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Original Article

Long non-coding RNA ZEB2-AS1 regulates osteosarcoma progression by acting as a molecular sponge of miR-107 to modulate SALL4 expression

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Abstract: Increasing evidence has confirmed long non-coding RNAs (lncRNAs) as important regulators involved in several pathophysiological processes in many diseases. The aim of this study was to investigate the roles of lncRNA ZEB2-AS1 (ZEB2-AS1) in osteosarcoma (OS). The levels of ZEB2-AS1 in OS tissues and cells were detected using RT-PCR. The clinical significance of ZEB2-AS1 expressions in OS patients was statistically analyzed. The functional effects of ZEB2-AS1 on the proliferation, apoptosis, invasion, and metastasis of OS cells was determined by a series of cellular experiments. Bioinformatic analysis, dual-luciferase reporter assays and pull-down assays were carried out for the confirmation of the molecular binding. We found that ZEB2-AS1 expression was distinctly upregulated in OS specimens and cell lines. Higher levels of ZEB2-AS1 in OS patients were associated with clinical stage, distant metastasis and unfavorable survivals. A multivariate Cox model revealed that ZEB2-AS1 expression was an independent prognostic factor for OS patients. Cellular experiments revealed that knockdown of ZEB2-AS1 inhibited proliferation and metastasis, and induced apoptosis *in vitro*. Mechanistic investigation revealed that ZEB2-AS1 acted as a sponge for miR-107 and blocked the inhibition of spalt like transcription factor 4 (SALL4) via miR-107 in OS cells. Rescue experiments suggested that up-regulation of ZEB2-AS1 could partly attenuate the miR-107 mediated inhibition of SALL4 expression in OS cells. To sum up, our data revealed that ZEB2-AS1 played an oncogenic role in OS progression, and could serve as a novel molecular target for treating this tumor.

Keywords: LncRNA ZEB2-AS1, miR-107, SALL4, prognosis, invasion, osteosarcoma

Introduction

Osteosarcoma (OS) is one of the most common primary high-grade bone malignancies frequently occurring in the metaphysis of long bones mainly in adolescents and young adults [1]. In China, the estimated incidence of this tumor is 5 per million per year [2]. Over the past decades, the treatment strategies of giving preoperative chemotherapy followed by clinical surgical operation and standard adjuvant treatments have resulted in great improvement in long-term survivals of OS patients with early stages [3, 4]. Unfortunately, despite the advancements in the diagnosis and treatment of OS, the clinical outcome of patients with metastases and recurrence is rather poor with a five-year survival rate of only 10-30% [5, 6]. Thus, a better understanding of the mechanisms involved in the progression of OS is urgently needed for the development of novel diagnostic and therapeutic strategies.

Non-coding RNAs lacking protein-coding abilities were previously regarded as 'junk' or 'noise' RNAs and occupied the majority in human genomes [7]. However, more and more evidences confirm that non-coding RNAs play a functional role in the regulation of gene expressions [8]. Long noncoding RNAs (lncRNAs) are a group of long RNA transcripts that are > 200 nucleotides in length [9]. LncRNAs have been demonstrated to exhibit functional effects in multiple biological processes, such as cellular growth, pyroptosis, apoptosis and differentiation [10, 11]. Emerging studies suggest that

Table 1. Primers used for qPCR in this study

Names	Sequences (5'-3')
ZEB2-AS1: F	ATGAAGAAGCCGCGAAGTGT
ZEB2-AS1: R	CACACCCTAATACACATGCCCT
miR-107: F	AGCAGCATTGTACAGGG
miR-107: R	TGCAGGGTCCGAGGT
SALL4: R	AGCACATCAACTCGGAGGAG
SALL4: F	CATTCCCTGGGTGGTTCACTG
GAPDH: F	CGAGATCCCTCCAAAATCAA
GAPDH: R	TTCACACCCATGACGAACAT
U6: F	CTCGCTTCGGCAGCACA
U6: R	AACGCTTCACGAATTTGCGT

lncRNAs could act as novel prognostic and diagnostic biomarkers and therapeutic targets and may mediate anti-oncogenic or tumor-promotive effects in various cancers, including OS [12-14]. However, a large number of tumor-related lncRNAs need to be identified and functionally characterized.

LncRNA ZEB2-AS1 (ZEB2-AS1) has attracted our attentions due to its dysregulation and potential effects in several tumors [15, 16]. However, the roles of ZEB2-AS1 in OS progression remain unknown. In this study, we firstly showed that ZEB2-AS1 expression was up-regulated in OS and may be a prognostic biomarker. Functional assays revealed that ZEB2-AS1 functioned as a tumor promoter in OS by sponging miR-107 to modulate spalt like transcription factor 4 (SALL4). Our findings identified a novel OS-associated lncRNA ZEB2-AS1, which may contribute to the advancement of lncRNAs-directed diagnostics and therapeutics against OS.

Materials and methods

Patient data and tissue samples

110 OS specimens and paired adjacent non-tumor specimens were collected from patients undergoing operations in the Linyi Cancer Hospital, between July 2011 and June 2013. The written informed consent and approval were provided by each patient prior to the study. The diagnosis of OS was histopathologically confirmed by two pathologists from our hospital. All patients did not receive any immunosuppressive therapeutics or conventional chemotherapy before clinical resection. Specimens samples were obtained from specimens frozen in liquid nitrogen and stored at -80°C. The histological grade of OS was determined based on the model of the World Health Organization. Our research was approved by the Bioethics Committee of Linyi Cancer Hospital.

Cell culture and transfection

Human OS cell lines (143B, Saos-2, MG-63, U2OS and HOS) and normal bone cell (Hfob1.19) were obtained from (Yaji Technology, Pudong, Shanghai, China). Cells were grown in DMEM medium (ATCC 30-2007, Gibo, Zhengjiang, Hangzhou, China) with 10% FBS (GIBCo/BRL1, Pudong, Shanghai, China), supplemented with 100 U/ml penicillin G (MedChem, Haidian, Biejing, China) and 100 μg/ml streptomycin (Sigma Corps, Madison, WI, USA). Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

To silence ZEB2-AS1 expression in MG63 and U2OS cells for functional assays, the target sequence for ZEB2-AS1 siRNA or scrambled siRNA was synthesized by JiMa Technology (Pudong, Shanghai, China). The pcDNA3.1-ZEB2-AS1 and empty vector (used as a negative control) were purchased from JiMa Technology (Pudong, Shanghai, China). MiR-107 mimics and inhibitors as well as their controls were purchased from Aoweiya Biotechnology (Haidian, Biejing, Chian). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for cell transfections.

RNA extractions and real-time quantitative PCR

Total cellular RNA from tumor specimens and cells was extracted using the TRIzol solution (Invitrogen, Tongzhou, Beijing, China). RNA concentration was assessed with a NanoDrop spectrophotometer (Life Technologies, Haidian, Biejing, China). The First Strand cDNA synthesis kit which was purchased from Takara (Hangzhou, Zhejiang, China) was used for teverse transcription. qRT-PCR was carried out on an the ABI PRISM® 7950HT Sequence Detection System (ThermoFisher Scientific, Waltham, MA, USA), along with a SYBR® Green Master Mix kit (ThermoFisher Scientific, Waltham, MA, USA). Transcripts were normalized to the amount of GAPDH expression. Comparative quantification was determined using the method of $2^{-\Delta\Delta CT}$. The specific primers are shown in **Table 1**.

Cell proliferation and colony formation assays

Twenty-four hours after transient transfection, cells were seeded into 48-well plates at a density of 4000 cells/well. CCK-8 solution was added 24, 48, 72, and 96 h after placing and incubated for 2 hours at 37°C. Then, optical density was assessed at 450 nm on a Synergy 3 microplate reader (Biotek, Xuhui, Shanghai, China).

