

Effects of ZEB1 on regulating osteosarcoma cells via NF- κ B/iNOS

X.-M. XU¹, W. LIU², Z.-H. CAO¹, M.-X. LIU¹

¹Hand and Foot Bone Surgery, Weifang People's Hospital, Weifang, Shandong, China

²Spinal Surgery, Weifang People's Hospital, Weifang, Shandong, China

Abstract. – **OBJECTIVE:** Osteosarcoma is one common malignant bone tumors, as it frequently has invasion, metastasis and recurrence, causing unfavorable prognosis of patients. Osteosarcoma has complicated pathogenesis, which has not been elucidated fully. Therefore, the identification of effective molecular target of osteosarcoma onset can help to improve treatment efficacy and prognosis of osteosarcoma. Zinc finger E-box binding homeobox 1 (ZEB1) protein is one member of zinc finger E-box binding protein family, and participates in embryonic genesis and development. A recent study found the participation of ZEB1 in mediating multiple tumor onset and its up-regulation of osteosarcoma. The regulatory mechanism of ZEB1 in osteosarcoma has not been illustrated yet.

MATERIALS AND METHODS: *In vitro* cultured osteosarcoma MG-63 cells were transfected with ZEB1 siRNA. Real-time PCR and Western blot were tested for ZEB1 mRNA/protein expression. MTT was used to test MG-63 cell proliferation, whilst cell invasion was used to describe the effect of ZEB1 on MG-63 cells. Caspase-3 activity assay was employed to test MG-63 cell apoptosis. Western blot was employed to detect nuclear factor kappa B (NF- κ B) and inducible nitric oxide synthase (iNOS) protein expression.

RESULTS: After transfecting with ZEB1 siRNA, MG-63 cell proliferation or invasion was inhibited accompanied with lower ZEB1 mRNA/protein expression. Caspase3 activity was also increased after transfection ($p < 0.05$), along with down-regulation of NF- κ B and iNOS proteins in MG-63 cells ($p < 0.05$).

CONCLUSIONS: Inhibition of ZEB1 can facilitate osteosarcoma cell apoptosis and inhibit cell proliferation or invasion via down-regulating NF- κ B/iNOS signal pathway.

Key Words:

Osteosarcoma, ZEB1, NF- κ B, iNOS, Cell proliferation

Introduction

Osteosarcoma is the most popular malignant bone tumor worldwide, as it occupies

more than 30% of primary bone cancer¹. Osteosarcoma mainly derives from mesenchymal tissues, and is frequently occurred in children or young people under 20 years². Tumor cells have rapid proliferation, and can directly affect immature bone or bone-like tissues, plus early and frequent occurrence of blood-borne metastasis, contributing to rapid progression^{3,4}. Osteosarcoma frequently occurs in distal femur or proximal humerus. Current treatment mainly includes amputation, which is assisted by chemo-, radio-therapy and bone reconstruction⁵. However, due to rapid progression of osteosarcoma, it has high malignancy, and is susceptible for invasion, metastasis or recurrence, leading to unfavorable prognosis, lower survival rate, and heavy economic/mental burdens for patients and families^{6,7}. Osteosarcoma has complicated pathogenesis mechanism, which has not been illustrated fully yet. Therefore, the identification of effective molecular targets of osteosarcoma pathogenesis can help to improve treatment efficacy⁸. Zinc finger E-box binding homeobox (ZEB) family belongs to zinc finger transcription factor superfamily, and mainly consists of ZEB1 and ZEB2⁹. ZEB1 protein exerts important functions in regulating transcription and is involved in embryonic formation and development, as its gene mutation causes sever malformation¹⁰. Recent studies^{11,12} indicated the role of ZEB1 in regulating occurrence of multiple tumors including breast cancer and prostate cancer. ZEB1 can also affect the expression of E-cadherin, and direct morphology and property transition of epithelial cells originally connected by polarity into mesenchymal cells, which can freely move across cellular matrix to facilitate epithelial-mesenchymal transition (EMT) process^{13,14}. EMT can cause metastasis and invasion of tumors¹⁵. A recent study showed elevated expression of ZEB1 in osteosarcoma cells, but leaving its regulatory

role or related mechanisms not fully illustrated¹⁶. This study thus aimed to investigate the expression of ZEB1 in osteosarcoma and manipulated its expression, to analyze the role and mechanism of ZEB1 for osteosarcoma cells.

