

ZEB1 promotes prostate cancer proliferation and invasion through ERK1/2 signaling pathway

X.-F. SONG, H. CHANG, Q. LIANG, Z.-F. GUO, J.-W. WU

Department of Urology, Minhang Hospital Affiliated to Fudan University, Shanghai, China

Abstract. – OBJECTIVE: Prostate cancer is a kind of malignancy with high occurrence in the male urogenital system. However, the mechanism of the occurrence, the progression, and the metastasis of prostate cancer are still unclear. Searching for the effective molecule target is of great significance to improve the curative effect on prostate cancer. Zinc finger E box binding protein-1 (ZEB1) protein is a member of the zinc finger transcription factor family that participates in the embryonic development and formation. ZEB1 was found to be involved in the occurrence and in the development of multiple cancers, while its role in prostate cancer still needs elucidation.

MATERIALS AND METHODS: Normal prostate cell line PC-3M and prostate cancer cell line DU145 were cultured in vitro and transfected by ZEB1 siRNA. ZEB1 mRNA and protein expressions were detected by real-time PCR and Western blot assay. Cell proliferation was determined by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cell migration was evaluated by transwell assay. Cell apoptosis was evaluated by caspase-3 activity. The impact of ZEB1 on extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathway was assessed by Western blot assay.

RESULTS: ZEB1 expression significantly increased in DU145 cells compared with PC-3M cells ($p < 0.05$). ZEB1 mRNA and protein obviously declined, cell proliferation inhibited, cell invasion suppressed, and Caspase-3 activity enhanced in DU145 cells after ZEB1 siRNA transfection ($p < 0.05$). ZEB1 siRNA markedly decreased ERK1/2 phosphorylation in DU145 cells compared with control ($p < 0.05$).

CONCLUSIONS: Inhibition of ZEB1 promoted prostate cancer apoptosis, restrained proliferation, and suppressed invasion through down-regulating ERK1/2 signaling pathway.

Key Words:

Prostate cancer, ZEB1, ERK1/2, DU145, Proliferation, Invasion.

Introduction

As a kind of malignancy occurring in prostate epithelium, prostate cancer accounts for the leading morbidity in the male urogenital system^{1,2}. Prostate cancer appears mostly in elderly and presents an increasing trend following the aging of population, the change of dietary and living habits³. Numerous factors can induce prostate cancer, leading to complex tumorigenesis and invasive mechanism. Genetic factors, physical and chemical factors, and the living environment can result in incidence increase⁴. Most patients tend to ignore the early screening because of its stealthiness. Therefore, prostate cancer patients are in an advanced stage when diagnosed, losing the chance of surgery and treatment. It increases the treatment's difficulty and leads to a poor prognosis, which seriously threatens the survival and the quality of life^{5,6}. Prostate cancer exhibits an increasing trend in our country year by year⁷. Inhibition of tumor invasion and metastasis is the key to improving the prognosis of patients with prostate cancer⁸. Recently, zinc finger transcription factor family is a hotspot in research, which mainly contains zinc finger E box binding protein-1 (ZEB1) and ZEB2⁹. ZEB1 protein participates in embryonic development and formation through regulating transcription. Its gene mutation may cause severe embryonic development malformation¹⁰. At present, it was found that ZEB1 was involved in multiple cancers' occurrence and development, such as osteosarcoma and non-small cell lung cancer^{11,12}. ZEB1 can influence E-cadherin expression and regulate the morphology and properties changes of epithelial cells connected by polarity. It accelerates epithelial cells transit to mesenchymal cells, thus freely moving in cell matrix to promote epithelial-mesenchymal transition (EMT)^{13,14}. EMT accelerates tumor metastasis and invasion¹⁵.

It was reported that ZEB1 overexpressed in several cancers, including osteosarcoma and non-small cell lung cancer¹⁶. However, the regulatory role of ZEB1 and related mechanism in prostate cancer has not been elucidated.

