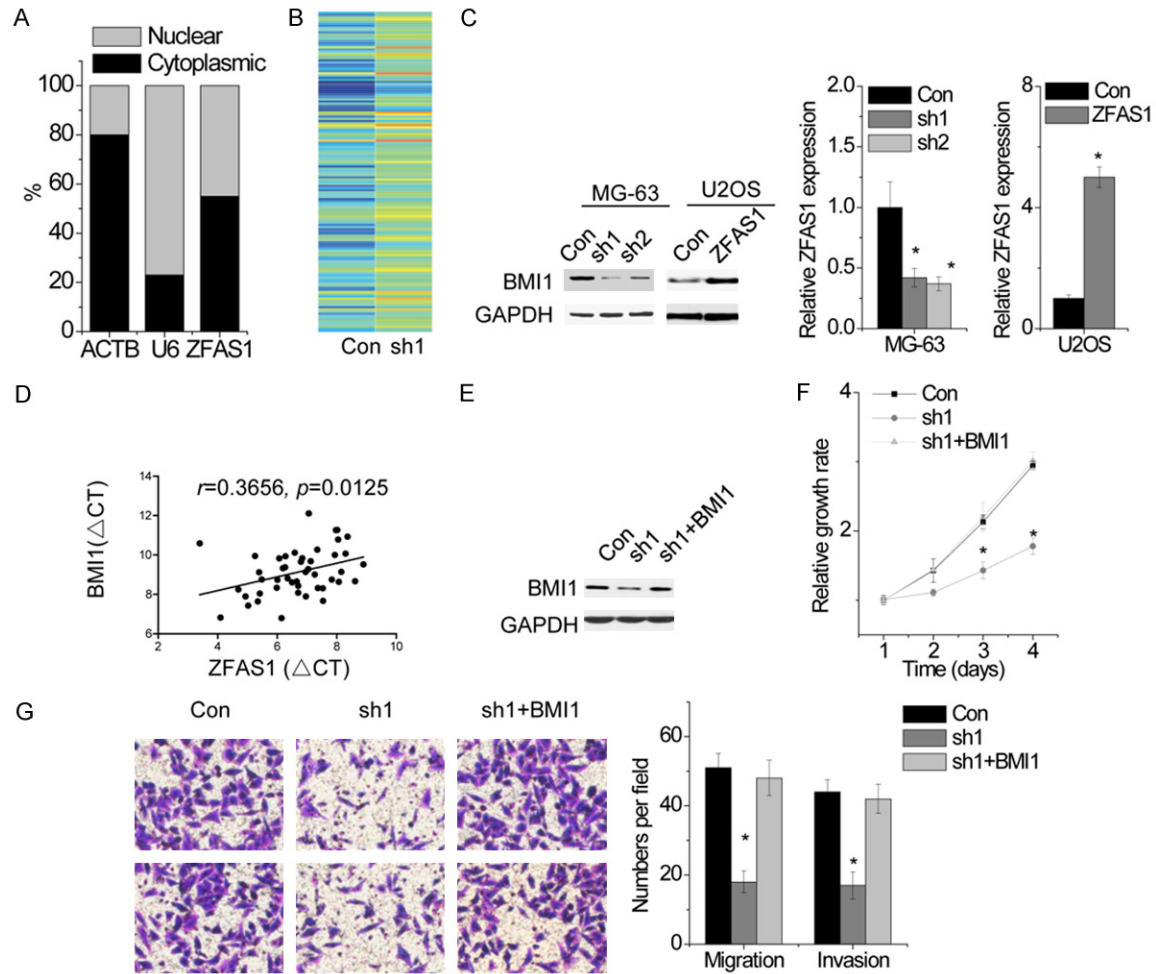
#### ZFAS1 increases BMI1 expression

We next explored how ZFAS1 functions in OS. We found that ZFAS1 localized in both the nucleus and cytoplasm of MG-63 cells through cellular fractionation assays (**Figure 4A**). The presence of ZFAS1 in both the cytoplasm and nucleus may suggest different functions. We performed microarray to identify the mRNA transcripts change mediated by ZFAS1 knock-down (**Figure 4B**). Among these target genes, *BMI1* arose our interest for its important oncogenic activity and remarkable change by ZFAS1. We found that overexpression of ZFAS1

