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Original Article

Long noncoding RNA ZEB1-AS1 predicts an unfavorable prognosis of non-small lung cancer and regulates epithelial to mesenchymal transition through reducing ZEB1 expression

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Abstract: Long non-coding RNAs (lncRNAs) are critical gene regulators with important roles in a wide variety of biological processes, including tumorigenesis. ZEB1-AS1 was significantly increased in NSCLC tissues and cell lines, and its expression levels were highly associated with aggressive tumor progression and poor prognosis. Down-regulation of ZEB1-AS1 suppresses NSCLC cell proliferation and invasion in vitro, as well as inhibits xenograft tumor growth in vivo. Mechanistically, we found that ZEB1-AS1 down-regulation effectively suppressed EMT process through reducing ZEB1 expression, and the ectopic expression of ZEB1 restored the migratory and invasive abilities of NSCLC cells inhibited by ZEB1-AS1 down-regulation. In conclusion, these findings revealed that ZEB1-AS1 plays regulatory roles in NSCLC and it might become a novel molecular indicator of prognosis and therapeutic target in NSCLC.

Keywords: Long non-coding RNA, ZEB1-AS1, non-small cell lung cancer, prognosis, cell cycle, epithelial to mesenchymal transition, ZEB1

Introduction

Lung cancer is a prevailing malignant disease and the leading cause of cancer-related mortality [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all cases of lung cancer, and adenocarcinoma is the most important histological types of NSCLC [2, 3]. Although prominent progresses have been achieved in the diagnosis and treatment of NSCLC, the prognosis of NSCLC patients remains dismal, with the 5-year survival rate less than 15% [4]. Thus, an understanding of the pathophysiological mechanisms contributing to NSCLC is of paramount importance for improving the diagnosis, prevention, prognosis, and treatment of NSCLC.

Long noncoding RNAs (lncRNAs) are a class of RNA molecules with more than 200 nucleotides in length and limited or no protein-coding capacity [5]. Recently, many lncRNAs have been reported to play imperative roles in a broad spectrum of physiological and pathological processes [6]. Pathologically, lncRNAs have been reported to be implicated in tumor initiation and progression, and the associations between lncRNAs and cancer have become a research focus, providing a new avenue to explore the cancer biology [7]. ZEB1-AS1 orients in antisense direction with respect to ZEB1, an important transcription factor functioning in many tumors [8]. Reports have been confirmed that elevated ZEB1-AS1 contributes to the development of many types of cancers. However, the relationship between ZEB1-AS1 and NSCLC remains largely unknown.

In the present study, we investigated the expression and function of ZEB1-AS1 in NSCLC. Further experiments were conducted to explore the biological function of ZEB1-AS1 with respect to NSCLC cell phenotypes in vitro and in vivo. Our novel findings provide a potential target for the treatment of NSCLC.

Materials and methods

Patient samples

122 pairs of fresh NSCLC tissue samples and corresponding normal adjacent tissues (>5 cm from the cancer tissues) were obtained from patients undergoing surgery at West China Hospital (Chengdu, China). All recruited patients did not receive chemotherapy or radiotherapy preoperatively. These tissues were frozen in liquid nitrogen after surgery immediately and then stored at -80°C. This study was conducted with the approval of the Ethics Committee of West China Hospital, and written formal approval was obtained from all the patients or their relatives.

Cell lines and cell culture

Four NSCLC adenocarcinoma cell lines (A549, H1975, H1299, and SPCA1), two NSCLC squamous carcinomas cell lines (H1703 and SK-MES-1), and a normal human bronchial epithelial cell line (16HBE), obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China), were cultured in RPMI 1640 medium (KeyGene, Nanjing, China) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) with 100 U/ml penicillin and 100 mg/ml streptomycin. All cell lines were grown in humidified air at 37°C with 5% CO_2.

Cell transfection

The sh-RNAs specifically targeting ZEB1-AS1 were subcloned into the pGIPZ-lentivirus vector (System Biosciences, Palo Alto, CA, USA). The sh-RNA sequences were listed as follows: sh-ZEB1-AS1-1: 5'-GGGCACTGCTGAATTTGAATT-3', sh-ZEB1-AS1-2: 5'-GGATGGGAAGTGACTTCAATG-3', sh-ZEB1-AS1-3: 5'-GCCAAACTGTGGACAAGTACC-3', sh-NC: 5'-CGUGGGUGGAUGCAUGGAUTT-3'. Plasmids pCDNA3FlagZEB1 and NC vector were obtained from RiboBio (Guangzhou, China). Cell transfection was performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). Transfection efficiency was measured by qRT-PCR.