In the colony formation assays, 1.5×10^3 cells were seeded in 48-well culture plates 24 h after transfection and then cultured in growth medium. 14 days later, the cells were fixed in 4% paraformaldehyde (CAS#: 30525-88, Dingguo, Haidian, Beijing, China). Then, crystal violet solution (Beyotime, cat#C0124, Pudong, Shanghai, China) was use to stain the cells. The number of colonies containing ≥80 MG63 and U2OS cells was calculated under a microscope.

Ethynyldeoxyuridine (EdU) assays

The effects of ZEB2-AS1 knockdown on cellular proliferation were also analyzed applying an EdU assays kit (#3381, RiboBio, Hangzhou, Zhejiang, China) following the manufacturer's protocol. A total of 2×10^5 cells was inoculated in a 48-well plate after transfection. Subsequently, fifty μM EdU labeling medium (ScienceDirect, Tangku, Tianjin, China) was added to the cells for two hours at 37°C under 5% CO_2. 4% paraformaldehyde (pH = 7.4) was used to fix cells and Glycine was used to neutralize the paraformaldehyde. Nucleus was double-stained with EdU and DAPI (Beyotime, Hiadian, Biejing, China) as cells of positive proliferation. The stained cells were visualized with a microscope.

Flow cytometry

Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (Beyotime, Haidian Biejing, China) was applied to explore the mechanisms of cell death after transfection. The cells were washed twice and centrifuged at 400 g with pre-cooled PBS, and then 100 μl of 1 × binding buffer was used to resuspend the cells. The cells were stained with Annexin V-FITC/PI based on the handbook of users. The assays were carried out in a BD FACSCantoo II (Biosciences, Nantong, Jiangsu, China).

Scratch-wound healing assays

Wound healing assays were applied to detect cellular abilities of migration. Briefly, the cells were pretreated with mitomycin C (Nanhu, Technology, Haidian, Biejing, China) for 4 h to suppress cell growth. The cells were serum-starved for 1 day for cell cycle synchronization, and pipette tips were used to scratch a confluent cell monolayer to further artificial creation of wounds. Drawing was amassed at the same locations of the plate at different time. The wound closure was photographed under a microscope.

Transwell assays

For the exploration of the effects of ZEB2-AS1 on cellular invasion, transwell chamber system (Biosystems, Hangzhou, Zhejiang, China) was used. Cells at 48 hr after transfection were washed, trypsinized and adjusted to 3×10^5 cells/ml. The collected cells seeded into the upper chamber of Boyden Chambers (Qinling, Haimen, Jiangsu, China) coated with Matrigel (Weijing, Pudong, Shanghai, China). After incubation, the nonmigrating cells on the upper chamber were wiped away using a cotton swab and the invasive cells were fixed in 4% paraformaldehyde and stained in 0.1% crystal violet solution. A microscope was used to count cells. Three independent experiments were carried out.

Luciferase reporter assays

The bioinformatics databases (Starbase v2.0 and miRcode) was used for the predication of potential miRNAs that can bind to ZEB2-AS1. We cloned wild-type ZEB2-AS1 with potential miR-107 binding sites or mutants of each site into a pmirGLO Dual-luciferase Targeting Vector (Promega, Zhejiang, Hangzhou, China). Similarly, the 3'UTR of SALL4 which wildtype (SALL4-WT) and mutated at the miR-107 binding-sites (SALL4-MT) was cloned into the pGL3-basic vector to synthetize a pGL3-SALL4-WT and pGL3-SALL4-MT vector, respectively. For reporter assays, U2OS cells were co-transfected with wild-type (mutant) reporter plasmids and miRNA-107 mimic or control mimics. The luciferase activity was examined by applying Dual-Luciferase Reporter Systems (Promega, Hangzhou, Zhejiang, China). Experiments were performed in triplicate.

RNA-pull down assays

ZEB2-AS1 RNA was transcribed from pcDNA-ZEB2-AS1 and was biotin-labeled with the T7 RNA polymerase (Roche, Pudong, Shanghai, China) and the Biotin RNA Labeling Mix (Roche, Pudong, Shanghai, China). 293T cells transfected with biotin-labelled ZEB2-AS1 were collected and sonicated 48 h after transfection. Then, cell lysates were added with beads and incubated for two h at room temperature. qRT-PCR assays were performed to further examine the levels of RNA in the collected materials.

Statistical analyses

Statistical analysis was performed using SPSS software 17.0 (SPSS Inc., Chicago, IL, USA). Data were tested using two-tailed Student's t-test or one-way ANOVA. Chi-square tests were used to compare the levels of ZEB2-AS1 and clinicopathological parameters of OS patients. Survival curves were delineated employing Kaplan-Meier method, and log-rank tests were used for the determination of the differences. Univariate and multivariate assays of factors influencing survivals were performed by the use of the Cox hazards models. $P < 0.05$ was considered statistically significant.

Results

ZEB2-AS1 expression in human OS specimens and cells

We firstly searched GEPIA which can analzye the expression pattern of genes in TCGA datets and observed that the increased expression of ZEB2-AS1 was a common event in several types of tumors (**Figure 1A**). However, the Chip data of OS is missing. To study the relation between ZEB2-AS1 expressions and malignant phenotype of OS, our group performed RT-PCR to detect the levels of ZEB2-AS1 in OS patients. As shown in **Figure 1B**, ZEB2-AS1 expression was upregulated in OS specimens compared with matched noncancerous tissues ($P < 0.01$). In addition, higher expression of ZEB2-AS1 was observed in tumor stages III-IV compared to tumor stages I-II (**Figure 1C**). Interestingly, the results of ROC assays indicated that high ZEB2-AS1 expression had an AUC value of 0.7133 (95% CI: 0.6445 to 0.7821) in distinguishing OS specimens from non-tumor bone specimens (**Figure 1D**). In addition, the results of real-time PCR indicated distinctly increased expressions of ZEB2-AS1 in five OS cell lines compared with Hfob1.19 cells (**Figure 1E**). These results revealed that ZEB2-AS1 may influence OS progression. For the subsequent cellular experiments, U2OS and MG63 cells were used due to the relative higher levels of ZEB2-AS1 in the above two cells.

ZEB2-AS1 upregulation associates with poor prognosis in patients with human OS

Given the frequent upregulation of ZEB2-AS1 in OS, we wondered whether ZEB2-AS1 had a clinical influence on OS patients. **Table 2** summarized the associations between ZEB2-AS1 expressions and clinicopathological parameters in OS. We showed that high levels of ZEB2-AS1 were positively associated with clinical stage ($P = 0.011$) and distant metastasis ($P = 0.026$). Moreover, the Kaplan-Meier analysis was performed to further explore the potential values of ZEB2-AS1 as a biomarker in OS patients. As shown in **Figure 1D**, the data showed that the high-ZEB2-AS1 group had a significantly reduced OS ($P = 0.0114$) and DFS ($P = 0.0020$) compared to the low-ZEB2-AS1 group. Moreover, univariate model indicated a statistically significant association between survivals and several factors, including distant metastasis, clinical stage and ZEB2-AS1 expression ($P < 0.05$, **Table 3**). Besides, multivariate assays enrolling above parameters suggested that high ZEB2-AS1 expression was an independent prognostic marker for both OS (HR = 2.857, 95% CI: 1.285-4.562, $P = 0.016$, **Table 3**) and DFS (HR = 2.985, 95% CI: 1.385-4.458, $P = 0.015$, **Table 4**) of OS patients, in addition to clinical stage and distant metastasis.