increased both mRNA and protein level of BMI1, whereas silence of ZFAS1 showed the opposite effect (**Figure 4C**). We also noticed that ZFAS1 expression was positively correlated with BMI1 expression in OS sample tissues ( $r = 0.3656$ ,  $P = 0.0125$ , **Figure 4D**). To determine whether ZFAS1 regulates cell phenotypes in a BMI1-dependent manner, we silenced BMI1 expression in ZFAS1-overexpressed OS cells (**Figure 4E**), the results showed that silence of BMI1 abolished the promotion of proliferation, migration and invasion induced by ZFAS1 overexpression (**Figure 4F and 4G**).

#### ZFAS1 interacts with miR-200b and miR-200c to regulate BMI1 expression

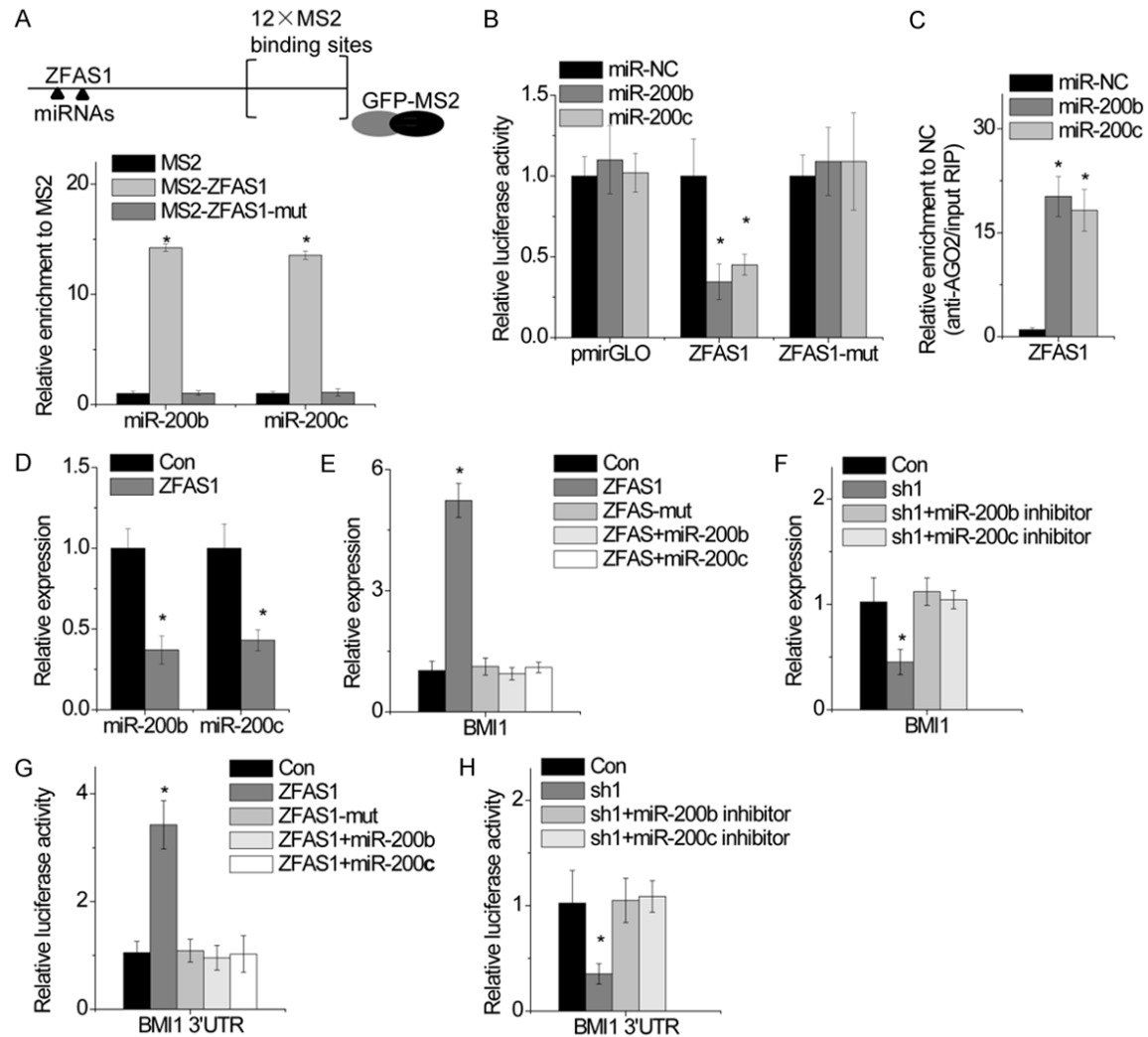
Recent studies showed that many RNA transcripts may act as competing endogenous RNAs (ceRNA) by competitively binding miRNAs. To explore the underlying mechanism responsible for ZFAS1-BMI1 in tumor growth and metastasis, we tested whether miRNAs are