Materials and Methods

Main Reagents and Instruments

Human prostate cancer cell line DU145 (HTB-81™) and normal prostate cell line PC-3M were purchased from ATCC Cell Bank (Manassas, VA, USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), ethylenediaminetetraacetic acid (EDTA), and penicillin-streptomycin were purchased from HyClone (South Logan, UT, USA). Dimethyl sulfoxide (DMSO) and MTT were purchased from Gibco BRL Co. Ltd. (Gaithersburg, MD, USA). Trypsin-EDTA was obtained from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene fluoride (PVDF) membrane was derived from Pall Life Sciences (Covina, CA, USA). Western blot related reagents were provided by Beyotime (Shanghai, China). Enhanced chemiluminescence (ECL) reagent was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Rabbit anti-human extracellular signal-regulated kinase 1 and 2 (ERK1/2), phosphorylated ERK1/2 (pERK1/2), ZEB1 monoclonal antibodies and mouse anti-rabbit horse-radish peroxidase (HRP) labeled IgG secondary antibody, were provided by Cell Signaling Technology Inc. (Danvers, MA, USA). RNA extraction kit and reverse transcription kit were obtained from Axygen (Tewksbury, MA, USA). Labsystem Version 1.3.1 microplate reader was provided by Bio-Rad Laboratories (Hercules, CA, USA). Thermo Forma 3110 cell incubator was bought from Thermo Scientific Pierce (Rockford, IL, USA). ABI 2720 PCR amplifier was derived from ABI (Vernon, CA, USA). Lipo2000 transfection reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA); other reagents from Sangon Bio. Ltd. (Shanghai, China).

Methods

DU145 Cell Culture and Grouping

DU145 cells were un-frozen at 37°C and centrifuged at 1000 r/min for 3 min. Then the cells were resuspended in 1 ml DMEM medium and cultured at 37°C and 5% CO₂ for 24 h to 48 h.

Next, the cells were seeded in dish at 1×10⁵/cm² in high-glucose DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells in logarithmic phase were used for the following experiments. DU145 cells were randomly divided into three groups, including control, empty plasmid, and ZEB1 siRNA groups.

ZEB1 siRNA Transfection

ZEB siRNA and Negative Control Were Transfected Into DU145 Cells

ZEB1 siRNA, 5'-GUUCCAAGUUGCUUCA-TAUAT-3', 5'-UAUAAUACAUUGGGGAAC-CT T-3'. ZEB1 siRNA negative control, 5'-ACAGCAUUGCCUGGUAGGAUG-3', 5'-ACUGUAUAGUAGGGACGCGUG-3'. DU145 cells in logarithmic phase were seeded in 6-well plate at 3×10⁶/ml for 12 h. A total of 5 µl lipo2000, ZEB1 siRNA, or ZEB1 siRNA negative control was added to 200 µl serum-free medium at room temperature for 15 min, respectively. Next, they were mixed and incubated at room temperature for 30 min. When the cell fusion reached 70% to 80%, they were used to transfect cells at 37°C and 5% CO₂ for 6 h. Then the medium was changed for further cultivation.

Real-Time PCR

Total RNA was extracted from hippocampus tissue by Trizol and reverse transcribed to cDNA. The primers were designed using Primer Premier 6.0 software and synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA) (Table I). Real-time PCR was performed at 56°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was selected as internal reference. The relative expression of mRNA was calculated by 2^{-ΔCt} method.

Western Blot Assay

The DU145 cells were added with RIPA (150 mM NaCl, 1% NP-40, 0.1% SDS, 2 µg/ml Aprotinin, 2 µg/ml Leupeptin, 1 mM PMSF, 1.5 mM EDTA, 1 mM NaVanadate) containing protease and cracked on ice for 15 min to 30 min. Next, the tissues were treated by ultrasound at 5 s for 4 times and centrifuged at 10000 ×g for 15 min. The protein was transferred to new Ep tube and stored at -20°C. The protein was separated by 10% lauryl sodium sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane at 100 mA for 1.5 h. After blocked by 5% skim milk for 2 h, the membrane was incubated

in ZEB1, pERK1/2, and ERK1/2 monoclonal antibodies (1:1000, 1:2000, and 1:1500, respectively) at 4°C overnight. Then, the membrane was incubated in goat anti-rabbit secondary antibody (1:2000) at room temperature for 30 min. Next, the membrane was treated by developer for 1 min and exposed to observe the result. The film was scanned by Quantity One software and analyzed by protein image processing system. Each experiment was repeated four times.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-Tetrazolium Bromide (MTT) Assay

DU145 cells in logarithmic phase were seeded in 96-well plate at 5×10^3 /well for 24 h. The cells were divided into four groups, including empty plasmid group, scramble group, and ZEB1 siRNA group. After 48 h incubation, the plate was added with 20 μ l MTT for 4 h. Then the plate was treated by 150 μ l dimethyl sulfoxide (DMSO) for 10 min and tested at 570 nm to measure the absorbance value. Each experiment was repeated for at least three times.

