

Ovol2 induces mesenchymal–epithelial transition via targeting ZEB1 in osteosarcoma

Jijun Liu¹
Qi Wu¹
Yonggui Wang¹
Yulong Wei²
Hong Wu³
Lijun Duan¹
Qiang Zhang¹
Yonggang Wu¹

¹Department of Orthopedics,

²Department of Pathology,

³Department of Ultrasound, Bayannaer City Hospital, Bayannaer, Inner Mongolia, People's Republic of China

Purpose: Osteosarcoma (OS) is the most common type of primary solid bone tumor. Ovo-like zinc finger 2 (Ovol2), a zinc finger transcription factor, is a mesenchymal–epithelial transition (MET) driver that induces miR-200 expression in prostate cancer, breast cancer, and hepatocellular carcinoma. However, little is known about the expression and function of MET in sarcomas, including OS. This study investigated the expression and clinicopathological significance of Ovol2 and its effect on MET in OS.

Patients and methods: The Ovol2 expression in the tumor samples from patients with OS was examined using immunohistochemistry (IHC). We then upregulated the Ovol2 expression in MG-63 and SW1353 cells, detected the expression of MET-associated proteins, and observed the effects of Ovol2 on OS cell proliferation, migration, and cytoskeleton reorganization using Cell Counting Kit-8, transwell invasion, and phalloidin dyeing assays, respectively. The correlation between zinc finger E-box-binding homeobox 1 (ZEB1) and Ovol2 was assessed using the luciferase gene reporter assay in the MG-63 and SW1353 cells and IHC in the human OS tissue samples.

Results: The Ovol2 protein overexpression was related to the clinical grade ($P=0.02$) and the recurrence and metastasis ($P=0.02$) of OS. Results of the in vitro experiments showed that Ovol2 overexpression can suppress cell migration and invasion and can regulate the expression levels of MET-associated proteins. Ovol2 suppresses ZEB1 expression by binding to the ZEB1 promoter. Ovol2 is concomitant with a reduced IHC expression of ZEB1 in human OS tissues.

Conclusion: Ovol2 expression is associated with MET in OS cells and suppresses ZEB1 expression and OS progression.

Keywords: Ovol2, mesenchymal–epithelial transition, osteosarcoma, ZEB1

Introduction

Osteosarcoma (OS) is the most common histological form of primary malignant bone tumor.¹ OS is the eighth leading cancer in children and adolescents.² Although the development of surgical and multiagent chemotherapy has improved the survival of patients with OS, 50% of patients with the disease exhibit pulmonary metastasis. Clinical prognosis remains poor.³ Therefore, OS treatment requires the understanding of the detailed mechanisms of metastasis and identifying reliable biomarkers.

Epithelial–mesenchymal transition (EMT) is the conversion from an epithelial to a mesenchymal cell type, resulting in morphological and biochemical changes.⁴ EMT and its reverse process, mesenchymal–epithelial transition (MET), are required for physical and pathological processes, such as embryonic development, wound healing, fibrosis, and tumor metastasis. EMT endows tumor cells the capability to gain metastatic morphology, stemness, and immunoresistance properties.^{5–7} Studies in the past 20 years have shown that EMT/MET plays a key role in various cancer-related processes.

Correspondence: Yonggang Wu
Department of Orthopedics, Bayannaer City Hospital, Bayannaer, Inner Mongolia 015000, People's Republic of China
Tel +86 133 0478 2211
Fax +86 047 8841 5386
Email bsyygk@163.com

Although there is evidence that some similar mechanisms may extend to the nonepithelial tumor types, much less is known about the role of phenotypic plasticity in tumors originating from sources other than the epithelium such as glioma, hematopoietic malignancy, and sarcoma. The mechanisms governing EMT/MET program in the onset and progression of sarcomas may shed light on the pathogenesis of these tumors, potentially paving the way to the identification of prognostic markers or therapeutic targets for future clinical management.