Quantitative real-time PCR

Total RNA from tissues or cells was obtained by using TRIzol reagent (Invitrogen). RNA was reversed transcribed into complementary DNA (cDNA) using Reverse Transcription Kit (Thermo Fisher Scientific). After reverse transcription, the quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR® Premix Ex TaqTM II (Takara, Dalian, China) on the ABI Prism 7500 (Applied Bio systems, Foster City, CA, USA). The housekeeping gene GAPDH was used as a loading control. The fold changes of individual genes were calculated by $2^{-\Delta\Delta Ct}$ methods [9]. The formula is as follows: $\Delta\Delta Ct=[Ct_{(target\ gene)}-Ct_{(GAPDH)}]_{experimental}-[Ct_{(target\ gene)}-Ct_{(GAPDH)}]_{control}$, and the amount of target genes in the control samples was set at 1.0. Sequences of primers used for qRT-PCR were designed as follows: ZEB1-AS1 forward: 5'-CCGTGGGCACTGCTGAAT-3', and reverse: 5'-CTGCTGGCAAGCGGAACT-3'; ZEB1 forward: 5'-TGCACTGAGTGTGGAAAAGC-3', and reverse: 5'-TGGTGATGCTGAAAGAGACG-3'; GAPDH: forward, 5'-GTCAACGGATTTGGTCTGTATT-3', and reverse: 5'-AGTCTTCTGGGTGGCAGTGAT-3'.

Western blotting

Total protein was extracted from tissues and cells using RIPA peptide lysis buffer (Beyotime, Shanghai, China). Protein concentration was measured by the BCA Protein Assay Kit (Vigorous Biotechnology, Beijing, China). Equal amounts of proteins were electrophoresed via SDS-PAGE, transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) and incubated with specific antibodies. The following primary antibodies were used: ZEB1 (Cell Signaling, Beverly, MA, USA), E-cadherin (Cell Signaling), N-cadherin (Cell Signaling), vimentin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (Sigma-Aldrich, St. Louis, MO, USA). Protein bands were detected using the enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) with imaging system (Bio-Rad, CA, USA). GAPDH was used as a loading control.

Cell proliferation assay

Cell proliferation was measured by Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Kumamoto, Japan). Briefly, cells were seeded in 96-well plates at 2×10^3 cells/well and cultured for 24, 48, 72 and 96 h after transfection. Cells were treated with 10 μl of CCK-8 reagent for another 4 h, and the absorbance at 450 nm was detected using a microplate reader.

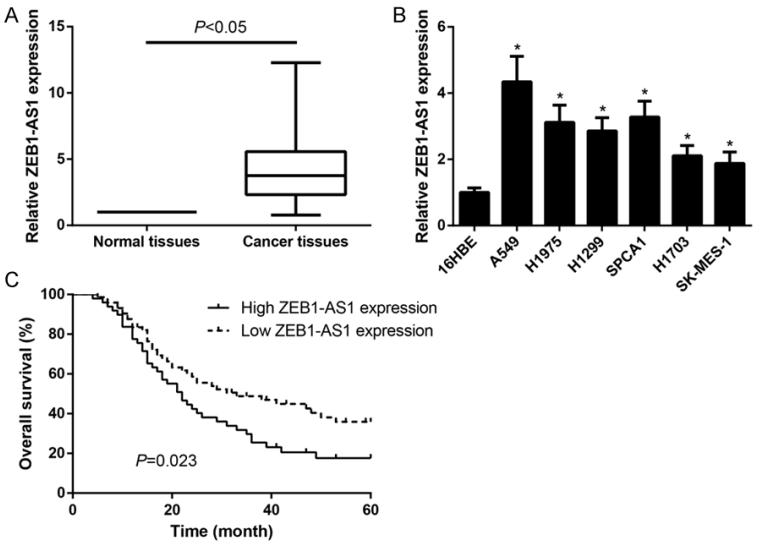

Figure 1. ZEB1-AS1 is upregulated in NSCLC tissues and cell lines. A: ZEB1-AS1 expression was assessed by qRT-PCR in NSCLC tissue samples. B: ZEB1-AS1 expression was assessed by qRT-PCR in NSCLC cell lines. The results are presented as the mean ± SD; **P*<0.05 by Student's t-test. C: Kaplan-Meier curves for overall survival of NSCLC patients categorized according to ZEB1-AS1 expression. *P*-value was determined using a log-rank test.