Transwell Assay

The cells were further cultured for 24 h and 48 h after transfection. A total of 50 mg/L Matrigel was used to coat the bottom of transwell chamber at 1:5. Next, 50 μ l serum free medium containing 10 g/L bovine serum albumin (BSA) was added to the upper chamber at 37°C for 30 min. Then, the chamber was put into the 24-well plate. A total of 500 μ l DMEM medium containing 10% FBS was added to the lower chamber, while 100 μ l tumor cell suspension was added to the upper chamber with serum free medium. After 48 h, the chamber was washed by PBS and fixed by absolute alcohol. At last, the membrane was stained by crystal violet and observed under the microscope. Each experiment was repeated for three times.

Caspase 3 Activity Detection

Caspase 3 activity was tested according to the manual. The cells were digested by trypsin and

centrifuged at 600 $\times$ g and 4°C for 5 min. Next, the cells were added with 2 mM Ac-DEVD-pNA and detected at 405 nm to calculate caspase 3 activity.

Statistical Analysis

All data were presented as mean $\pm$ standard deviation and compared by Student's *t*-test or ANOVA. All data analyses were performed on SPSS11.5 software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was depicted as statistical significance.

Results

ZEB1 mRNA and Protein Expressions in Prostate Cells and Prostate Cancer Cells

Real-time PCR and Western blot were applied to test ZEB1 mRNA and protein expressions in PC-3M and DU145 cells. ZEB1 mRNA and protein levels significantly increased in DU145 cells compared with that in PC-3M cells ($p < 0.05$, Figure 1).

The Impact of ZEB1 Regulation on ZEB1 mRNA and Protein Expressions in DU145 Cells

Real-time PCR and Western blot were adopted to detect ZEB1 mRNA and protein in DU145 cells transfected by ZEB1 siRNA. ZEB1 siRNA transfection significantly suppressed ZEB1 mRNA and protein expressions compared with control ($p < 0.05$, Figure 2).

Effects of ZEB1 on DU145 Cell Proliferation

MTT assay was selected to evaluate the impact of ZEB1 on DU145 cell proliferation. ZEB1 siRNA markedly inhibited NPC cell proliferation in DU145 cells compared with control ($p < 0.05$, Figure 3). It suggested that ZEB1 siRNA is in favor of suppressing prostate cancer cell proliferation.

Effects of ZEB1 on DU145 Cell Invasion

Transwell assay was used to test the influence of ZEB1 on DDU145 cell invasion. ZEB1 siRNA apparently restrained DU145 cell invasion compared with control ($p < 0.05$, Figure 4). It indicated

Table I. Primer sequences.