Materials and Methods

Major Reagents and Equipment

Human osteosarcoma cell line MG-63 (CRL-1427TM) was purchased from ATCC cell bank (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) culture medium, fetal bovine serum (FBS) Gibco (Rockville, MD, USA) and streptomycin-penicillin were purchased from Hyclone (Logan, UT, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) powders were purchased from Gibco (Rockville, MD, USA). Trypsin-EDTA lysis buffer was purchased from Sigma-Aldrich (St. Louis, MO, USA). Caspase 3 activity assay kit and polyvinylidene fluoride (PVDF) membrane were purchased from Pall Life Sciences Inc. (Pensacola, FL, USA). Ethylene Diamine Tetraacetic Acid (EDTA) was purchased from Hyclone (Logan, UT, USA). Western blotting reagent was purchased from Beyotime Co. Ltd. (Beijing, China). ECL reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-human nuclear factor kappa B (NF- κ B) monoclonal antibody (Catalogue No. 76041), rabbit anti-human ZEB1 monoclonal antibody (Catalogue No. 3396), rabbit anti-human inducible nitric oxide synthase (iNOS) monoclonal antibody (Catalogue No. 13120) and mouse anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody (Catalogue No. 5127) were all purchased from Cell Signaling Technology (Beverly, MA, USA). Transwell chamber was purchased from Corning (Corning, NY, USA). ZEB1 siRNA negative control (scramble) and ZEB1 siRNA sequences were synthesized by Gimma Co. Ltd. (Beijing, China). RNA extraction kits and reverse transcription kit were purchased from Axygen (Tewksbury, MA, USA). Other common reagents were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China). Lab System Version 1.3.1 microplate reader was purchased from Bio-Rad Laboratories (Hercules, CA, USA). ABI7700 Fast fluorescent quantitative PCR cycler was purchased from ABI (Foster City, CA, USA). Ultrapure workstation was pur-

chased from Sutai High-Tech Materials Co. Ltd. (Shanghai, China). Thermo Scientific Forma CO₂ incubator was purchased from Thermo Electron Corp. (Burlington, Ontario, Canada). PCR cycler model 2400 was purchased from PE Gene Applied Biosystems. (Foster City, CA, USA).

Osteosarcoma MG-63 Cell Culture and Grouping

MG-63 cells stored in liquid nitrogen were re-suscitated in 37°C water-bath until fully thawing. Cells were centrifuged at 1000 r/min for 3 min, and were re-suspended in 1 ml fresh medium and were removed from 5 ml culture flask containing 3 ml fresh culture medium. Cells were kept in a humidified chamber with 5% CO₂ at 37°C for 24-48 h. Cells were seeded in culture dish at 1×10⁶/cm². The culture medium contained 10% fetal bovine serum (FBS), and 90% high-glucose Dulbecco's Modified Eagle Medium (DMEM) medium (containing 100 U/ml penicillin, 100 µg/ml streptomycin). Cells were kept in a humidified chamber with 5% CO₂ at 37°C. The medium was changed every other day and cells were passed every 2-3 cells. Cells at log-phase with 2nd to 8th generation were randomly divided into control group, which was cultured under normal condition; ZEB1 siRNA group, which was transfected by ZEB1 siRNA negative control sequence and ZEB1 siRNA group, which was transfected with ZEB1 siRNA sequence.

Liposome Transfection of ZEB1 siRNA Into MG-63 Cells

ZEB1 siRNA sequence (5'-GUUUG UCGCU UCCAA ATAUA T-3' and 5'-UAUAG GGAUA CAAAU UGCCT T-3') or ZEB1 siRNA negative control sequence (5'-AUGCC AUCCU GAGGU AGGAU G-3' and 5'-ACAGG GAUGU AUAGU CGCGU G-3') were transfected into MG-63 cells. In brief, cells were cultured until reaching 70-80% confluence. ZEB1 siRNA or negative control liposome were mixed with 200 µl serum-free medium for 15 min room temperature incubation. Lipo2000 reagent was then mixed with ZEB1 siRNA or negative controlled dilutions for 30 min room temperature incubation. Serum was removed, followed by phosphate buffered saline (PBS) rinsing gently and the addition of 1.6 ml serum-free culture medium. Cells were then kept in a humidified chamber with 5% CO₂ at 37°C for 6 h, followed by the application of 10% FBS-containing medium in 48 h continuous incubation for further experiments.