ZEB2-AS1 promotes OS cell proliferation, migration and invasion

The overexpression of ZEB2-AS1 and its clinical values in OS patients promoted us to further explore the function of ZEB2-AS1 in OS cellular behaviors. Using small interfering RNA, we down-regulated the levels of ZEB2-AS1 in U2OS and MG63 cells and the results of RT-PCR confirmed si-ZEB2-AS1-1 and si-ZEB2-AS1-2 could reduce ZEB2-AS1 expression (**Figure 2A**). The results of CCK-8 assays revealed that cell growth was significantly impaired in si-ZEB2-AS1-transfected U2OS and

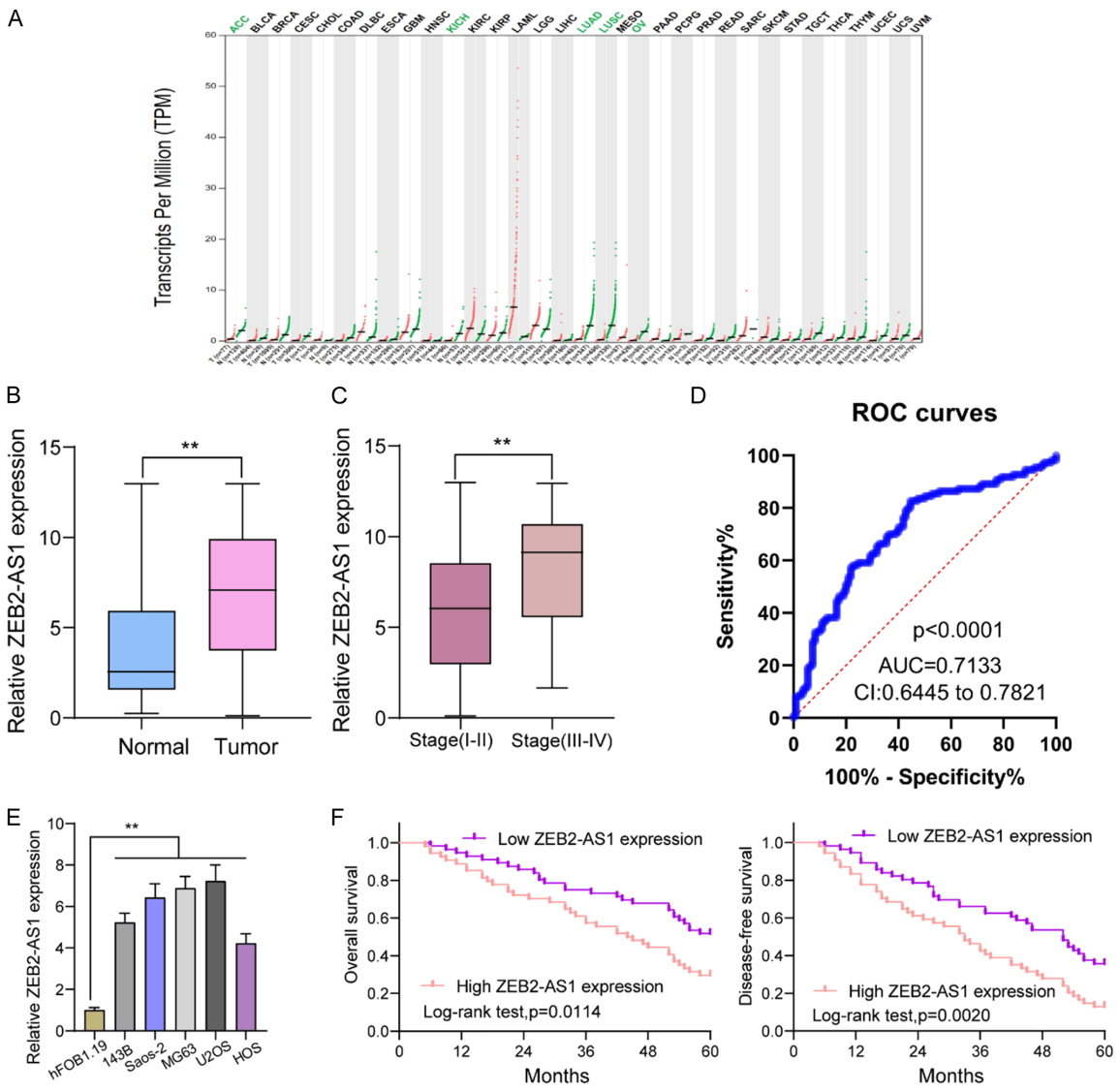


Figure 1. The levels of ZEB2-AS1 in OS tissues and cell line, and its clinical values. A. The pan-cancer expression of ZEB2-AS1 based on TCGA datasets. B. The relative expression level of ZEB2-AS1 expression in OS tissues and adjacent normal specimens was determined by qRT-PCR. C. Higher levels of ZEB2-AS1 were observed in patients with advanced stages. D. ROC assays for ZEB2-AS1 as a diagnostic marker for OS patients. E. ZEB2-AS1 expression in five OS cells and Hfob1.19 cells was measured by RT-PCR. F. Kaplan-Meier methods suggested that OS patients with high levels of ZEB2-AS1 expression were correlated with a poor overall survival and disease-free survival. $^{**}P < 0.01$, $^{*}P < 0.05$.

MG63 cells (**Figure 2B**). Similar findings were obtained using colony-formation assays, ZEB2-AS1 knockdown decreased clonogenic survival of U2OS and MG63 cells (**Figure 2C**). Moreover, EDU staining was further carried out to demonstrate the effects of ZEB2-AS1 knockdown on cellular ability. As shown in **Figure 2D**, fewer EDU-positive cells were observed in the si-ZEB2-AS1 group compared to the si-NC group. In order to further explore the mechanisms by which ZEB2-AS1 contributed to cellular growth, Flow cytometric assays were carried out, and the results suggested that the percentage of apoptotic cells was increased with ZEB2-AS1 knockdown (**Figure 2E**). Then, we further explored whether ZEB2-AS1 had a functional effect on metastasis abilities of OS cells. The results of wound healing assays indicated that cell migration was enhanced when ZEB2-AS1 was suppressed (**Figure 3A**). Consistent with this result, we also found that knockdown of ZEB2-AS1 led to a sig-

Table 2. Correlation of ZEB2-AS1 expression with clinicopathological features of osteosarcoma

Clinicopathological features	Number of cases	ZEB2-AS1 expression		*p* value
		High	Low	
Gender				0.436
Male	59	31	28	
Female	51	23	28	
Age (years)				0.708
<25	57	27	30	
≥25	53	27	26	
Tumor size (cm)				0.129
<8	65	28	37	
≥8	45	26	19	
Anatomic location				0.574
Tibia/femur	52	27	25	
Elsewhere	58	27	31	
Clinical stage				0.011
I/II	72	29	43	
III-IV	38	25	13	
Distant metastasis				0.026
Absence	78	33	45	
Presence	32	21	11	

nificant decrease in the capability of OS cell invasion using Transwell assays (**Figure 3B** and **3C**). Overall, our findings revealed that ZEB2-AS1 acted as a tumor promoter in OS cells.