**Figure 4.** ZFAS1 increases BMI1 expression. A. The cellular locatoin of ZFAS1 in MG-63 cells. B. The mRNA expression prolifes was detected by microarray in control and ZFAS1 knockdown MG-63 cells. C. The protein and mRNA change induced by ZFAS1 downregulation or upregulation was analyzed by wetern blot or qPCR. D. The correlation between BMI1 and ZFAS1 expression in OS tissues. E. BMI1 was transfected into ZFAS1 knockdown MG-63 cells. F. Restoring BMI1 expression resuced the proliferation inhibition induced by ZFAS1 downregulation. G. Restoring BMI1 expression resuced the migration and invasion inhibition induced by ZFAS1 downregulation. Data are shown as mean  $\pm$  s.d. (n = 3) and are representative of three independent experiments. \*P<0.05.

involved in the process. We predicted the miRNAs that may interact with ZFAS1 using StarBase and TargetScan. Bioinformatics analysis showed that ZFAS1 contains binding sites of miR-200b and miR-200c, which have been reported to suppress BMI1 expression [16]. To validate the direct binding between miR-200b/c and ZFAS1, we performed anti-MS2 RIP assay [17] to pull down endogenous microRNAs interacted with ZFAS1. The ZFAS1 RIP in MG-63 cells was significantly enriched for miR-200b/c compared to the empty vector (MS2), IgG and mut-ZFAS1 with mutations in miR-200b/c binding sites (Figure 5A). In addition, we constructed luciferase reporters containing wild-type

ZFAS1 or ZFAS1-mut. We found that transfection of miR-200b/c reduced the luciferase activities of the wild-type ZFAS1 reporter vector, but not empty vector or mutant reporter vector (Figure 5B). The microRNAs degrade mRNA by AGO2-dependent manner [18]. To investigate whether ZFAS1 was also regulated by miR-200b/c in this manner, we performed anti-AGO2 RIP in U2OS cells with overexpression of miR-200b or miR-200c. Endogenous ZFAS1 pull-down by AGO2 was specifically enriched in both miR-200b and miR-200c over-expressing cells (Figure 5C), which suggest that miR-200b and miR-200c are ZFAS1-targeting microRNAs. Finally, ectopically expressed ZFA-