Transwell assay

To determine cell migration and invasion, after transfection, 5 × 10^3 cells in serum-free medium were placed into the upper chamber of a transwell insert (8-μm pore size; Corning, NY, USA) pre-coated with or without Matrigel (Chemicon, CA, USA). The lower chamber contained medium with 10% FBS as chemoattractant. After cultured for 48 h, the cells remaining in upper membrane were wiped off, while cells in the lower chamber were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet, imaged and counted under a microscope.

Flow-cytometric analysis of apoptosis and cell cycle

Cells were harvested 48 h after transfection by trypsinization. Following the double staining with FITC-Annexin V and Propidium Iodide (PI), the cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) equipped with CellQuest software (BD Biosciences). For the cell cycle analysis, the cells were stained with PI using the CycleTEST™ PLUS DNA reagent kit (BD Biosciences).

In vivo tumorigenesis

Five-week-old BALB/C-nu nude male mice, purchased from Shanghai Laboratory Animals Center (Shanghai, China), were randomly divided into two groups, with five mice in each group. 2 × 10^6 A549 cells stably transfected with sh-NC or sh-ZEB1-AS1 were injected subcutaneously in the right flanks of mice. Tumor diameters were measured every five days, and the tumor volumes were calculated using the equation: Volume=0.5 × L (length) × W^2 (width). The mice were sacrificed after four weeks, and the tumors were removed and weighed. All animal procedures were approved by the Animal Care and Use Committee of West China Hospital, and all efforts were made to minimize suffering.

Statistical analysis

All statistical analyses were executed by using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA) and SPSS 20.0 software (IBM, Chicago, IL, USA). All data from three independent experiments were presented as mean ± standard deviation (SD). The significance of the differences between groups was estimated using the Student *t*-test or Chi-squared (X^2) test. The Kaplan-Meier method was used for survival curves, and a log-rank test was used for comparison. Differences were considered statistically significant according to *P*<0.05.

Results

ZEB1-AS1 is upregulated in NSCLC tissues and cell lines

The expression level of ZEB1-AS1 was examined by carrying out qRT-PCR in NSCLC tissues and pair-matched adjacent normal tissues in a cohort of 122 NSCLC patients. As shown in **Figure 1A**, compared with pair-matched adjacent normal tissues, ZEB1-AS1 expression was increased significantly in NSCLC tissues. In addition, the expression of ZEB1-AS1 was remarkably upregulated in multiple NSCLC cell lines compared to normal 16HBE cells (**Figure 1B**).

The relationships between the ZEB1-AS1 expression levels and clinicopathological characteristics of NSCLC patients were further ana-

Table 1. Correlation between clinicopathological features and the expression of ZEB1-AS1 in NSCLC patients

Characteristics	Total number	ZEB1-AS1 expression		*P* value
		Low (n=73)	High (n=49)	
Age				0.741
<65	57	35	22	
≥65	65	38	27	
Gender				0.675
Male	82	48	34	
Female	40	25	15	
Smoking history				0.371
Never	78	49	29	
Yes	44	24	20	
Tumor size				0.017
<5 cm	80	54	26	
≥5 cm	42	19	23	
Primary location				0.911
Left lung	59	35	24	
Right lung	63	38	25	
Histology type				0.481
Adenocarcinoma	70	40	30	
Squamous carcinoma	52	33	19	
TNM stage				<0.001
I/II	82	59	23	
III/IV	40	14	26	
Lymph nodes metastasis				0.026
No	72	49	23	
Yes	50	24	26	

lyzed. The 122 NSCLC patients were classified into two groups: the high ZEB1-AS1 expression group (n=49); and the low ZEB1-AS1 expression group (n=73). As listed in **Table 1**, we found that the expression level of ZEB1-AS1 was closely associated with tumor size (*P*=0.017), TNM stage (*P*<0.001) and lymph nodes metastasis (*P*=0.026). However, no correlation was observed between the ZEB1-AS1 expression and other features such as age and gender. Moreover, Kaplan-Meier overall survival analysis showed that NSCLC patients with higher ZEB1-AS1 expression had the worse overall survival after surgical procedure (*P*=0.023; **Figure 1C**).