ZEB2-AS1 binds to miR-107 and represses its expressions

Some functional lncRNA has been demonstrated to communicate with miRNAs via shared common miRNA binding sites and further result in the suppression of the activity of miRNAs. Firstly, we performed subcellular fractionation to verify ZEB2-AS1 localization in OS cells, finding that ZEB2-AS1 was mainly expressed in cytoplasm, suggesting that ZEB2-AS1 may act as a ceRNA (**Figure 4A**). Then, our group checked two bioinformatic for the exploration of the potential miRNAs targeting ZEB2-AS1. Interestingly, miR-107, a tumor suppressor in OS, could bind to ZEB2-AS1 (**Figure 4B**). Then, we examined the expression of miR-107 in our cohort, further confirming that miR-107 was highly expressed in OS specimens compared to matched non-tumor tissues (**Figure 4C**). Moreover, the ROC assays revealed that high miR-107 expression had an AUC value of 0.7269 (95% CI: 0.6585 to 0.7954) in distinguishing OS specimens from non-tumor bone specimens (**Figure 4D**). Furthermore, RNA-pull down assays directly proved that ZEB2-AS1 could interact with miR-107 in both U2OS and MG63 cells (**Figure 4E**). To further demonstrate the association between ZEB2-AS1 and miR-107, we subcloned full-length ZEB2-AS1 or ZEB2-AS1 harboring a site-directed mutation in the miR-107-binding site into the pmirGLO Dual-luciferase Targeting Vector (referred to as ZEB2-AS1-WT or ZEB2-AS1-MUT, respectively). Then, the results of Luciferase reporter assays revealed that co-transfetion of miR-107 mimics and pmirGLO-ZEB2-AS1-wt distinctly decreased the luciferase activity, whereas co-transfection of control mimics and pmirGLO-ZEB2-AS1-wt remained unchanged (**Figure 4F**). Similarly, cells co-transfected with miR-107 and pmirGLO-ZEB2-AS1-mt exhibited no dramatical changes in luciferase activity (**Figure 4F**). Finally, the potential effects of the dysregulation of ZEB2-AS1 on the expressions of miR-107 were analyzed using RT-PCR. As shown in **Figure 4F**, overexpression of ZEB2-AS1 suppressed the levels of miR-107, while knockdown of ZEB2-AS1 showed an opposite trend (**Figure 4G**). In addition, we also observed that overexpression of miR-107 could suppress the levels of ZEB2-AS1 in U2OS and MG63 cells (**Figure 4H**). Overall, our findings suggested that ZEB2-AS1 was targeted by miR-107.

miR-107 directly targeted SALL4

To further study the molecular mechanisms underlying miR-107 displayed its tumor-suppressive roles in OS cells, our group searched three online tools to explore the potential targeting genes of miR-107 (**Figure 5A**). Among these potential candidates, SALL4 attracted our attentions due to its important roles in several tumor progression via promoting tumor cells proliferation and metastasis (**Figure 5B**) [17, 18]. Subsequently, our group constructed two types of plasmids containing the luciferase

Table 3. Univariate and multivariate analysis of overall survival in osteosarcoma patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age	1.275	0.662-1.994	0.217	-	-	-
Gender	1.446	0.723-2.216	0.179	-	-	-
Tumor size	1.216	0.989-2.014	0.188	-	-	-
Anatomic location	1.385	0.785-1.958	0.137	-	-	-
Clinical stage	2.986	1.325-4.782	0.013	2.786	1.185-4.542	0.025
Distant metastasis	3.015	1.472-5.127	0.005	2.893	1.285-4.628	0.011
ZEB2-AS1 expression	3.174	1.385-4.775	0.008	2.857	1.285-4.562	0.016

Table 4. Univariate and multivariate analysis of disease-free survival in osteosarcoma patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Age	0.873	0.5438-2.213	0.238	-	-	-
Gender	1.213	0.784-1.982	0.177	-	-	-
Tumor size	1.543	0.742-2.213	0.358	-	-	-
Anatomic location	1.448	0.832-2.041	0.213	-	-	-
Clinical stage	3.137	1.375-5.428	0.008	2.876	1.284-4.887	0.011
Distant metastasis	3.427	1.485-5.338	0.001	3.015	1.238-4.978	0.003
ZEB2-AS1 expression	3.231	1.533-4.785	0.008	2.985	1.385-4.458	0.015

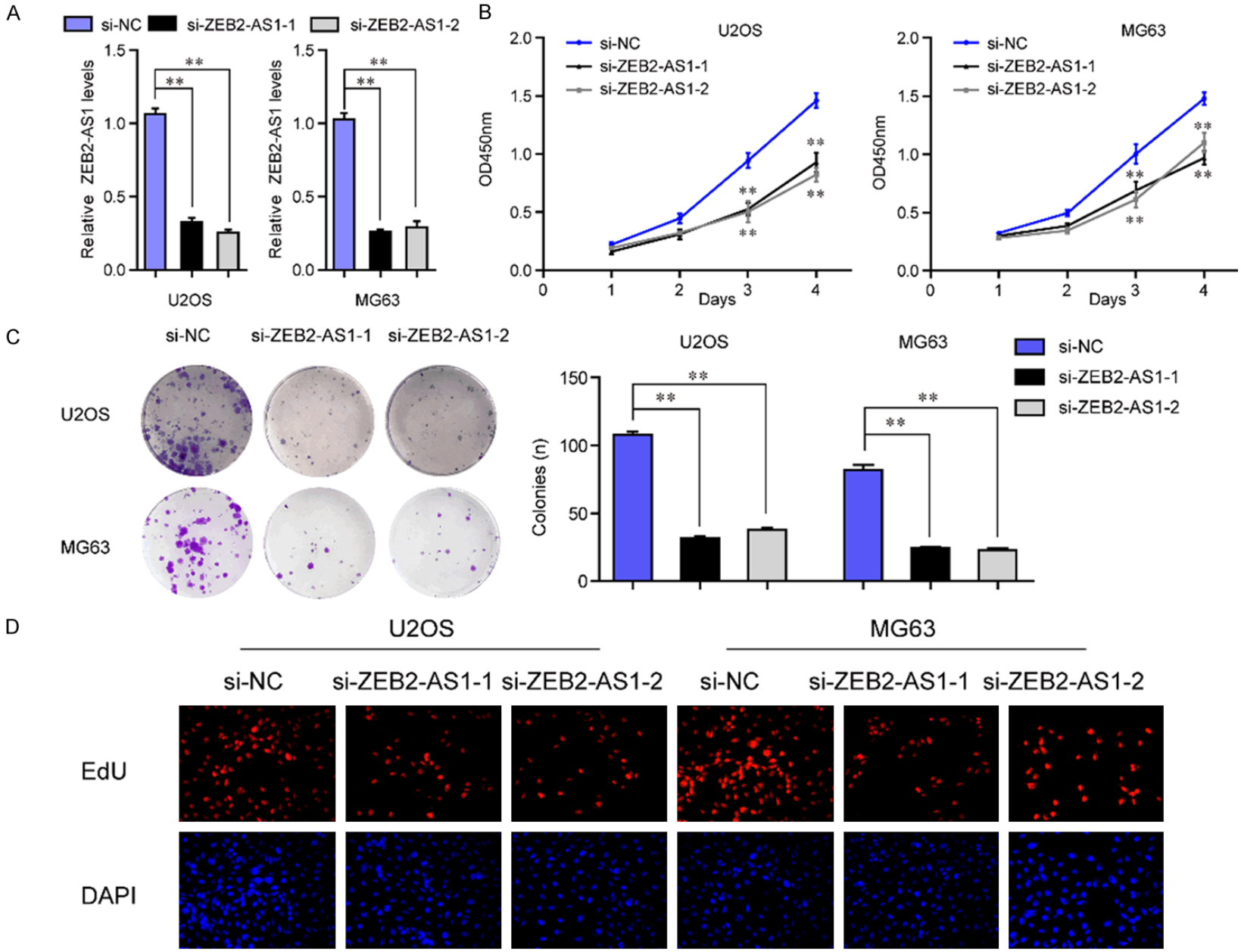
reporting gene and wild-type-1/2 or mutant-1/2 SALL4 3'UTR and co-transfected miR-107 mimics or miR-107 inhibitors into U2OS cells. As shown in **Figure 5C**, we observed that the transfection of miR-107 mimics suppressed the luciferase activity in U2OS cells of the wild-type 3'-UTR of SALL4 and the transfection of miR-107 inhibitors increased the luciferase activity of the wild-type 3'-UTR of SALL4. However, upregulation or downregulation of miR-107 did not distinctly influence the luciferase activity in U2OS cells of the mutant SALL4 3'-UTR (**Figure 5C**). Besides, we observed that SALL4 expression was distinctly upregulated in OS tissues and cell lines (**Figure 5D** and **5E**). Moreover, we performed RT-PCT to explore the possible effects of dysregulation of miR-107 on the expressions of SALL4, finding that knockdown of miR-107 promoted the expressions of SALL4. In contrast, upregulation of miR-107 distinctly suppressed the expressions of SALL4 in U2OS and MG63 cells (**Figure 5F**). Previously, SALL4 has been reported to invovled in the regulation of AKT and MAPK pathways. Thus, we further performed western blot to explore the influence of ZEB2-AS1 on the activity of AKT and MAPK pathways, finding that knockdown of ZEB2-AS1 suppressed the expressions of p-ERK, p-AKT and p-MAPK (**Figure 5G**). Taken together, our data indicated that miR-107 may displayed its tumor-suppressive effects by directly targeting SALL4.