Various EMT-regulating transcription factors, such as Snail, Slug, Twist, and zinc finger E-box-binding homeobox 1 (ZEB1), have been widely recognized as important players in tumor development and progression. A number of studies have reported the involvement of these transcription factors in the complex pathogenesis of OS. Snail is overexpressed in OS, represses the receptor for the vitamin D gene, inhibits $1,25(\text{OH})_2\text{D}_3$, and increases OS progression.⁸ Sharili et al⁹ reported that Slug is overexpressed in OS and upregulated Runx2, resulting in the abnormal differentiation of osteoblasts. Okamura et al¹⁰ investigated the interaction between Twist1 and tissue inhibitor of metalloproteinase 1, which increases the invasion and bone metastasis of OS. Twist1 is a predictor of resistance to neoadjuvant chemotherapy in OS.¹¹ ZEB1 and ZEB2 are overexpressed in OS. OS cells transfected with ZEB1 siRNA show decreased invasion capabilities.^{12,13} Despite the widely recognized implications of these transcription factors in tumor progression, research on the role of MET transcription factors in OS remains far less understood.

The Ovo-like zinc finger (Ovol) family (including Ovol1, Ovol2, and Ovol3) comprises zinc finger transcription factors that act as regulators of epithelial development. Ovol2 regulate the formation of a wide variety of organs, including the epidermis, hair follicles, and kidneys.^{14–16} Ovol2 reside downstream of many signaling pathways, including those of Wnt, epidermal growth factor, and bone morphogenetic protein/transforming growth factor (TGF)- β .^{17–19} In prostate and breast cancer cell lines, Ovol2 induces miR-200 expression and consequently acts as a MET driver.²⁰ Ovol2 can regulate the tumor progression and can affect the MET of hepatocellular carcinoma, lung adenocarcinoma, and colorectal, prostate, and breast cancers.^{17,21,22} However, Ovol2 expression in sarcoma and its association with sarcoma progression have not been systematically elucidated.

In this study, we detected the Ovol2 expression in human OS tissues and analyzed its clinicopathological significance. We also studied the effects of the ectopic expression of

Ovol2 in OS cell lines on MET-associated proteins and cellular behavioral phenotype and investigated the relationships between Ovol2 and ZEB1 in OS cell lines and human tissues.

Patients and methods

Clinical samples

Twenty-six primary OS tissue samples and lung metastatic foci samples were obtained from the Inner Mongolia Bayannaer City Hospital (Bayannaer, China). None of the patients had received any chemotherapy or radiotherapy before their operation. This study has been approved by the institutional review board of the hospital. Consent for participation in the study was obtained from all the participants or, where participants are children, a parent or guardian. Written informed consent was obtained from the patients and parents/guardians. All these samples were confirmed by pathologists. The clinicopathological information was obtained from patients' clinical records and pathological reports.

Immunohistochemical (IHC) staining

The sections were hydrated with gradient alcohol, pretreated with microwaves for antigen repairment, blocked with sheep serum, incubated with primary antibodies anti-Ovol2 (1:50; Novus Biologicals, Littleton, CO, USA) or anti-ZEB1 (1:100; Santa Cruz Biotechnology Inc., Dallas, TX, USA) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated immunoglobulin G (dilution 1:200; Zhongshan, Beijing, China) for 40 min. The signals were revealed, with 3,3'-diaminobenzidine buffer used as the substrate. PBS was used for negative control in place of the primary antibodies.

Cell lines

OS cell lines SW1353 and MG-63 were from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences/Peking Union Medical College (Beijing, China). MG-63 and SW1353 cells were cultured in Leibovitz Medium and Minimum Essential Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA), respectively. These cells were all maintained at 37°C in 5% CO₂.

Cell transfection

Ovol2 and controlled scrambled plasmids were purchased from GeneChem (Shanghai, China). Transfection was performed with Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions.

Western blot

Cells were lysed in ice-cold modified RIPA buffer. Proteins were harvested and determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). Antibodies to Ovol2, ZEB1, and β -actin and the secondary antibodies were from Santa Cruz Biotechnology Inc. Antibodies to N-cadherin, Snail, Slug, and Twist were from Abcam (Cambridge, UK). Antibody to E-cadherin was from BD Biosciences (San Jose, CA, USA). Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skim milk for 1 h, incubated with primary antibodies at 4°C overnight, incubated with secondary antibodies, detected with chemiluminescence reagent (EMD Millipore, Billerica, MA, USA), and visualized on an autoradiography film. The relative density of bands was analyzed using an Odyssey infrared scanner (LI-COR Biosciences, Lincoln, NE, USA).