Real-time PCR for ZEB1 mRNA Expression in MG-63 Cells

Trizol reagent was used to extract RNA from MG-63 cells. Reverse transcription was performed according to the manual instruction of test kit, using primers designed by PrimerPremier 6.0 and synthesized by Invitrogen/Life Technologies (Carlsbad, CA, USA) as shown in Table I. Real-time polymerase chain reaction (PCR) was performed on target genes under the following conditions: 56°C for 1 min, followed by 35 cycles each containing 92°C for 30 s, 58°C for 45 s and 72°C for 35 s. Data were collected and calculated for CT values of all samples and standards based on fluorescent quantification using GAPDH as the reference. Standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis by $2^{-\Delta Ct}$ method.

Western Blot for ZEB1, NF- κ B and iNOS Protein Expression

Total proteins were extracted from MG-63 cells. In brief, cells were mixed with RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 2 μ g/ml Aprotinin, 2 μ g/ml Leupeptin, 1 mM PMSF, 1.5 mM EDTA and 1 mM NaVanadate) (Sigma-Aldrich, St. Louis, MO, USA) on ice for 15~30 min, with ultrasound treatment (5 s, 4 times). After centrifugation at 10000 \times g for 15 min, the supernatant was saved, quantified and was stored at -20°C for Western blot assay. Proteins were separated in 10% SDS-PAGE, and were transferred to PVDF membrane by semi-dry method (100 mA, 1.5 h). Non-specific binding sites were removed by 5% defatted milk powder for 2 h. Anti-ZEB1 monoclonal antibody (1:1000), anti-NF- κ B monoclonal antibody (1:2000) and anti-iNOS monoclonal antibody (1:1500) were added for 4°C overnight incubation. After PBST washing, goat anti-rabbit secondary antibody (1:2000) was added for 30 min incubation at room temperature. ECL reagent was then added for developing the membrane for 1 min after PBST rinsing, followed by X-ray exposure. The film was scanned and analyzed by protein imaging system and Quantity

One software for measuring band density. Each experiment was replicated for four times (n=4) for statistical analysis.

MTT for Testing Cell Proliferation

MG-63 cells at log-phase were seeded into 96-well plate which contained DMEM medium with 10% FBS at 5×10^3 density. After 24 h incubation, the supernatant was removed. Cells were randomly divided into control, scramble and ZEB1 siRNA groups as treated above. After 48 h incubation, 20 μ l sterile MTT was added into each test well in triplicates. After 4 h continuous culture, the supernatant was completely removed, with the addition of 150 μ l DMSO for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values were measured at 570 nm in a microplate reader. The proliferation rate was calculated in each group.

Transwell Chamber Assay for Cell Invasion

Following the manual instruction, serum-free DMEM medium was changed for another 24 h cell culture. Transwell chamber was pre-coated using 1:5 50 mg/L Matrigel dilutions on the bottom and upper layer of the membrane, followed by 4°C air-dry. 500 μ l DMEM medium containing 10% FBS was added outside the chamber. Inside the chamber, 100 μ l cell suspension was added using serum-free culture medium. Each group was tested in triplicate. Control cells were cultured in Transwell chamber without Matrigel. After 48 h, PBS was used to rinse Transwell chamber to remove membrane-fixed cells. Chambers were then fixed in cold ethanol and stained with crystal violet. Cells at the lower surface of the membrane were counted. Each experiment was repeated for more than three times.

Caspase3 Activity Assay

Caspase 3 activity in cells was evaluated using test kit from all groups. In brief, cells were digested with trypsin, and were centrifuged at 600 \times g for 5 min under 4°C. The supernatant was discarded, followed by the addition of cell lysis buffer and iced incubation for 15 min. The mixture was then

Table I. Primer sequences.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
ZEB1	ACCTCTCTAGATCTGCCCAT	TAGAATGGTGTAAACCTCTT

centrifuged at $20\,000 \times g$ for 5 min under 4°C , followed by the addition of 2 mM Ac-DECD-pNA. Optical density (OD) values at 450 nm wavelength was measured to reflect caspase 3 activity.