Silence of ZEB2-AS1 decreases the OS progression by regulating miR-107/SALL4 axis

Then, we explored whether ZEB2-AS1 promoted the progression of OS via regulating miR-107/SALL4 axis. As shown in **Figure 6A**, we found that the mRNA levels of SALL4 were significantly decreased by ZEB2-AS1 knockdown in both U2OS and MG63 cells, and this reduction could be restored by down-regualtion of miR-107. More importantly, the results of rescue experiments revealed that knockdown of SALL4 markedly inhibited the proliferation, colony formation, invasion of U2OS and MG63 cells, while the effects were abolished by miR-107 down-regulation (**Figure 6B-E**). These results indicated that ZEB2-AS1 promoted the cell proliferation, migration, and invasion by regulating miR-107/SALL4 in OS cells (**Figure 7**).

Discussion

The advancements of High throughput sequencing demonstrate the fact that many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si-NC
si-ZEB2-AS1-1
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Relative ZEB2-AS1 levels
U2OS
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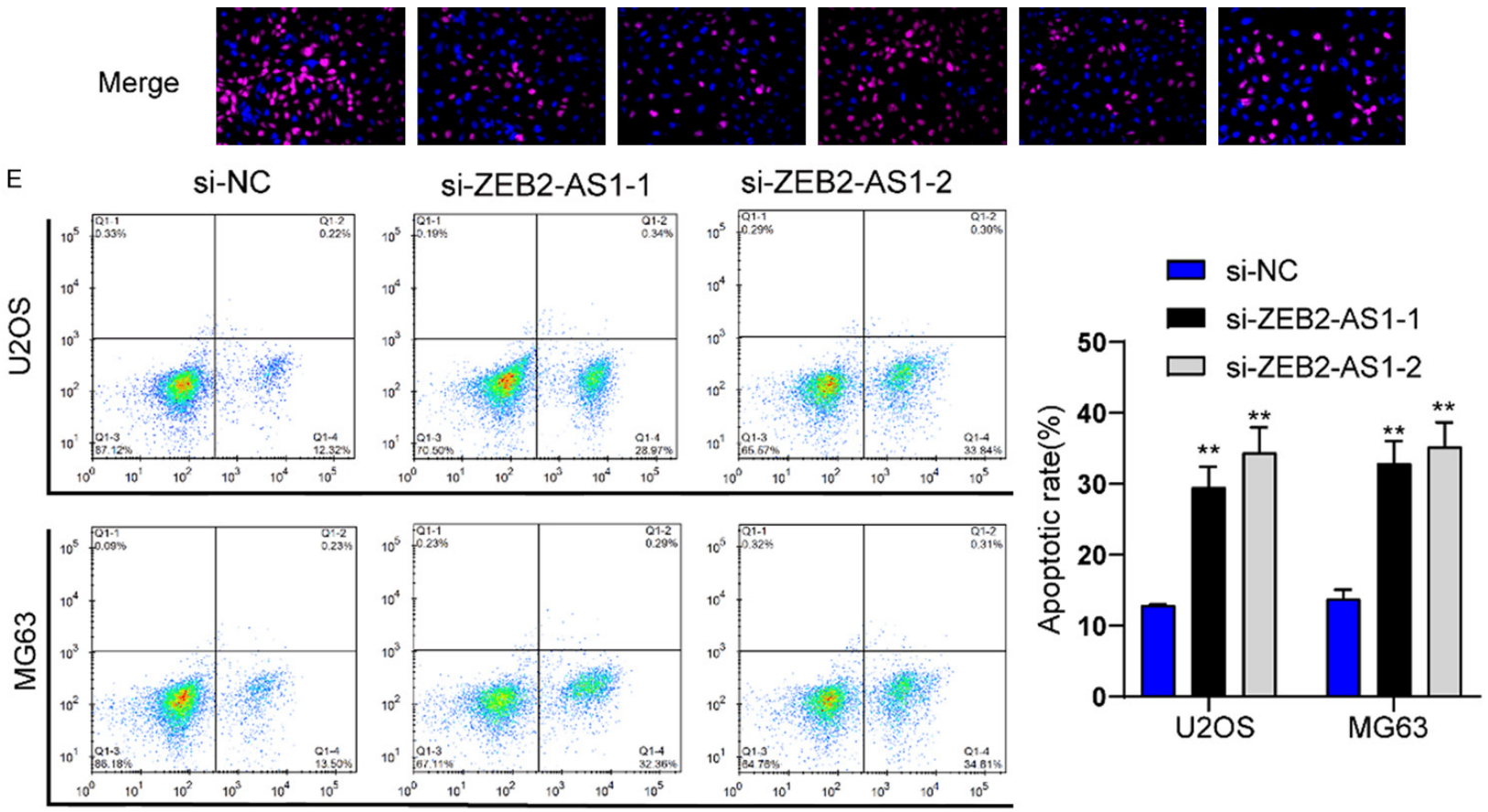

Figure 2. Knockdown of ZEB2-AS1 expression inhibits OS cell growth, colony formation, and promoted apoptosis. A. QPCR analysis of ZEB2-AS1 knockdown efficiency in U2OS and MG63 OS cells. B. The CCK-8 assay revealed that ZEB2-AS1 down-regulation distinctly decreased cell proliferation. C. The effects of knockdown of ZEB2-AS1 on cell proliferation cells by colony formation assays. D. EdU assays were performed to further examine the inhibition of G1/S transition in U2OS and MG63 cells. E. Knockdown of ZEB2-AS1 promoted the apoptosis of U2OS and MG63 cells. $^{**}P < 0.01$, $^{*}P < 0.05$.