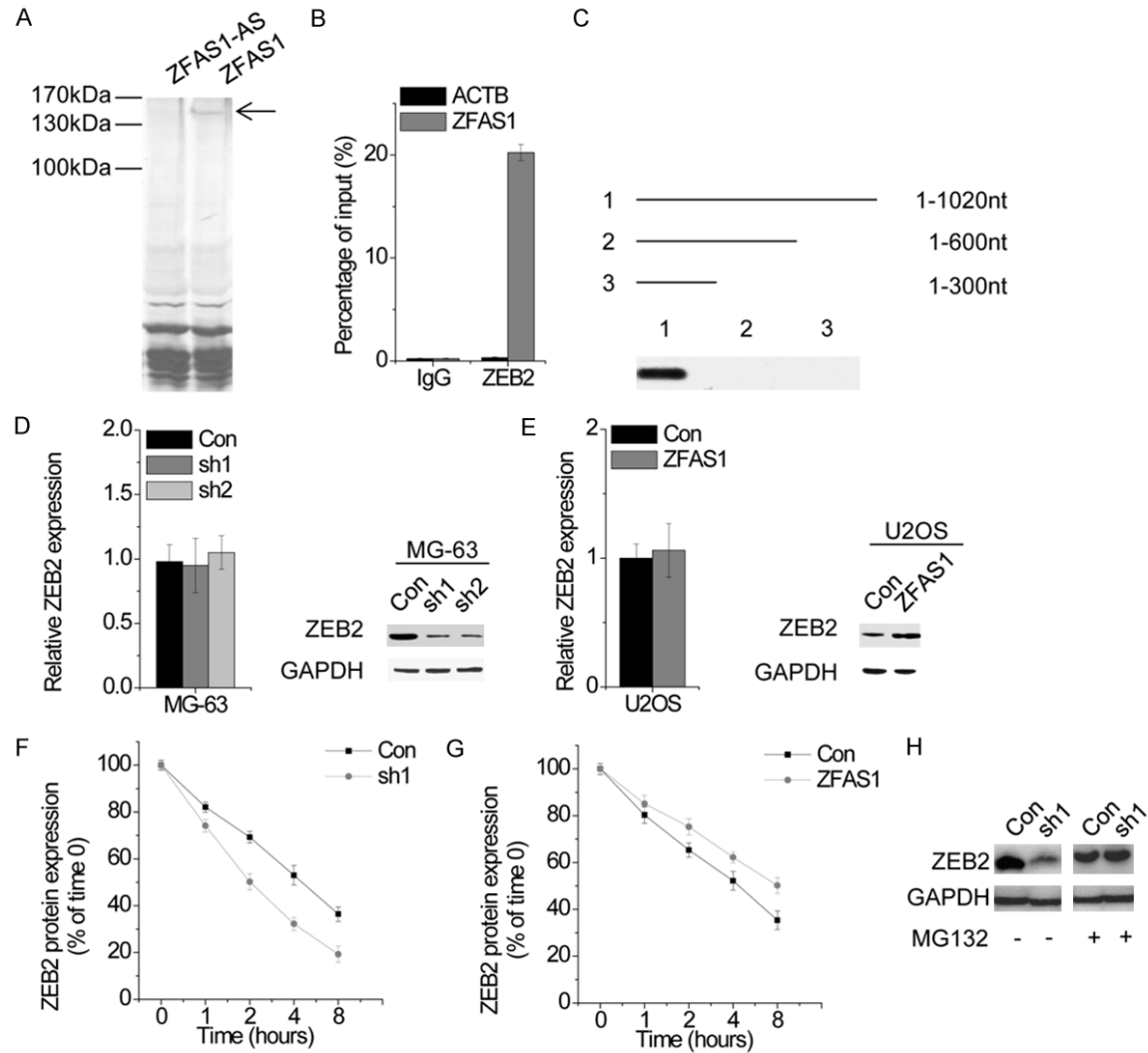
**Figure 5.** ZFAS1 interacts with miR-200b and miR-200c to regulate BMI1 expression. A. The RIP assay was performed to pull down the miRNAs associated with ZFAS1. B. Luciferase activity in MG-63 cells cotransfected with miR-200b or miR-200c and luciferase reporters containing nothing, ZFAS1 or ZFAS1 mutant transcript. Data are presented as the relative ratio of firefly luciferase activity to renilla luciferase activity. C. Anti-AGO2 RIP was performed in MG-63 cells transiently overexpressing miR-200b or miR-200c, followed by qPCR to detect ZFAS1 associated with AGO2. D. The expression of miR-200b and miR-200c expression in ZFAS1 overexpressed U2OS cells. E. The mRNA level of BMI1 expression in ZFAS1 or mutant ZFAS1 overexpressed U2OS cells cotransfected with miR-200b or miR-200c was analyzed by qPCR. F. The mRNA level of BMI1 expression in ZFAS1 silenced U2OS cells cotransfected with miR-200b or miR-200c inhibitor was analyzed by qPCR. G. The relative luciferase activity of BMI1 3'UTR in ZFAS1 or mutant ZFAS1 overexpressed U2OS cell cotransfected with miR-200b or miR-200c was analyzed. H. The relative luciferase activity of BMI1 3'UTR in ZFAS1 silenced U2OS cells cotransfected with miR-200b or miR-200c inhibitor was analyzed. Data are shown as mean  $\pm$  s.d. (n = 3) and are representative of three independent experiments. \*P<0.05.