Downregulation of ZEB1-AS1 inhibits cell proliferation and invasion in NSCLC cells

As increased ZEB1-AS1 expression in NSCLC is a common molecular incident, we hypothesize that ZEB1-AS1 knockdown in NSCLC can exert inhibitory effects on cell malignant phenotypes. To validate the hypothesis, three ZEB1-AS1-specific shRNAs were used to reduce ZEB1-AS1 expression in A549 cells. As shown in **Figure 2A**, among them, sh-ZEB1-AS1-1 got the most powerful knockdown efficiency, and thus was chosen for subsequent studies. Subsequently, CCK-8 assay was performed to evaluate the effect of ZEB1-AS1 on NSCLC cell proliferation. Data showed that ZEB1-AS1 knockdown significantly inhibits the proliferation rate in A549 cells (**Figure 2B**).

Subsequently, we investigated the effect of ZEB1-AS1 on NSCLC cell migration and invasion. The results of transwell assay were shown in **Figure 2C** and indicated that reduced ZEB1-AS1 expression levels significantly hindered the migration and invasion of A549 cells compared with controls.

Downregulation of ZEB1-AS1 induces cell cycle arrest and apoptosis in NSCLC cells

Flow cytometry analysis was then performed to determine the effects of ZEB1-AS1 on NSCLC cell apoptosis and cell cycle progression. As exhibited in **Figure 3A**, ZEB1-AS1 knockdown caused cell cycle arrest at the G0/G1 phase in A549 cells. Simultaneously, the proportion of cells in the S phase reduced. Additionally, the results shown in **Figure 3B** revealed a remarkably higher percentage of apoptotic cells for sh-ZEB1-AS1-treated A549 cells compared to NC cells.

Downregulation of ZEB1-AS1 inhibits NSCLC tumor growth in vivo

To further test the oncogenic activity of ZEB1-AS1, A549 cells stably expressing control shRNA or sh-ZEB1-AS1 were injected subcutaneously into nude mice. As shown in **Figure 4A**, the growth of tumors generated from the sh-ZEB1-AS1 group was significantly suppressed at each time point compared to those in the

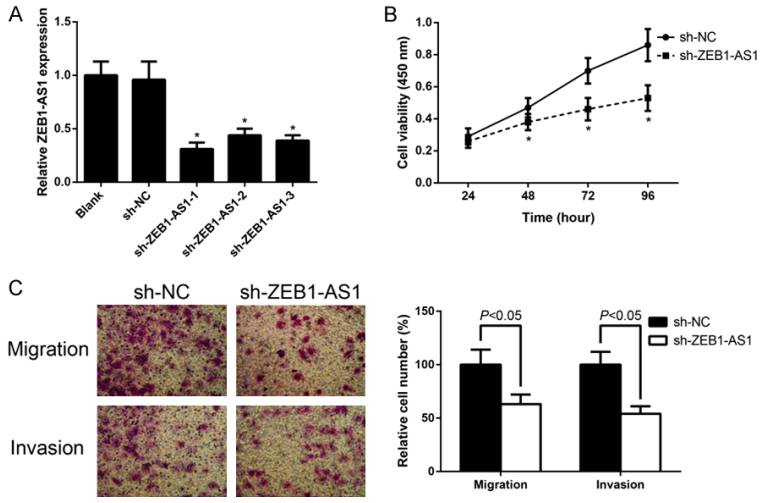

Figure 2. Downregulation of ZEB1-AS1 inhibits cell proliferation and invasion in NSCLC cells. A: Transfection efficacy was determined by qRT-PCR in A549 cells. B: Reduced expression of ZEB1-AS1 in A549 cells significantly promoted their proliferative capacities, as determined by CCK-8 assay. C: Transwell assay confirmed that ZEB1-AS1 knockdown significantly inhibited A549 cell migration and invasion. The results are presented as the mean ± SD; **P*<0.05 by Student's t-test.

sh-NC group. Besides, as expected, the tumor weights for the sh-ZEB1-AS1 group were remarkably lower than those in the sh-NC group (**Figure 4B**). Furthermore, qRT-PCR analysis showed that the expression of ZEB1-AS1 in the sh-ZEB1-AS1 group was lower than its expression in the sh-NC group (**Figure 4C**).