Cell Counting Kit-8 (CCK8) assay

CCK8 was from Dojindo Laboratories (Kumamoto, Japan). Cells (8×10^3 cells/well) were seeded into 96-well flat-bottomed plates in 100 μ L of complete medium. The cells were incubated overnight to allow cell attachment and recovery. Cells were incubated with CCK8 solution (10 μ L) for 3 h. Absorbance was measured at 450 nm using a microplate reader.

Colony formation assay

The cells were trypsinized and replated at a density of 500 cells/well in six-well plates. Plates were incubated at 37°C and 5% CO₂. After 14 days, plates were fixed in 4% paraformaldehyde for 15 min, washed with PBS, and stained with a solution of 1% rhodamine B and 1% Nile Blue before washing and air drying. The number and size of colonies (>50 μ m) were then scored under a light microscope.

Wound healing assay

Cells were grown in 35 mm dishes to form a confluent monolayer. After making a straight scratch with a 10 μ L pipette tip and washing with PBS for three times, cells were incubated in a minimum medium at 37°C with 5% CO₂. We used 0.5% serum (containing 0.5% FBS) to minimize cell proliferation. The speed of wound closure was measured at intervals to assess migratory ability. The results were analyzed three times.

Matrigel invasion chamber assay

The cell invasion assay was performed using transwell cell culture inserts (8 μ m pore size, Transwell Assay System; Corning

Incorporated, Corning, NY, USA). Matrigel (1.5 mg/mL) was added to the upper surface of each transwell chamber filter and incubated for 1 h at 37°C. The cells were trypsinized, and 200 μ L of cell suspension (5×10^5 cells/mL) contained in a serum-free culture medium was added to the upper chamber. A total of 300 μ L of culture medium supplemented with 20% FBS was added to the lower chamber. After incubation at 37°C with 5% CO₂ for 48 h, the passed cells were fixed with 4% PFA and stained with 0.5% crystal violet. The number of invading cells was counted using an inverted light microscope (Olympus Corporation, Tokyo, Japan).

Phalloidin staining

Cells were cultured on sterile glass cover slips in serum-free medium 1 day prior to staining. Slips were fixed with 4% PFA for 10 min and then incubated with the phalloidin conjugated to Alexa 594 (Sigma-Aldrich Co., St Louis, MO, USA) for 40 min in the dark. Cells were washed for five times with PBS and mounted and visualized under a confocal laser scanning microscopy (Leica TCS SP5; Leica Microsystems, Wetzlar, Germany).

Co-immunoprecipitation (Co-IP) assay

The cells were lysed in ice-cold-modified Co-IP lysis buffer and protease inhibitor cocktail and then incubated on ice for 30 min. The insoluble material was pelleted at 12,000 \times g for 15 min at 4°C, precleared by incubation with protein A/G PLUS-Agarose (Santa Cruz Biotechnology Inc.). The aliquots were co-immunoprecipitated with anti-Ovol2 primary antibody or IgG followed by incubation with protein A/G PLUS-Agarose beads for 1 h at 4°C. After the immunoprecipitated complexes were washed and separated by 10% SDS-PAGE, Western blot was performed as described earlier.

Luciferase reporter gene assay

Luciferase reporter gene assays were conducted using the pGL3-basic luciferase reporter cassette. All plasmids were transfected into cells using Lipofectamine 2000 according to the manufacturer's instructions. Light units were measured using the luminometer. Data were normalized for transfection efficiency by co-transfecting with the β -galactosidase levels.

Chromatin immunoprecipitation (ChIP) assay

Briefly, the cells were fixed and lysed with SDS lysis buffer. The lysates were sonicated to reduce the DNA lengths to between 300 and 500 bp. The lysates were immunoprecipitated

with anti-Ovol2 antibody or control IgG. The DNA product was analyzed using real-time PCR. The following primers were used to detect ZEB1 promoter regions: F: 5'-AGGCTATAAACGCTTTACCTCTCTG-3', R: 5'-TTACGATTA CACCCAGACTGCGT-3'.