S1, but not the ZFAS1-mut reduced the miR-200b and miR-200c expression (Figure 5D). Our results suggested that ZFAS1 is associated with the miR-200b/c and may serves as a ceRNA.

To investigate whether ZFAS1 can regulate BMI1 targeted by miR-200b/c, we detected the

expression of BMI1 in ZFAS1 WT and ZFAS1-mut overexpressed U2OS cells. Overexpression of ZFAS1 but not ZFAS1-mut increased BMI1 expression (Figure 5E). For the rescue experiment, we transfected miR-200b or miR-200c in ZFAS1 overexpressed cells. Overexpression of miR-200b or miR-200c abrogated the increase of BMI1 by ZFAS1 overexpression (Figure 5E).





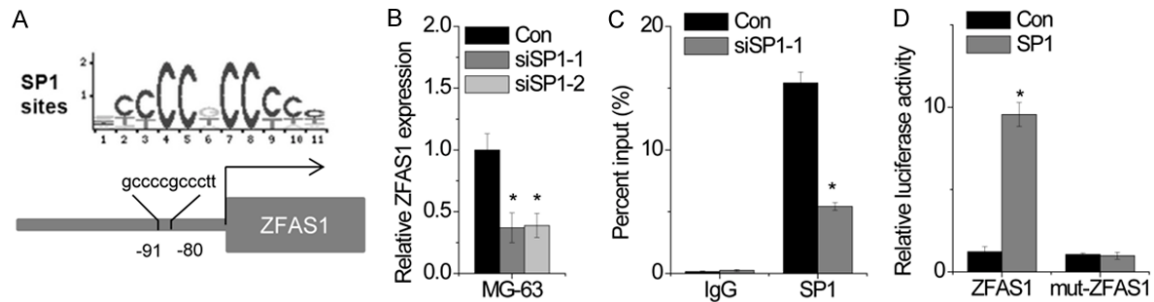
**Figure 6.** ZFAS1 regulates ZEB2 stability. A. Biotin-RNA pull-downs were performed with extracts of MG-63 cells using full-length ZFAS1 transcript (ZFAS1) and antisense ZFAS1 (ZFAS1-AS). This was followed by mass spectrometry. B. The interaction of ZFAS1 with ZEB2 was confirmed by an RNA immunoprecipitation (RIP) assay. C. Deletion mapping analysis of ZEB2-binding domains of ZFAS1. Shown are the following: schematic diagram of ZFAS1 full-length and truncated fragments (top panel) and western blot of ZEB2 in RNA pull-down samples by different ZFAS1 fragments (bottom panel). D. The ZEB1 mRNA and protein expression in control and ZFAS1 overexpression U2OS cells. E. The ZEB1 mRNA and protein expression in control and ZFAS1 knockdown MG-63 cells. F. The stability of ZEB2 protein over time was measured by western blot relative to time 0 after blocking new protein synthesis with CHX in control and ZFAS1 knockdown MG-63 cells. G. The stability of ZEB2 protein over time was measured by western blot relative to time 0 after blocking new protein synthesis with CHX in control and ZFAS1 overexpressed U2OS cells. H. ZEB2 protein expression in control and ZFAS1 knockdown MG-63 cells treated with vehicle control or MG132. Data are shown as mean  $\pm$  s.d. (n = 3) and are representative of three independent experiments. \*P<0.05.