Statistical Analysis

All data were presented as mean \pm standard deviation (SD). Comparison of means between groups was performed by *t*-test. SPSS 11.5 software (SPSS Inc. Chicago, IL, USA) was used for analyzing data. Between-group difference was tested by analysis of variance (ANOVA). Statistical significance was defined when $p < 0.05$.

Results

ZEB1 mRNA Expression in Osteosarcoma Cells

Real-time PCR was used to test the effect of siRNA interference on ZEB1 mRNA expression in osteosarcoma cells. Results found that ZEB1 siRNA transfection significantly inhibited ZEB1 mRNA expression in osteosarcoma cells ($p < 0.05$ compared to control group). Another transfection using ZEB1 siRNA negative control sequence did not affect mRNA expression ($p > 0.05$, Figure 1).

ZEB1 Protein Expression in Osteosarcoma Cells

Western Blot was used to test the effect of siRNA interference on ZEB1 protein expression in osteosarcoma cells. Results found that ZEB1 siRNA transfection significantly inhibited ZEB1 protein expression in osteosarcoma cells ($p < 0.05$ compared to control group), whilst transfection using ZEB1 siRNA negative control sequence did not affect its protein expression ($p > 0.05$, Figure 2).

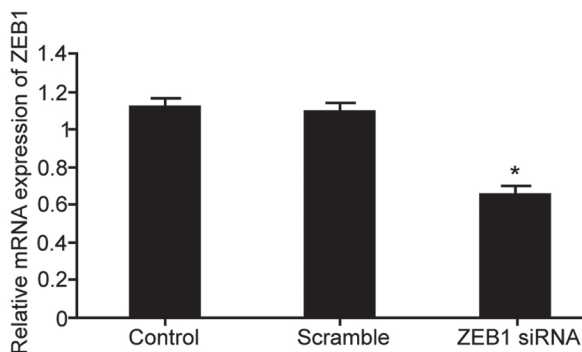


Figure 1. Regulation of ZEB1 on mRNA expression in osteosarcoma cells. *, $p < 0.05$ compared to control group.

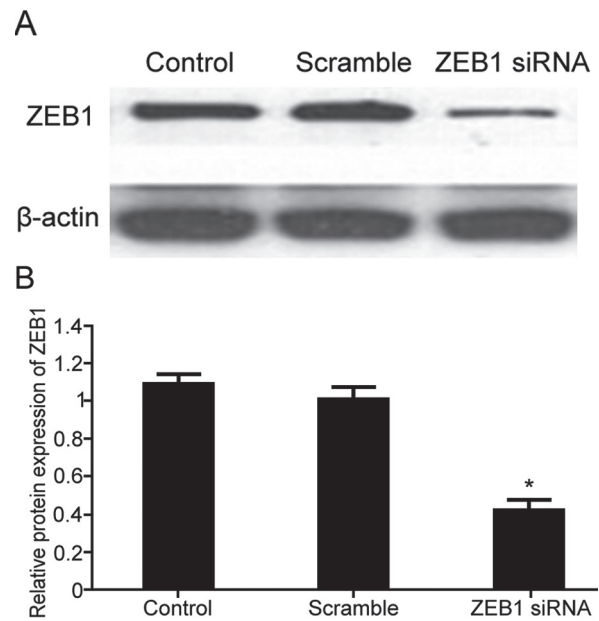


Figure 2. ZEB1 protein expression in osteosarcoma cells. (A) ZEB1 protein bands in osteosarcoma cells; (B) Analysis of ZEB1 protein relative expression. *, $p < 0.05$ compared to control group.

Effects of ZEB1 Regulation on Osteosarcoma MG-63 Cells Proliferation

MTT was used to test the effect of siRNA interference on proliferation of osteosarcoma cell line MG-63. Results showed that down-regulation of ZEB1 expression by ZEB1 siRNA interference into MG-63 cells significantly inhibited MG-63 cell proliferation ($p < 0.05$ compared to control group, Figure 3). These results indicated that ZEB1 expression was correlated with abnormal proliferation of osteosarcoma cells.