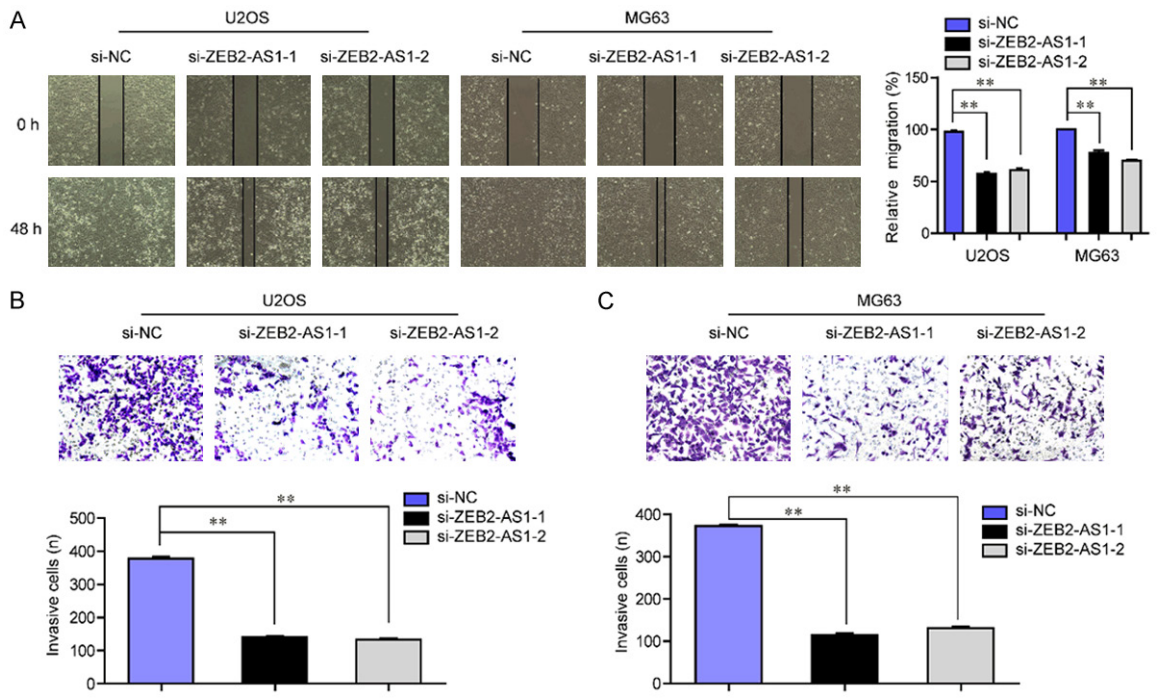

Figure 3. The effects of ZEB2-AS1 on OS cell migration and invasion in vitro. A. Representative micrographs of wound healing assays of U2OS and MG63 cells transfected with si-ZEB2-AS1 or si-NC. B, C. The effects of ZEB2-AS1 knockdown on invasion of U2OS and MG63 cells were analyzed using Transwell assays. $^{**}P < 0.01$, $^{*}P < 0.05$.

lncRNAs are abnormally expressed in tissues and blood, indicating potential values of lncRNAs in development and diseases and highlighting them as novel biomarkers and therapeutic targets [19, 20]. In this study, we identified a novel OS-related oncogene, ZEB2-AS1 which was distinctly highly expressed in both OS samples and cell lines using RT-PCR. ROC assays confirmed the diagnostic value of high ZEB2-AS1 expression in distinguishing OS specimens from normal bone specimens. Then, we performed clinical assays, finding that patients with higher ZEB2-AS1 expressions exhibited an advanced clinical stage and positively distant metastasis. Moreover, upregulation of ZEB2-AS1 predicted shorter OS and DFS of OS patients. Further multivariate analyses confirmed ZEB2-AS1 as an unfavorable prognostic factor in OS patients, which highlighted the potential value of ZEB2-AS1 used as a new diagnsoitc and prognostic biomarker for OS patients.

Growing studies showed that lncRNAs were involved in the tumor progression by modulating cell growth and metastasis [21]. Recently, the potential functions of ZEB2-AS1 in several tumors have been reported. For instance, Wu and his group reported that ZEB2-AS1 levels were upregulated in both bladder cancer cells and specimens and its knockdown using si-ZEB2-AS1 displayed tumor-suppressive roles in tumor cells by suppressing cell proliferation via modulating miR-27b [16]. Gao et al [15] suggested ZEB2-AS1 as a novel oncogenic lncRNA whose upregulation was obvious in pancreatic cancer. In their functional experiments, silencing ZEB2-AS1 was observed to inhibit pancreatic cancer cell growth and invasion via modulating miRNA-204/HMGB1 axis. Recent findings by Wang et al [22] indicated that overexpression of ZEB2-AS1 promoted the proliferation, migration and invasion of gastric cancer cells by the regulation of the Wnt/β-catenin pathway, suggesting it as a tumor promoter in this disease. However, the roles of ZEB2-AS1 in OS remained to be explored. In this study, functional analysis revealed knockdown of ZEB2-AS1 inhibited OS cell proliferation, migration and invasion. In addition, we also found that ZEB2-AS1 may protect OS cells from apoptosis. It has been demonstated that AKT and MAPK pathways are invovled in the tumor progression [23]. Mechanistic inves-

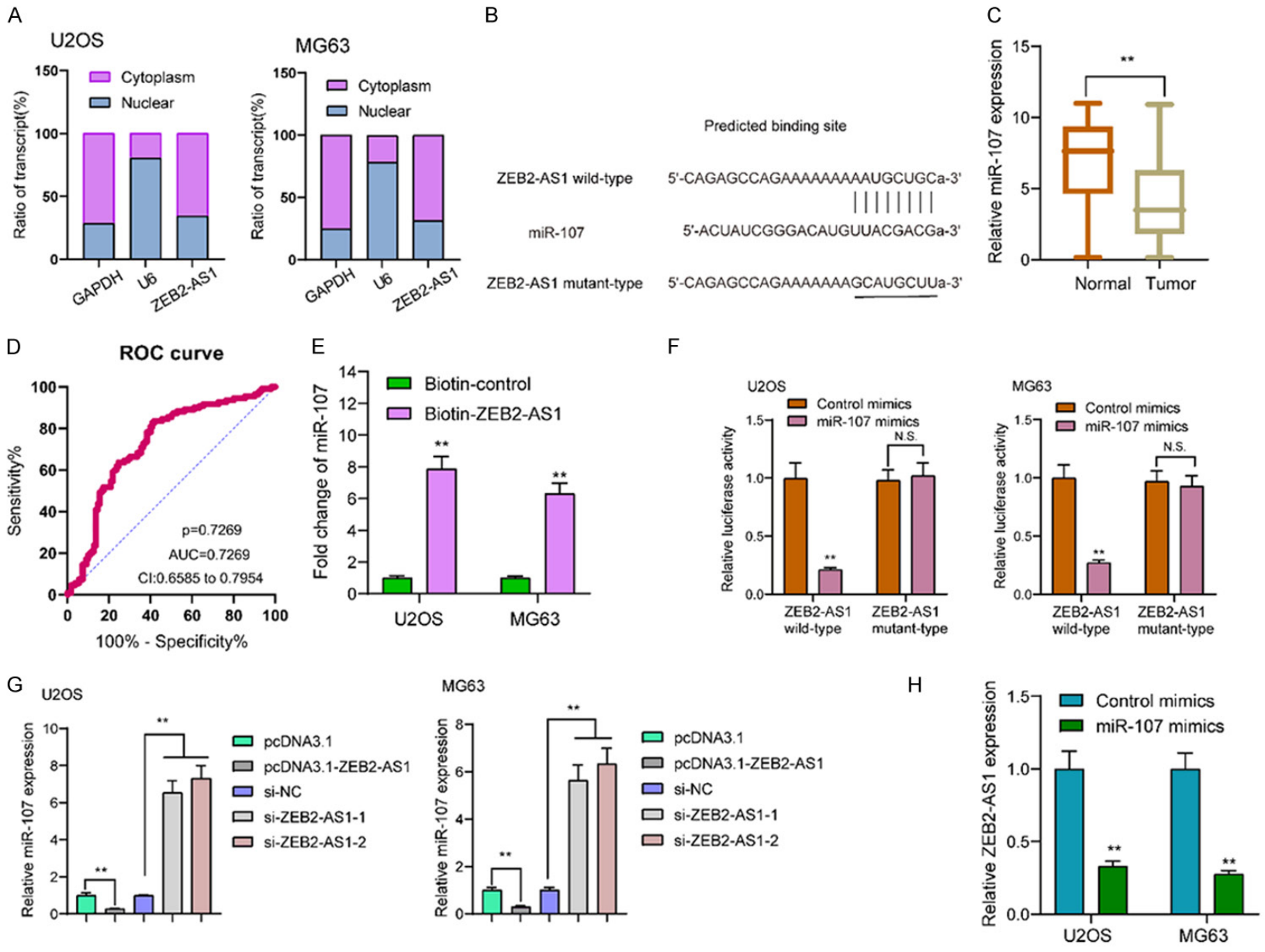
A
U2OS
MG63
Cytoplasm
Nuclear
Ratio of transcript(%)
GAPDH
U6
ZEB2-AS1
B
Predicted binding site
ZEB2-AS1 wild-type
5'-CAGAGCCAGAAAAAAAAAUGCUGCa-3'
miR-107
5'-ACUAUCGGGACAUGUUACGACGa-3'
ZEB2-AS1 mutant-type
5'-CAGAGCCAGAAAAAAAGCAUGCUUa-3'
C
Relative miR-107 expression
Normal
Tumor
D
ROC curve
Sensitivity%
100% - Specificity%
p=0.7269
AUC=0.7269
CI:0.6585 to 0.7954
E
Biotin-control
Biotin-ZEB2-AS1
Fold change of miR-107
U2OS
MG63
F
U2OS
MG63
Control mimics
miR-107 mimics
N.S.
Relative luciferase activity
ZEB2-AS1 wild-type
ZEB2-AS1 mutant-type
G
U2OS
MG63
pcDNA3.1
pcDNA3.1-ZEB2-AS1
si-NC
si-ZEB2-AS1-1
si-ZEB2-AS1-2
Relative miR-107 expression
H
Control mimics
miR-107 mimics
Relative ZEB2-AS1 expression
U2OS
MG63