In contrast, inhibition of miR-200b or miR-200c overcame the suppression of BMI1 by ZFAS1 knockdown (Figure 5F). To confirm BMI1 3'UTR is involved in this regulation, we constructed luciferase reporters containing BMI1 3'UTR. The luciferase activity of BMI1 3'UTR is increased by ZFAS1, while abolished by miR-200b or miR-200c overexpression (Figure 5G). In contrast, the knockdown of ZFAS1 decreased the luciferase activity of BMI1 3'UTR, which

were rescued by miR-200b or miR-200c silence (Figure 5H). All these results suggest an important role of ZFAS1 in regulating BMI1 by competitively binding miR-200b/c.

#### ZFAS1 regulates ZEB2 stability

LncRNA also interacted with proteins to exerts its function. To determine associated proteins of ZFAS1, we performed RNA pull-down assays



**Figure 7.** SP1 Activates ZFAS1 Transcription. A. SP1 binding site prediction in the ZFAS1 promoter region using JASPAR. B. The relative expression of ZFAS1 in MG-63 cells transfected with siRNAs against SP1 was determined by qPCR. C. ChIP-qPCR analysis of SP1 occupancy in the ZFAS1 promoter in control and SP1 knockdown MG-63 cells. D. Luciferase assays of the cells indicated that were transfected with pGL3 vector containing ZFAS1 promoter region or its mutant, the SP1 vector, or an empty vector. Data are shown as mean  $\pm$  s.d. (n = 3) and are representative of three independent experiments. \*P<0.05.

followed by mass spectrometry in MG-63 cells. One differential band between 170 kDa and 130 kDa appeared by silver staining (Figure 6A) and identified to be ZEB2 by mass spectrometry. To confirm the direct interaction between ZFAS1 and ZEB2, we performed anti-ZEB2 RIP assay in MG-63 cells. We found that endogenous ZFAS1 could be significantly enriched by ZEB2 antibody compared with negative IgG and unrelated RNA ACTB mRNA (Figure 6B). Furthermore, through domain mapping and RNA pull-down assay, we found that the 3'-end fragment of ZFAS1 (600 to 1020 nt) was essential to bind ZEB2 (Figure 6C). Taken together, we demonstrated a specific association between ZFAS1 and ZEB2.

Next, we determined the functional association between ZFAS1 and ZEB2. Silence of ZFAS1 significantly suppressed the protein level of ZEB2, but had no effect on the ZEB2 mRNA level (Figure 6D). In contrast, overexpression of ZFAS1 increased the protein level, but not mRNA level, of ZEB2 (Figure 6E). These results strongly indicated that ZFAS1 may influence the degradation of ZEB2. To further confirm this regulation, control and ZFAS1-silenced MG-63 cells were treated with protein synthesis inhibitor, cycloheximide (CHX). We found that the half-life of ZEB2 was much shorter in ZFAS1 knockdown cells than that in control cells (Figure 6F). In contrast, ZFAS1 upregulation significantly elongated the half-life of ZEB2 (Figure 6G). When MG132, an inhibitor of proteasome degradation, was used, the ZEB2 protein level in ZFAS1 knockdown MG-63 cells was markedly upregulated and reached a level that was

comparable to that in control cells (Figure 6H). Taken together, these data suggested that ZFAS1 is important for the stability of ZEB2 protein.