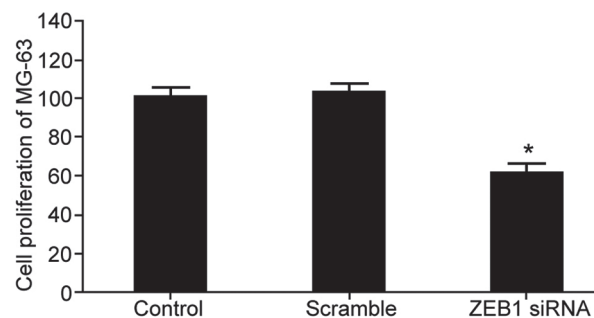


Figure 3. Effects of ZEB1 regulation on MG-63 cell proliferation. *, $p < 0.05$ compared to control group.

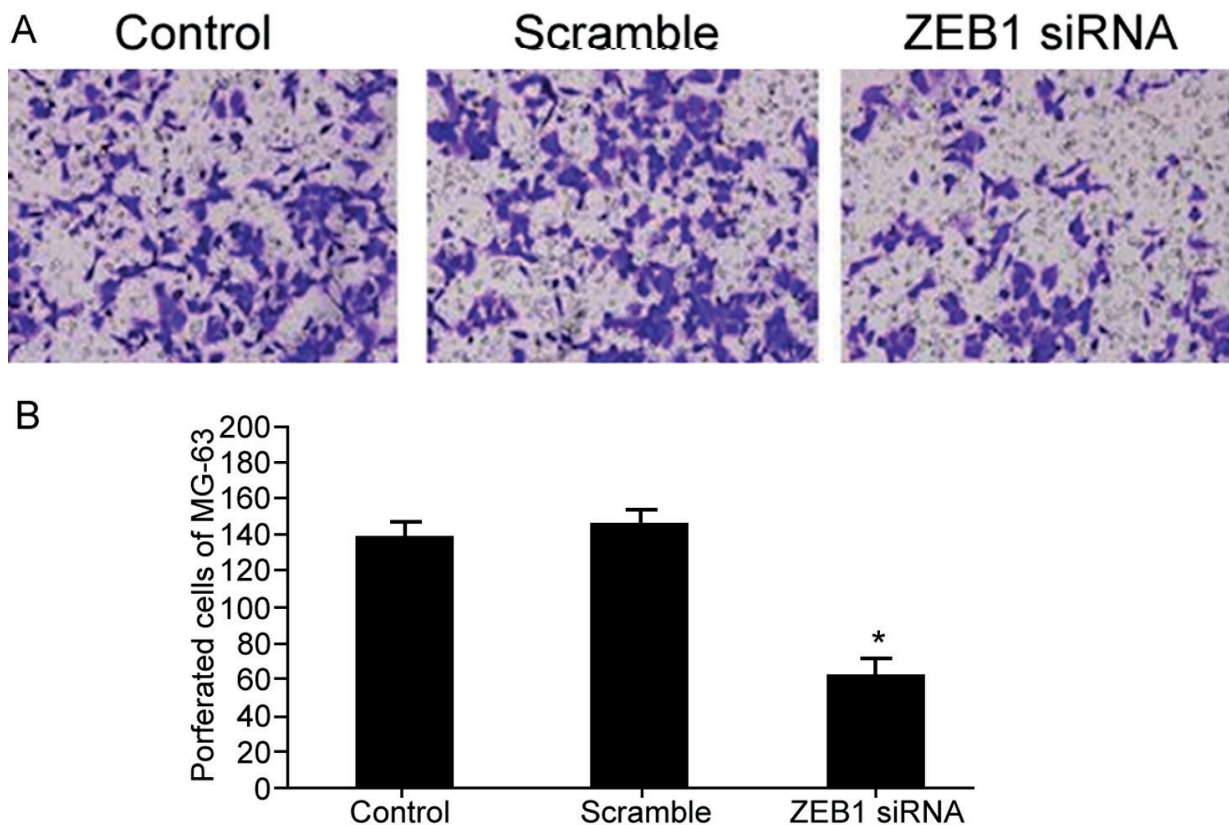


Figure 4. Effects of ZEB1 modulation on invasion potency of osteosarcoma MG-63 cells. *, $p < 0.05$ compared to control group.

Effects of ZEB1 on MG-63 Cell Invasion

Transwell chamber assay was used to test the effect of siRNA interference on invasion of osteosarcoma cell line MG-63. Results showed that down-regulation of ZEB1 expression by ZEB1 siRNA interference into MG-63 cells significantly inhibited cell invasion ($p < 0.05$ compared to control group, Figure 4). These results indicated that ZEB1 expression was correlated with invasion ability of osteosarcoma cells.