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
ZEB1	ACCTGCCTCTCTAGATCCAT	TAGGACCTCAGTGTTAATTT

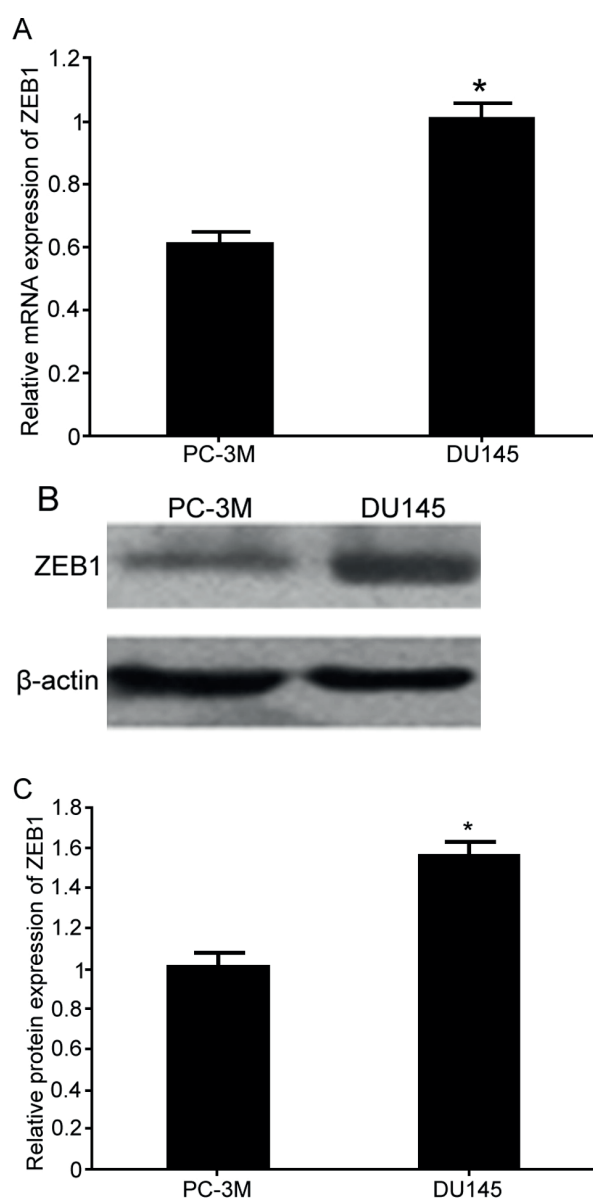


Figure 1. ZEB1 mRNA and protein expressions in prostate cells and prostate cancer cells. **A**, Real-time PCR detection of ZEB1 mRNA expression; **B**, Western blot detection of ZEB1 protein expression; **C**, ZEB1 protein expression analysis. * $p < 0.05$, compared with PC-3M.

that ZEB1 siRNA is in favor of suppressing prostate cancer cell invasion.

Effect of ZEB1 on Caspase 3 Activity in DU145 Cells

Caspase 3 activity detection kit was used to determine the impact of ZEB1 on caspase 3 activity in DU145 cells. ZEB1 siRNA significantly enhanced caspase 3 activity in DU145 cells compared with

control ($p < 0.05$, Figure 5). It revealed that ZEB1 siRNA facilitates prostate cancer cell apoptosis.

Effect of ZEB1 on ERK1/2 Expression in DU145 cells

Western blot assay was adopted to analyze the impact of ZEB1 on ERK1/2 protein expression in DU145 cells. ZEB1 siRNA down-regulated ERK1/2 protein phosphorylation in DU145 cells compared with control ($p < 0.05$, Figure 6). It indicated that upregulation of ZEB1 siRNA restrained NPC proliferation and invasion through ERK1/2 phosphorylation.