Figure 4. ZEB2-AS1 acts as a sponge for miR-107 in OS cells. A. Relative ZEB2-AS1 expression levels in nuclear and cytosolic fractions of U2OS and MG63 cells. B. Bioinformatics analysis showed the binding region between ZEB2-AS1 and miR-107. C. The expression of miR-107 in our cohort. D. ROC assays for ZEB2-AS1 as a diagnostic marker for OS patients. E. RNA pull-down assays were used to determine the direct combination of ZEB2-AS1 and miR-107. F. The luciferase reporter constructs containing the wildtype (WT-ZEB2-AS1) or mutant ZEB2-AS1 (MUT-ZEB2-AS1). G. The effects of ZEB2-AS1 overexpression or knockdown on the expression of miR-106 by RT-PCR. H. Overexpression of miR-107 resulted in down-regualtion of ZEB2-AS1 in U2OS and MG63 cells. $**P < 0.01$, $*P < 0.05$.

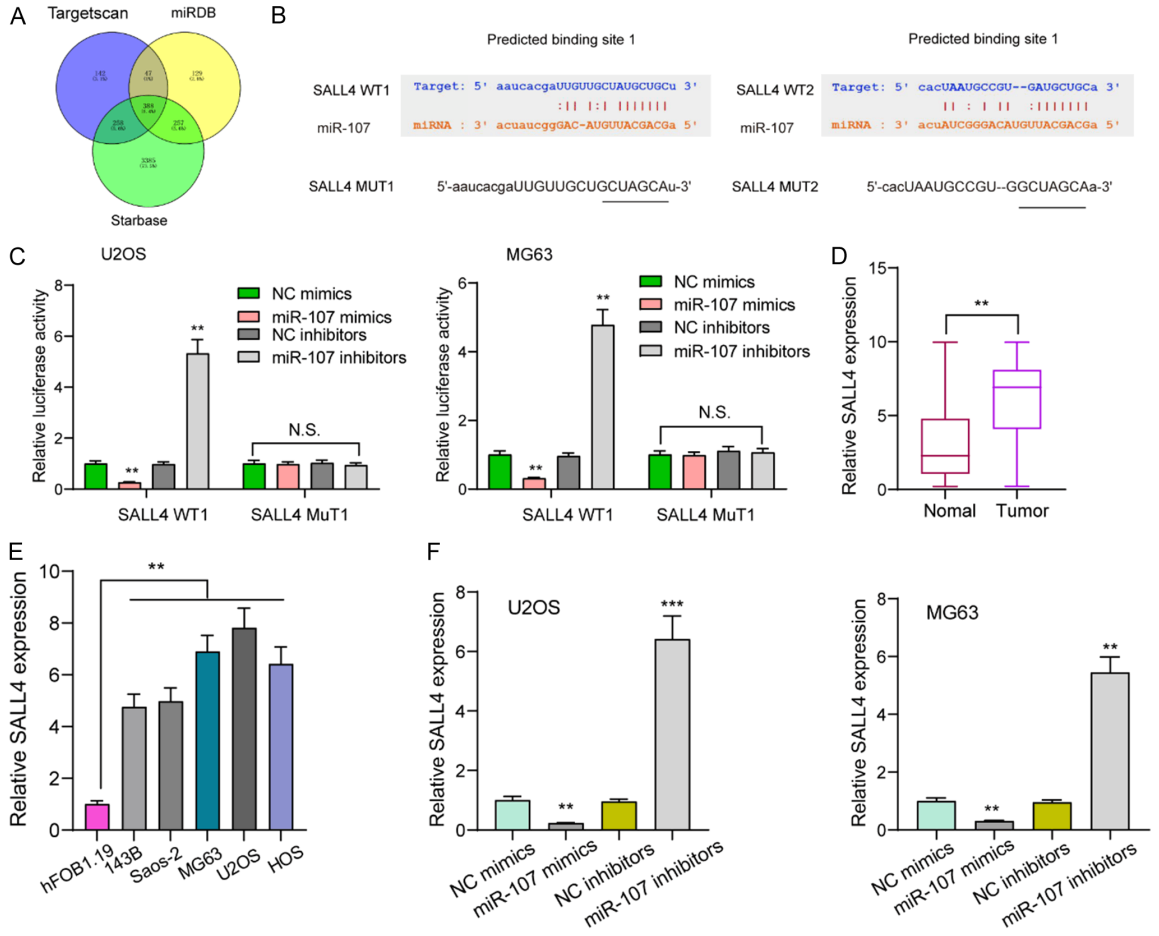

Figure 5. ZEB2-AS1 regulates SALL4 expression by acting as a molecular sponge. A. Three online bases predicted the potential targets of miR-107. B. Position of the miR-107 target site in 3'-UTR of SALL4 mRNA was shown using microRNA target databases. C. Dual-luciferase reporter assays of the Wt and Mut SALL4 3'-UTR reporter constructs in the presence of miR-107 inhibitors or miR-107 mimics. D. RT-PCR for the expression of mRNA SALL4 in our cohort. E. RT-PCR for the expression of mRNA SALL4 in five OS cell lines and Hfob1.19. F. The effects of miR-107 overexpression or knockdown on the expression of SALL4 by RT-PCR. G. Western blto examined the activity of AKT and MAPK pathways after ZEB2-AS1 knockdown. $**P < 0.01$, $*P < 0.05$.

tigation showed knockdown of ZEB2-AS1 suppressed the activity of AKT and MAPK pathways. Our findings implicated that ZEB2-AS1 acted as a tumor promoter and drives carcinogenesis via the promotion of cell proliferation and metastasis in OS.

The competing endogenous RNA hypothesis believed that some functional lncRNAs could compete against miRNAs for the regulation of miRNAs, thus further modulating the expression of targeting proteins of miRNAs [24, 25]. Recently, ZEB2-AS1 was also reported to act as a ceRNA for several miRNAs in several tumors, such as gastric cancer and pancreatic cancer [15, 26]. In this study, we found that ZEB2-AS1 mainly expressed in Cytoplasm, suggesting it may have potential to be a ceRNA.