#### SP1 activates ZFAS1 transcription

Finally, we explored the upstream of ZFAS1. Using the JASPAR online database, we selected transcription factor SP1, which is predicted to be bound to the ZFAS1 promoter region (Figure 7A). We transiently transfected MG-63 cells with siRNAs against SP1. Interestingly, we found that knockdown of SP1 significantly inhibited ZFAS1 expression (Figure 7B). Moreover, we designed one primer that covered the SP1 binding site and performed ChIP assays followed by qPCR to validate whether SP1 could bind to this site. The ChIP results showed that SP1 bind to this site, and downregulation of SP1 suppressed the SP1 binding level (Figure 7C). Next, we constructed the luciferase reporter plasmids containing ZFAS1 promoter region with wild-type or mutant SP1 binding site. Dual luciferase reporter assays showed that SP1 increased the luciferase activity of ZFAS1 promoter with SP1 binding site, but had no effect on ZFAS1 promoter with mutant SP1 binding site (Figure 7D). These results demonstrated that ZFAS1 transcription is regulated by SP1.

#### Discussion

In the present study, for the first time, we revealed a functional role of ZFAS1 on OS growth and metastasis. The expression of ZFAS1 was significantly overexpressed in OS

samples and cell lines, and upregulation of ZFAS1 is significantly associated with unfavorable prognosis of OS patients. Functional assays also demonstrated that ZFAS1 enhanced the growth and metastatic ability of OS cells *in vitro* and *in vivo*. Mechanistic investigation demonstrated that ZFAS1 positively regulated malignant phenotypes by competitively binding the miR-200b and miR-200c and upregulating BMI1. ZFAS1 also interacted with ZEB2 to regulate its stability. Furthermore, SP1 functions as an activated factor of ZFAS1. ZFAS1 may be a potential therapeutic target for OS tumorigenesis and progression.

lncRNAs can regulate gene expression through different mechanisms, and play important roles in biological processes. Many studies have shown that lncRNAs act as oncogenes or cancer suppressor genes to affect tumorigenesis, metastasis, prognosis or diagnosis [19-21]. The mechanism and function of ZFAS1 have been studied in some kinds of cancers. For example, ZFAS1 increased p53 stability and interacted with CDK1/cyclin B1 complex to promote cell cycle progression and inhibit cell apoptosis in colorectal cancer [22]. Moreover, ZFAS1 could simultaneously interact with EZH2 and LSD1/CoREST to repress underlying targets KLF2 and NKD2 transcription, and then enhance the tumor growth in gastric cancer [23]. A previous study showed a closely relationship between ZFAS1 expression and EMT markers, and ZFAS1 could increase ZEB1 mRNA level [11]. Another study also demonstrated that ZFAS1 upregulated ZEB1 expression through interaction with miR-150 [8]. Aberrant overexpression of BMI1 is associated with a number of malignancies and correlated with poor prognosis [24, 25]. BMI1 serves as the key regulatory component of the PRC1 (polycomb repressive complex-1) complex. BMI expression is thought to promote stem state, proliferation and invasion [26, 27]. In our present study, for the first time, we found that ZFAS1 could function as a ceRNA of BMI1. ZFAS1 competed with BMI1 mRNA 3'UTR region for common miRNAs, miR-200b and miR-200c. Furthermore, ZFAS1 transcript level was significantly correlated with BMI1 mRNA level in OS tissues. All results strongly supported that ZFAS1 functions as a ceRNA. Furthermore, we determined the associated protein of ZFAS1 through RNA pull-down and mass spectrometry

analysis. We found that ZFAS1 directly interacted with ZEB2 to regulate its stability. For the first time, we revealed the interaction and regulatory relationship between ZFAS1 and ZEB2. We also explored the upstream of ZFAS1. We found that SP1 could directly bind to ZFAS1 promoter and activate its transcription. These findings indicate that some transcription factors can contribute to human cancer development and progression not only through affecting protein coding genes expression but also through regulating lncRNAs transcription.

Taken together, our research demonstrated that transcription factor SP1 binds directly to the ZFAS1 promoter and activates its transcription. ZFAS1 acts as a critical regulator of OS growth and metastasis. As direct targets of ZFAS1, miR-200b/c and BMI1 and ZEB2 mediated the role of ZFAS1 in growth and metastasis, indicating that ZFAS1 could be an effective target for OS therapies.

#### Disclosure of conflict of interest

None.

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