Statistical analysis

Statistical analyses were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Fisher's exact test or Pearson's χ^2 test was used to analyze the relationship between Ovol2 expression and clinicopathological characteristics and between Ovol2 and ZEB1 protein expression in OS tissues. Data are expressed through at least triplicate independent determinations. Data were presented as mean \pm SD. Differences between groups were assessed by the Mann-Whitney *U*-test and the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Ovol2 protein expression significantly correlates with clinical stages of OS

To clarify the clinical significance of Ovol2 expression in OS, we analyzed its expression in human OS tissue samples from 26 patients by using IHC staining. Ovol2 protein expression was mainly found in the cytoplasm and nucleus. The IHC staining reaction was evaluated by the percentage of positive (PP) cells and staining intensity (SI). SI was scored 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). PP was scored 0 (negative), 1 (1%–10% positive cells), 2 (11%–50% positive cells), 3 (51%–80% positive cells), and 4 (>80% positive cells). The IHC scores were calculated by multiplying SI with PP. A final score of ≥ 4 was considered a positive staining result.

Among the samples, eight (30.8%) samples showed positive Ovol2 expression, whereas the remaining 18 (69.2%) samples showed negative Ovol2 expression (Figure 1A). The tumors were categorized as positive or negative for Ovol2. The relationships between the clinicopathological characteristics and Ovol2 expression in OS were separately analyzed (Table 1). The frequency of positive immunostaining for individual pathological stages was 6/11 (54.5%) for stage I/II and 2/15 (13.3%) for stage III/IV. The Ovol2 protein expression was significantly correlated with clinical stage ($P=0.02$). Twelve (46.2%) patients with OS experienced metastasis or recurrence. Ovol2 protein expression was detected in 1/12 (8.3%) OS specimens from patients with metastasis; these rates were significantly lower than those in cases

without metastasis or recurrence ($P=0.02$) (7/14, 50.0%). No statistically significant difference in the tumor Ovol2 expression was observed among the groups in terms of age, gender, tumor size, and histological type.

We further analyzed the Ovol2 expression in six sets of matched specimens (including OS primary foci and lung metastatic foci) obtained from one patient. Among these specimens, only one sample showed an Ovol2-positive expression. Figure 1B shows that the Ovol2 expression in the primary foci was positive, whereas that in the lung metastatic foci was negative (Figure 1B).

Overexpression of Ovol2 impairs the migration and invasion of OS cells

We established the Ovol2 overexpression in SW1353 and MG-63 cells to observe the effects of Ovol2 on the cellular morphology and functional phenotypes (Figure 2A). No significant differences in growth were observed between the cells overexpressing Ovol2 and control cells in the CCK8 assays (MG-63, $P=0.058$; SW1353, $P=0.097$) (Figure 2B) and colony formation assays (MG-63, $P=0.304$; SW1353, $P=0.119$) (Figure 2C). Through a wound healing experiment, we observed the inhibited migration ability in the cells overexpressing Ovol2 (MG-63, $P=0.016$; SW1353, $P=0.046$) (Figure 2D). In the invasion assays, less cells overexpressing Ovol2 passed through the transwell chamber filter compared with the control cells (MG-63, $P=0.043$; SW1353, $P=0.006$) (Figure 2E).

Ovol2 overexpression induces epithelial phenotype and decreases the expression of ZEB1 in OS cells

Using phalloidin to dye fibrous actin (F-actin), the Ovol2 overexpression was observed to induce the SW1353 and MG-63 cells to form structures with regular shape and uniform composition (Figure 3A).

Western blot analysis showed that the SW1353 and MG-63 cells with Ovol2 overexpression presented significantly increased epithelial E-cadherin and decreased mesenchymal N-cadherin expression levels compared with the control cells (Figure 3B).