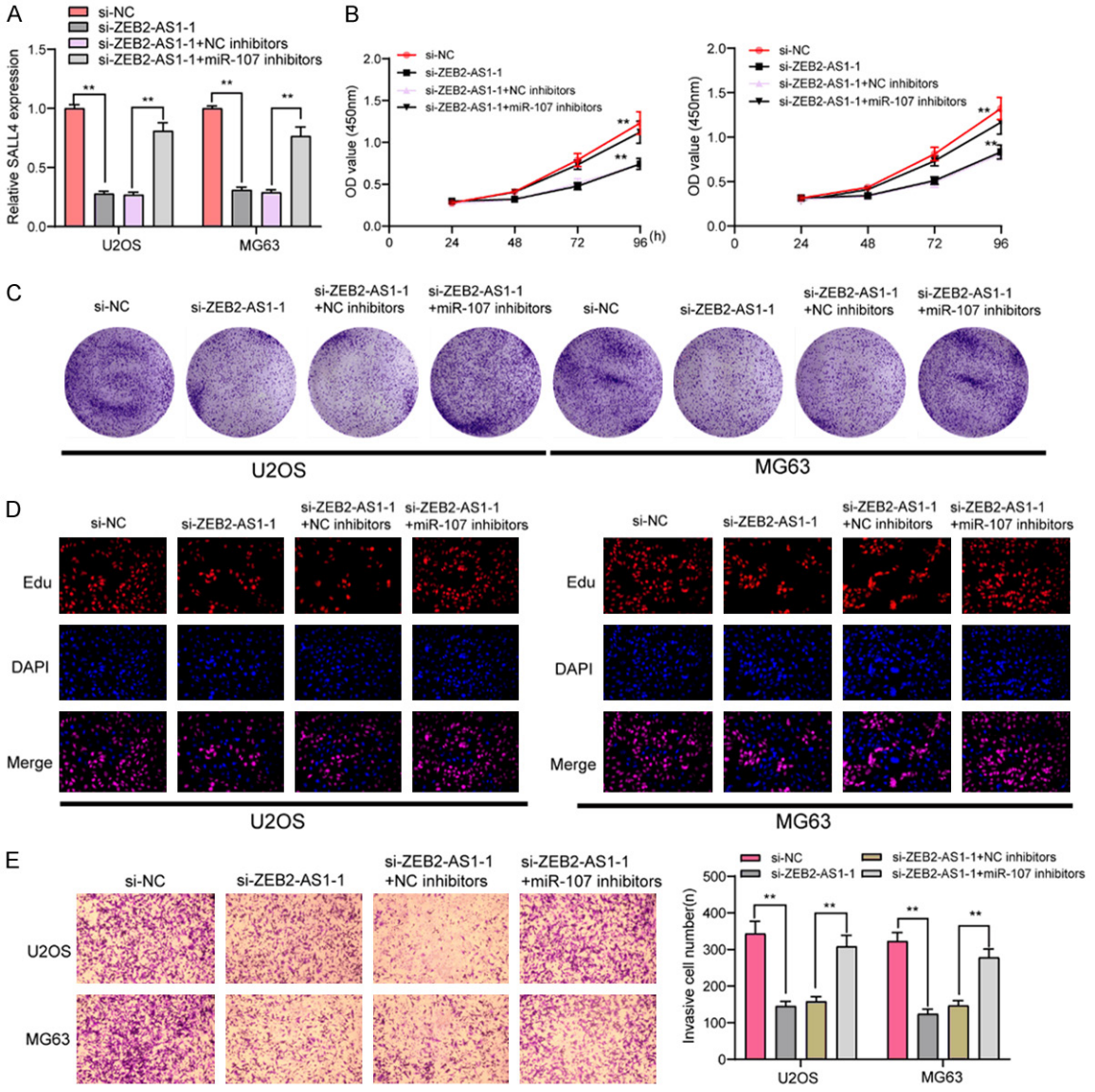

Figure 6. Deficiency of miR-107 attenuates the regulatory effect of ZEB2-AS1 knockdown on the cellualr progress of OS cells. (A) The expression of SALL4 following knockdown of ZEB2-AS1 and/or inhibition of miR-107. (B-E) The CCK-8 assays (B), colony formation assays (C), Edu assays (D) and transwell assays (E) following knockdown of ZEB2-AS1 and/or inhibition of miR-107. $^{**}P < 0.01$, $^{*}P < 0.05$.

Then, the results of bioinformatics analysis and Dual-luciferase reporter assays further confirmed that ZEB2-AS1 functioned as an endogenous sponge via flatly binding to miR-107 and, consequently suppressing the expressions of miR-107. Previously, miR-107 had been reported to play an important role in the progression of tumors, including OS [27, 28]. Importantly, Zhang et al [29] reported that miR-107 was highly expressed in OS, and its overexpression distinctly inhibited Proliferation and metastasis of OS cells via targeting Dkk-1. Thus, our finding suggested that ZEB2-AS1 may contribute to tumor progression via targeting miR-107.

SALL4, located in 20q13.2 with 3,162 bp of coding sequence, is a member of the mammalian homologs of Drosophila homeotic gene spalt and has been functionally identified to be involved in the maintainability of self-renewal abilities of embryonic stem cells [30, 31]. SALL4 encodes for a zinc-finger transcription factor whose roles were important during embryo-fetal development. In recent years, dysregulation of SALL4 expression was fre-

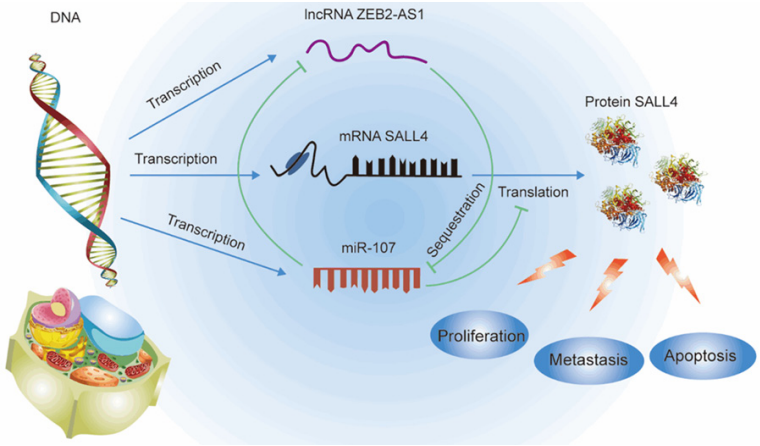

Figure 7. Hypothetical model for ZEB2-AS1 function in OS. ZEB2-AS1 promoted the proliferation, migration and invasion of OS cells via regulating miR-107/SALL4 axis.

quently reported in various tumors, and the studies of its epigenetic status and various functional assays have suggested it as an oncogene in several tumors, including OS [17, 32]. In this study, we found that SALL4 was one of miR-107's target using bioinformatics analysis, which was confirmed by luciferase reporter assays and western blot assays. In addition, we also showed that knockdown of miR-107 could increase the protein levels of SALL4 which was decreased by ZEB2-AS1 down-regualtion. More importantly, the results of rescue experiments revealed that knockdown of SALL4 markedly inhibited the proliferation, colony formation, invasion of U2OS and MG63 cells, while the effects were abolished by miR-107 down-regulation. Our findings revealed that ZEB2-AS1 displayed its tumor-promotive effects partially by increasing SALL4 expression.

In conclusion, ZEB2-AS1 is upregulated in OS tissues and correlates with unfavorable clinical outcomes, thereby potentially representing a novel marker for OS patients. In addition, ZEB2-AS1 may function as a ceRNA to promote SALL4 expressions via sponging miR-107, which consequently contributes to OS growth and invasion. Our findings may facilitate the identification of new predictive indicators and drug targets for OS.

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Disclosure of conflict of interest

None.

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