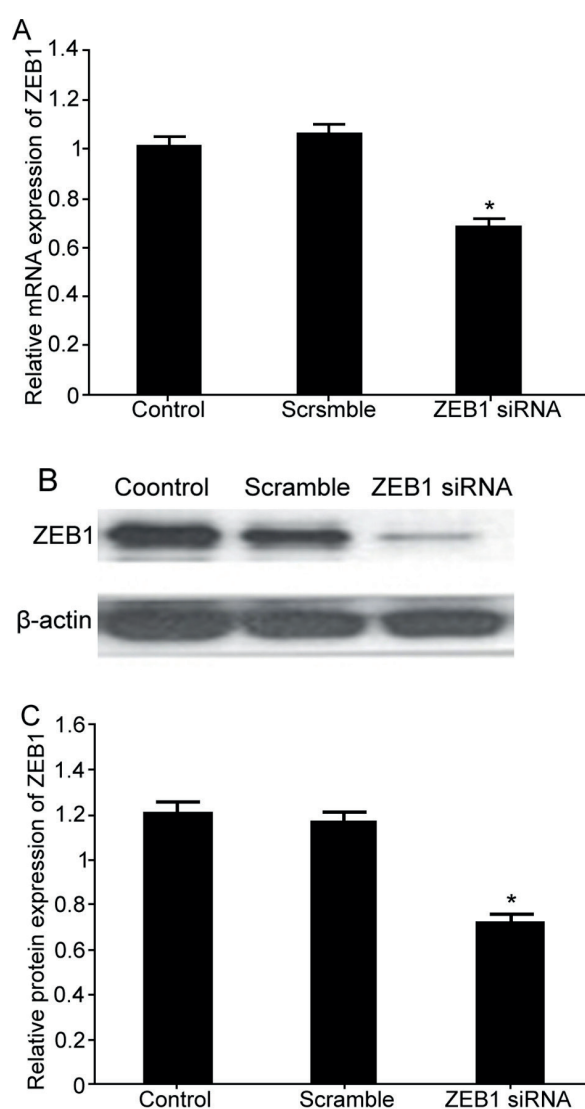


Figure 2. The impact of ZEB1 regulation on ZEB1 mRNA and protein expressions in DU145 cells. **A**, Real-time PCR detection of ZEB1 mRNA expression; **B**, Western blot detection of ZEB1 protein expression; **C**, ZEB1 protein expression analysis. * $p < 0.05$, compared with control.

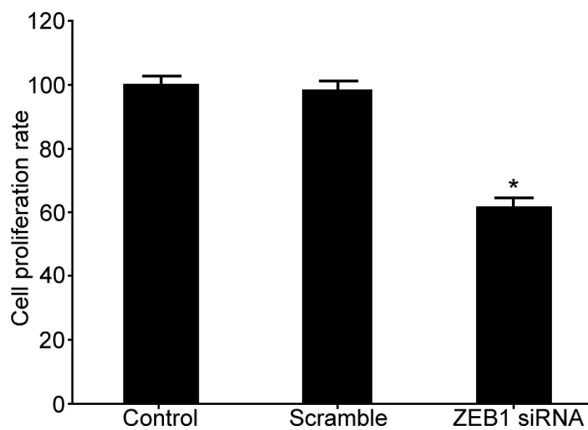


Figure 3. The impact of ZEB1 on DU145 cell proliferation. * $p < 0.05$, compared with control.

Discussion

In spite of rapid improvement of current treatment method, the survival rate of prostate cancer patients has not been substantially increased. High incidence and poor prognosis are still the conundrum of prostate cancer in clinic^{17,18}. Tumor occurrence, development, and metastasis is a multi-stage, multi-factor, and multi-step process regulated by numerous factors and genes. EMT plays a critical role in tumor invasion. Therefore, ZEB1 exhibits the strongest correlation with EMT originated ma-

lignant tumors¹⁹. ZEB1 can directly or indirectly suppress E-cadherin expression. E-cadherin is an important inhibitor of EMT. E-cadherin can form dimer to maintain normal cell alignment mediated by calcium ion^{13,14}. E-cadherin mediates cell adhesion and serves as cytoskeleton¹⁵. ZEB1 can specifically bind with E-cadherin to inhibit its expression²⁰. This study showed that ZEB1 mRNA and protein increased in prostate cancer, while its role and related mechanism in prostate cancer has not been elucidated. We used ZEB1 siRNA to interpose ZEB1 expression. ZEB1 mRNA and protein declined, cell proliferation inhibited, cell invasion suppressed, and caspase-3 activity enhanced in DU145 cells after ZEB1 siRNA transfection. As the strongest member of caspase family, caspase 3 is the executor of apoptosis that can induce tumor cell apoptosis²¹. ERK, also known as extracellular regulated kinase, has five subgroups. ERK1/2 is an important member belonging to serine/threonine protease family²². ERK1/2 can be activated under cell adhesion, stress, and hormone to regulate transcription factor phosphorylation^{23, 24}. Sustained activation and phosphorylation of ERK1/2 may lead to cell excessive proliferation and malignant transfor-

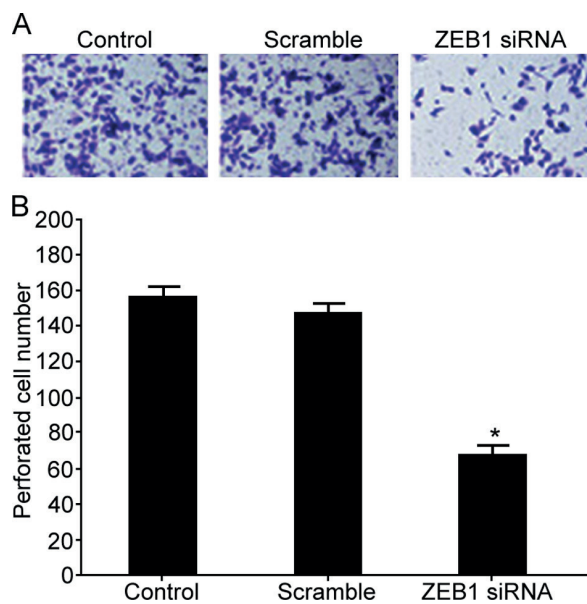


Figure 4. The influence of ZEB1 on DU145 cell invasion. *A*, transwell assay detection of cell invasion; *B*, cell invasion analysis. * $p < 0.05$, compared with control.