Downregulation of ZEB1-AS1 inhibits EMT process by reducing ZEB1 expression

ZEB1-AS1 could positively regulate the ZEB1 expression in hepatocellular carcinoma [10]. In the present study, we also found that ZEB1-AS1 knockdown could significantly decrease the expression of ZEB1 both at mRNA and protein levels in A549 cells (**Figure 5A**, **5B**). ZEB1 is a repressor of EMT [11]. Western blotting showed the expression of the epithelial marker E-cadherin was greatly increased and meanwhile the expression of the mesenchymal marker vimentin and N-cadherin was obviously reduced in A549 cells treated with sh-ZEB1-AS1 compared with the controls (**Figure 5C**).

To further verify the speculation that the effect of ZEB1-AS1 on NSCLC cell phenotypes is partially mediated by its regulatory effect on ZEB1, sh-ZEB1-AS1 along with pCDNA3FlagZEB1 was co-transfected into A549 cells. As anticipated, the inhibitory effects of ZEB1-AS1 downregulation on A549 cell migration and invasion were remarkably impaired when ZEB1 was overexpressed (**Figure 5D**).

Discussion

As critical players in cancer biology, lncRNAs have gradually moved to the forefront of NSCLC research. Up to now, researchers have identified a number of NSCLC-associated lncRNAs, including GAS5-AS1 [12], FOXD2-AS1 [13] and XLOC_008466 [14]. As the antisense RNA of ZEB1, ZEB1-AS1 (NCBI no. NR_024284.1) is a lncRNA transcript which maps on chromosome 10p-11.22. Evidence has validated that the elevated expression of ZEB1-AS1 is detected in a wide variety of human malignancies, such as hepatocellular carcinoma [10], esophageal squamous cell carcinoma [15], osteosarcoma [16] and bladder cancer [17]. However, data on the regulatory role of ZEB1-AS1 in NSCLC are limited.

To our knowledge, this report might be the first direct investigation of the relationship between ZEB1-AS1 expression and NSCLC. In the present study, we validated the upregulation of ZEB1-AS1 in NSCLC tissue samples and a panel of NSCLC cell lines. Increased expression of ZEB1-AS1 was more frequently correlated with aggressive tumor characteristics and unfavorable prognosis of NSCLC patients. Moreover, we found that knockdown of ZEB1-AS1 in NSCLC cells led to the significant inhibited cell proliferation, migration, invasion, and the promotion of apoptosis in vitro. In vivo studies also showed that knockdown of ZEB1-AS1 greatly impaired xenograft tumor formation of NSCLC cells. All these results further confirmed the oncogene role of ZEB1-AS1 in NSCLC and suggested the critical function of ZEB1-AS1 in NSCLC tumourigenesis.

Tumor metastasis is the major cause of death in NSCLC patients. Epithelial-to-mesenchymal transition (EMT) has been recognized as a main cause for metastasis in various cancers [18]. Loss of the epithelial marker E-cadherin and gain of mesenchymal marker vimentin are con-