Effects of ZEB1 on Caspase3 Activity in MG-63 Cells

Caspase3 activity was used to test the effect of ZEB1 on apoptosis of osteosarcoma cell line MG-63. Results showed that down-regulation of ZEB1 expression by ZEB1 siRNA interference into MG-63 cells significantly enhanced Caspase-3 activity ($p < 0.05$ compared to control group, Figure 5). These results indicated that ZEB1 down-regulation could facilitate Caspase 3 activity, further accelerating apoptosis of osteosarcoma cells.

ZEB1 Modulation and NF- κ B/iNOS Signal Pathway in MG-63 Cells

Western blot was used to test the effect of ZEB1 modulation on NF- κ B/iNOS signal pathway in osteosarcoma cell line MG-63. Results showed that down-regulation of ZEB1 expression by ZEB1 siRNA interference into MG-63

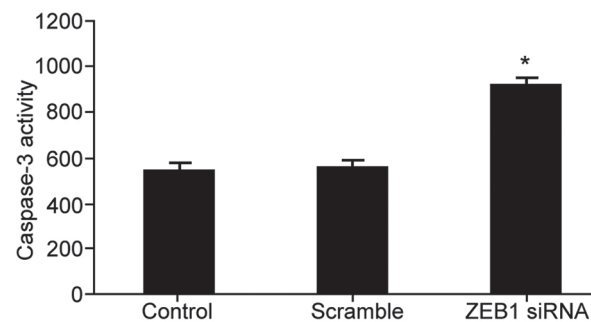


Figure 5. Regulation of ZEB1 on Caspase3 activity in osteosarcoma MG-63 cells. *, $p < 0.05$ compared to control group.

cells significantly inhibited NF- κ B or iNOS protein expression ($p < 0.05$ compared to control group, Figure 6). These results indicated that ZEB1 could regulate proliferation and apoptosis of MG-63 osteosarcoma cells via mediating NF- κ B/iNOS pathway.

Discussion

Osteosarcoma has a high incidence and unfavorable prognosis. Although treatment methods are continuously being improved, patient survival rate has not been significantly increased, making it one major challenge^{17,18}. Tumor occurrence, progression and metastasis involve multiple steps and factors, under the regulation of various genes and external factors. In those malignant tumors with EMT origin, ZEB1 is strongly correlated with tumor pathogenesis, and progression¹⁹. E-cadherin is one important metastatic inhibitor. ZEB1 can directly or indirectly inhibit E-cadherin expression. E-cadherin can form protein dimers with adjacent molecules to assisting normal alignment of cells¹³. The adhesion between E-cadherin and epithelium can work as cytoskeleton component¹⁴. ZEB1 specifically bind with E-cadherin to suppress its expression²⁰. Previous work¹⁶ demonstrated elevated expression of ZEB1 in osteosarcoma, whilst leaving its regulation and mechanism on osteosarcoma unillustrated. This study utilized siRNA approach to down-regulate ZEB1 expression, and found inhibition of osteosarcoma cell proliferation or invasion, plus potentiated Caspase3 activity. As one of the most potent members of apoptotic family, Caspase3 has enhanced activity that can induce tumor cell apoptosis²¹. NF- κ B as one nuclear transcriptional factor, can participate in various physiological processes including inflammation and immunity. It can activate different immune or inflammation related genes such as iNOS²². A previous research²³ indicated significantly enhanced iNOS expression in osteosarcoma tissues, as it may facilitate tumor angiogenesis, resistance to tumor cell apoptosis, and accelerating cell proliferation. This study showed that interference of ZEB1 expression decreased its mRNA and protein expression in osteosarcoma cells, and further inhibited cell proliferation and induced apoptosis possibly via suppressing NF- κ B/iNOS pathway. This work demonstrates the functional role and mechanism of ZEB1 in