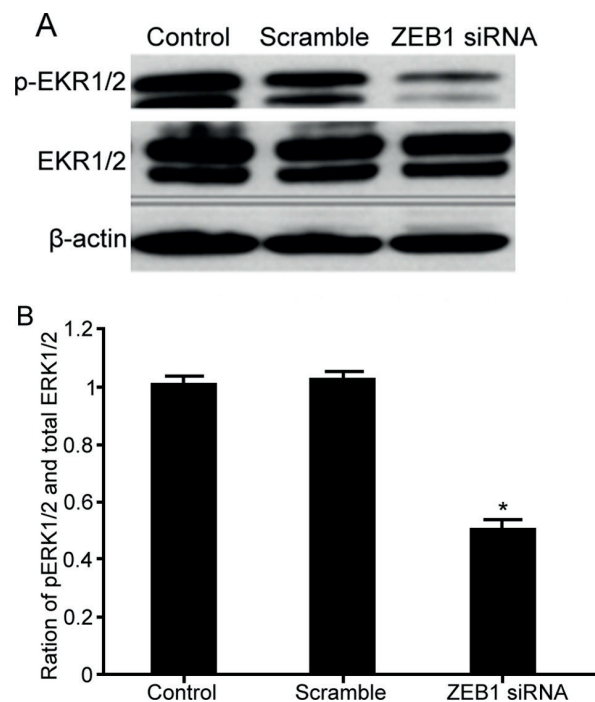


Figure 5. The effect of ZEB1 on ERK1/2 activity in DU145 cells. * $p < 0.05$, compared with control.

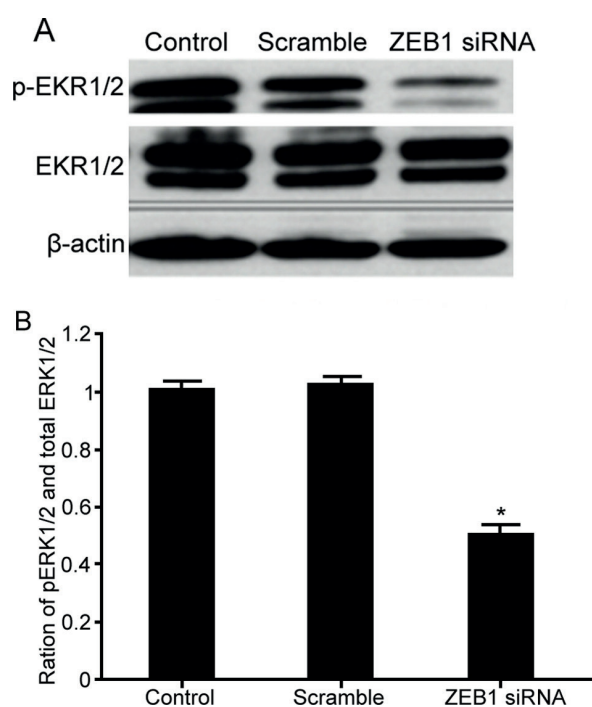


Figure 6. The impact of ZEB1 on ERK1/2 expression in DU145 cells. * $p < 0.05$, compared with control.

mation²⁵. This study revealed that ZEB1 siRNA markedly decreased ERK1/2 phosphorylation in DU145 cells, thus accelerating cell apoptosis, inhibiting cell proliferation, and restraining cell invasion.

Conclusions

Inhibition of ZEB1 promoted prostate cancer apoptosis, restrained proliferation, and suppressed invasion through down-regulating ERK1/2 signaling pathway. ZEB1 can be treated as a new molecular biomarker for the treatment of prostate cancer, providing bases to elucidate the mechanism of prostate cancer tumorigenesis.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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