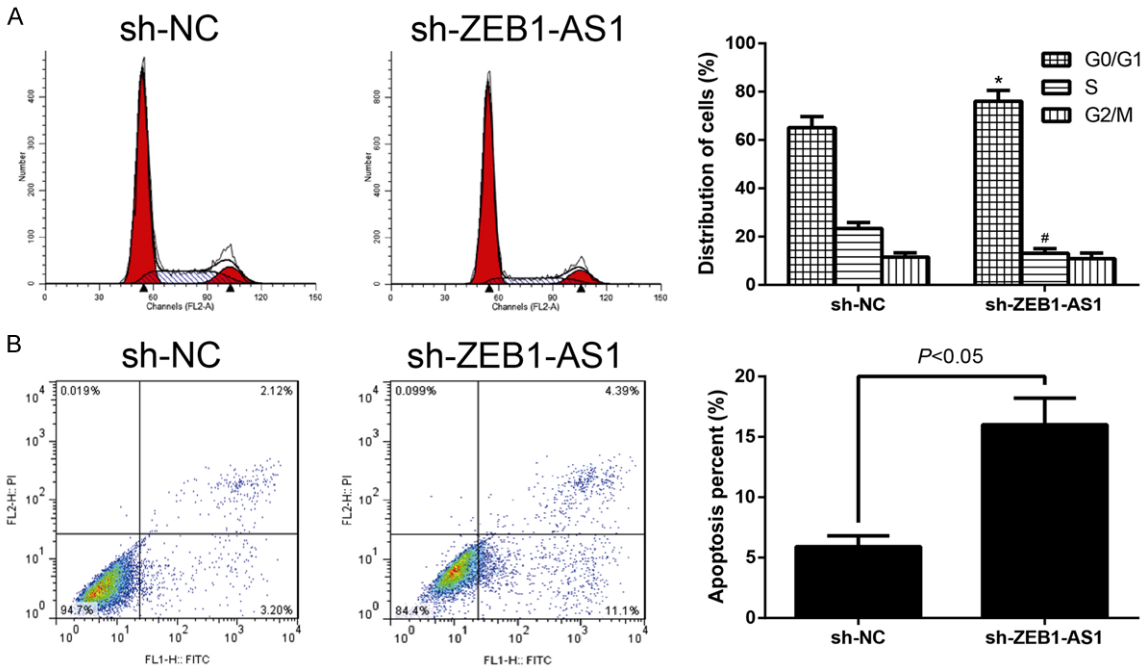

Figure 3. Downregulation of ZEB1-AS1 induces cell cycle arrest and apoptosis in NSCLC cells. A: Reduced expression of ZEB1-AS1 induces A549 cell cycle arrest at the G0/G1 phase. B: Reduced expression of ZEB1-AS1 promotes A549 cell apoptosis. The results are presented as the mean ± SD; **P*<0.05, #*P*<0.05 by Student's t-test.

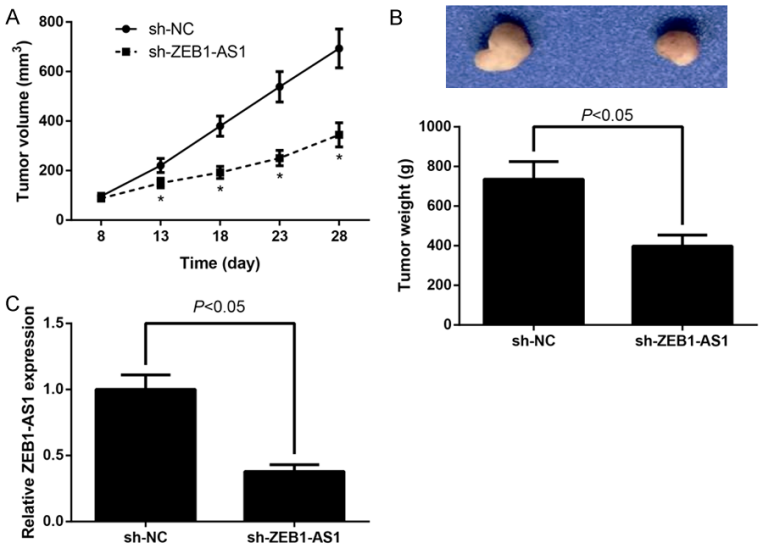

Figure 4. Downregulation of ZEB1-AS1 inhibits NSCLC tumor growth in vivo. A: Tumor volume was calculated every three days after the injection of cells. B: Tumors were excised and tumor weights were calculated. C: qRT-PCR analysis of ZEB1-AS1 expression in tumor tissues. The results are presented as the mean ± SD; **P*<0.05 by Student's t-test.

sidered as the fundamental events in EMT process [19]. Our results demonstrated that ZEB-AS1 knockdown inhibits EMT process in NSCLC cells. Antisense lncRNAs often positively or negatively regulate their sense mRNA partners through controlling their stability and translatability [20, 21], and we observed that ZEB-AS1 expression was positively correlated with its sense partner, ZEB1 in NSCLC cells. Zinc finger E-box binding homeobox 1 (ZEB1), one of the most common pro-metastasis factors, is an activator of EMT in many human cancers, including lung cancer [22]. Consistent with our findings, LV et al. reported that ZEB1-AS1 might enhance glioma cell migration and invasion through ZEB1-EMT axis [23].