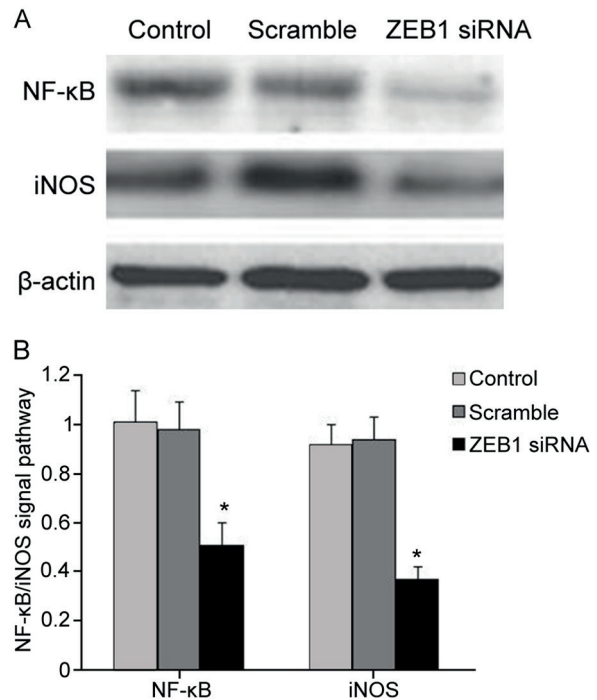


Figure 6. ZEB1 regulation and NF- κ B/iNOS signal pathway in MG-63 cells. (A) Western blot for NF- κ B/iNOS signal pathway in MG-63 cells; (B) Analysis of expression level of NF- κ B/iNOS signal pathway. *, $p < 0.05$ compared to control group.

regulating osteosarcoma, although further animal *in vivo* studies can be performed to illustrate detailed mechanism.

Conclusions

Inhibition of ZEB1 can facilitate osteosarcoma apoptosis via down-regulating NF- κ B/iNOS signal pathway, thus inhibiting cell proliferation or invasion, suggesting its potential role as novel molecular target for clinical treatment of osteosarcoma. This study provides evidence for illustrating pathogenesis of osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

- LIAN D, WANG ZZ, LIU NS. MicroRNA-1908 is a biomarker for poor prognosis in human osteosarcoma. *Eur Rev Med Pharmacol Sci* 2016; 20: 1258-1262.

- 2) RUDNICK-GLICK S, COREM-SALKMON E, GRINBERG I, MARGEL S. Targeted drug delivery of near IR fluorescent doxorubicin-conjugated poly (ethylene glycol) bisphosphonate nanoparticles for diagnosis and therapy of primary and metastatic bone cancer in a mouse model. *J Nanobiotechnology* 2016; 14: 80.
- 3) LOPEZ C, CORREA A, VAPORCIYAN A, AUSTIN M, RICE D, HAYES-JORDAN A. Outcomes of chest wall resections in pediatric sarcoma patients. *J Pediatr Surg* 2017; 52: 109-114.
- 4) REN XF, MU LP, JIANG YS, WANG L, MA JF. LY2109761 inhibits metastasis and enhances chemosensitivity in osteosarcoma MG-63 cells. *Eur Rev Med Pharmacol Sci* 2015; 19: 1182-1190.
- 5) ECHCHIKHI Y, LOUGHLIMI H, TOUIL A, KEBDANI T, BENJAAFAR N. Radiation-induced osteosarcoma of the skull base after radiation therapy in a patient with nasopharyngeal carcinoma: a case report and review of the literature. *J Med Case Rep* 2016; 10: 334.
- 6) DONG C, DU Q, WANG Z, WANG Y, WU S, WANG A. MicroRNA-665 suppressed the invasion and metastasis of osteosarcoma by directly inhibiting RAB23. *Am J Transl Res* 2016; 8: 4975-4981.
- 7) HLAVATY J, WOLFESBERGER B, HAUCK M, OBERMAYER-PIETSCH B, FUCHS-BAUMGARTINGER A, MILLER I, WALTER I. Ezrin and moesin expression in canine and feline osteosarcoma. *Histol Histopathol* 2016; 11848. Epub ahead of print]
- 8) ZHAN C, LI C, ZHANG H, TANG H, JI F, QIAO SC, XU WD, WANG ZW. MicroRNA-150 upregulation reduces osteosarcoma cell invasion and metastasis by downregulating Ezrin. *Oncol Lett* 2016; 12: 3457-3462.
- 9) OTSUKA Y, SATO H, OIKAWA T, ONODERA Y, NAM JM, HASHIMOTO A, FUKUNAGA K, HATANAKA KC, HATANAKA Y, MATSUNO Y, FUKUDA S, SABE H. High expression of EPB41L5, an integral component of the Arf6-driven mesenchymal program, correlates with poor prognosis of squamous cell carcinoma of the tongue. *Cell Commun Signal* 2016; 14: 28.
- 10) PAZOS MC, ABRAMOVICH D, BECHIS A, ACCIALINI P, PARBORELLI F, TESONE M, IRUSTA G. Gamma secretase inhibitor impairs epithelial-to-mesenchymal transition induced by TGF-beta in ovarian tumor cell lines. *Mol Cell Endocrinol* 2017; 440: 125-137.
- 11) HAN ML, ZHAO YF, TAN CH, XIONG YJ, WANG WJ, WU F, FEI Y, WANG L, LIANG ZQ. Cathepsin L upregulation-induced EMT phenotype is associated with the acquisition of cisplatin or paclitaxel resistance in A549 cells. *Acta Pharmacol Sin* 2016; 37: 1606-1622.
- 12) LIU C, LIN J. Long noncoding RNA ZEB1-AS1 acts as an oncogene in osteosarcoma by epigenetically activating ZEB1. *Am J Transl Res* 2016; 8: 4095-4105.
- 13) LIU M, ZHAO YY, YANG F, WANG JY, SHI XH, ZHU XO, XU Y, WEI D, SUN L, ZHANG YG, YANG K, QU YC, WANG X, LIANG SY, CHEN X, ZHAO CX, ZHU L, TANG L, ZHENG CG, YANG Z. Evidence for a role of GPRCC6A in prostate cancer metastasis based on case-control and in vitro analyses. *Eur Rev Med Pharmacol Sci* 2016; 20: 2235-2248.
- 14) OHAYON D, GARCES A, JOLY W, SOUKKARIEH C, TAKAGI T, SABOURIN JC, AGIUS E, DARLING DS, DE SANTA BARBARA P, HIGASHI Y, STOLT CC, HUGNOT JP, RICHARDSON WD, CARROLL P, PATTYN A. Onset of spinal cord astrocyte precursor emigration from the ventricular zone involves the zeb1 transcription factor. *Cell Rep* 2016; 17: 1473-1481.
- 15) QIAN C, DANG X, WANG X, XU W, PANG G, CHEN Y, LIU C. Molecular mechanism of microRNA-200c regulating transforming growth factor-beta (TGF-beta)/SMAD family member 3 (SMAD3) pathway by targeting zinc finger e-box binding homeobox 1 (ZEB1) in hypospadias in rats. *Med Sci Monit* 2016; 22: 4073-4081.
- 16) SHEN A, ZHANG Y, YANG H, XU R, HUANG G. Overexpression of ZEB1 relates to metastasis and invasion in osteosarcoma. *J Surg Oncol* 2012; 105: 830-834.
- 17) ARMAKOLAS N, ARMAKOLAS A, ANTONOPOULOS A, DIMAKAKOS A, STATHAKI M, KOUTSILIERIS M. The role of the IGF-1 Ec in myoskeletal system and osteosarcoma pathophysiology. *Crit Rev Oncol Hematol* 2016; 108: 137-145.
- 18) CHEN SP, TANG JL, ZHU XL. Primary intracerebral osteosarcoma: a rare case report and review. *Springerplus* 2016; 5: 1997.
- 19) JIA X, WANG Z, QIU L, YANG Y, WANG Y, CHEN Z, LIU Z, YU L. Upregulation of LncRNA-HIT promotes migration and invasion of non-small cell lung cancer cells by association with ZEB1. *Cancer Med* 2016; 5: 3555-3563.
- 20) LEE YJ, CHOI SY, YANG JH. AMP-activated protein kinase is involved in perfluorohexanesulfonate-induced apoptosis of neuronal cells. *Chemosphere* 2016; 149: 1-7.
- 21) POLYAK K, WEINBERG RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 2009; 9: 265-273.
- 22) AL-SADI R, GUO S, YE D, RAWAT M, MA TY. TNF-alpha modulation of intestinal tight junction permeability is mediated by NIK/IKK-alpha axis activation of the canonical NF-kappaB pathway. *Am J Pathol* 2016; 186: 1151-1165.
- 23) JAHANI-ASL A, BONNI A. iNOS: a potential therapeutic target for malignant glioma. *Curr Mol Med* 2013; 13: 1241-1249.