In summary, this study provides the first evidence that ZEB1-AS1 was downregulated in NSCLC and the increased expression of ZEB1-AS1 significantly associated with poor prognosis. Downregulation of ZEB1-AS1 suppressed the EMT process in NSCLC cell lines by reducing ZEB1 expression. Our results implied that ZEB1-AS1 may be a novel diagnostic and prog-

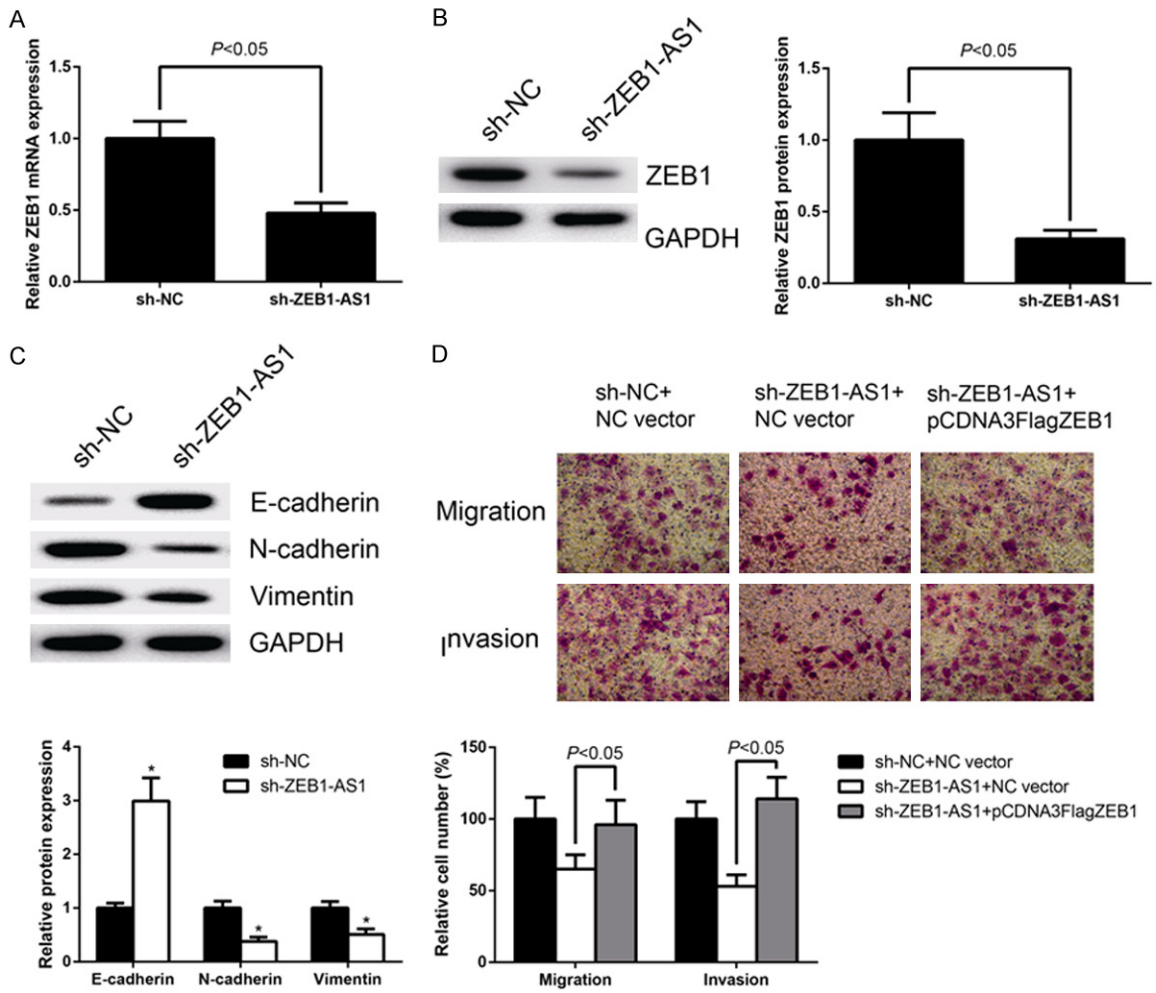

Figure 5. Downregulation of ZEB1-AS1 inhibits EMT process by reducing ZEB1 expression. A: qRT-PCR analysis of ZEB1 mRNA in A549 cells. B: Western blot analysis of ZEB1 protein in A549 cells. C: Western blot analysis of EMT-associated proteins in A549 cells. D: Transwell assay confirmed that the inhibitory effects of ZEB1-AS1 downregulation on A549 cell migration and invasion were remarkably impaired by overexpressed ZEB1. The results are presented as the mean ± SD; *P<0.05 by Student's t-test.

nostic biomarker, and a potential therapeutic target for NSCLC.

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Disclosure of conflict of interest

None.

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