In addition to classical MET markers, we checked the expression levels of MET-regulated transcription factors, such as Twist, Snail, Slug, and ZEB1, which can repress E-cad expression by binding to the E-box in the E-cad gene promoter.²³ All these transcription factors are involved in the complex pathogenesis of OS.⁵ Among these factors,

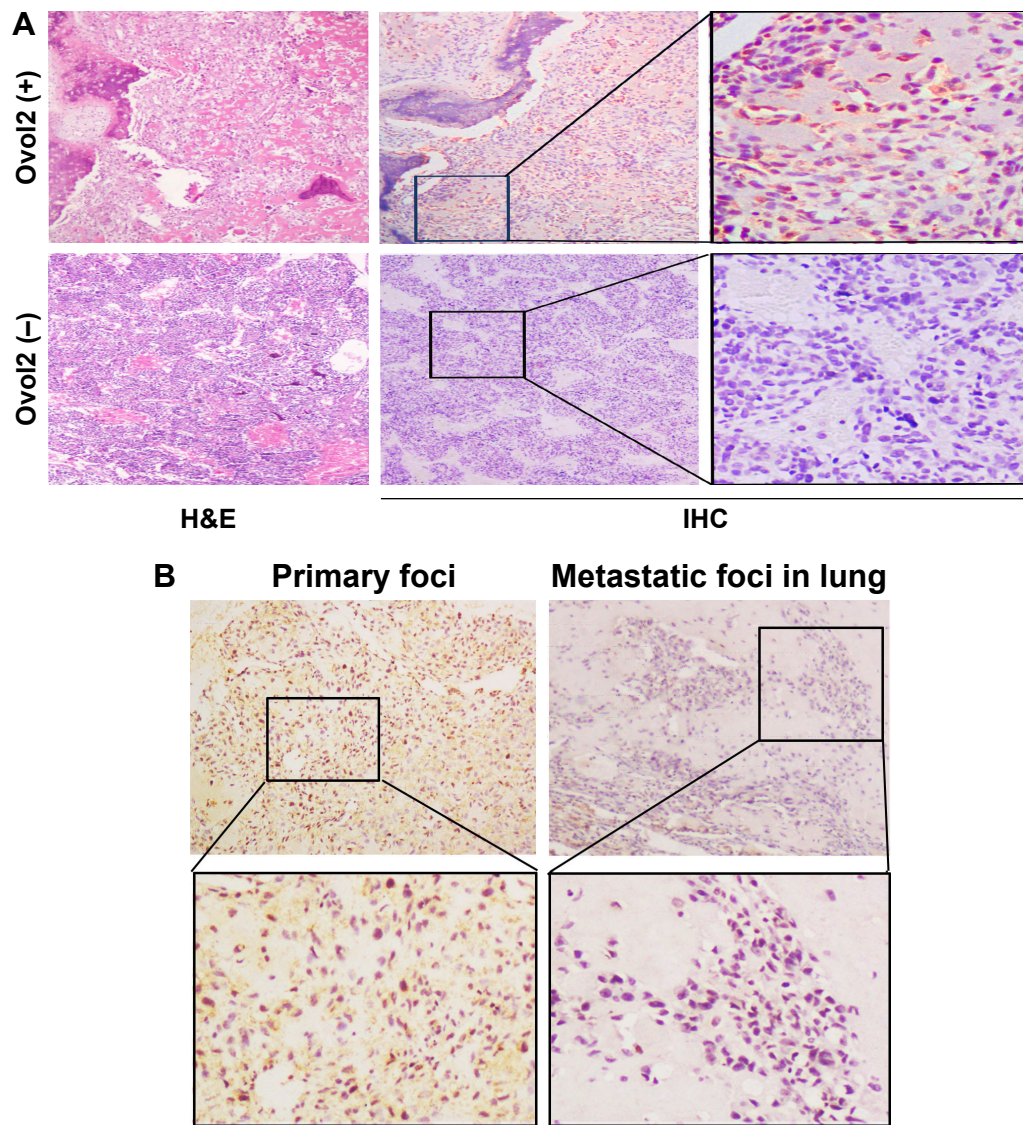


Figure 1 Expression of Ovol2 in human OS tissue samples.

Notes: (A) Representative Ovol2-positive sample with staining mainly in the nucleus and Ovol2-negative sample showing almost no appreciable staining, H&E staining, and IHC staining (magnification $\times 100$). (B) Different Ovol2 levels in OS primary foci and lung metastatic foci from the same patient (magnification $\times 100$).

Abbreviations: H&E, hematoxylin and eosin; IHC, immunohistochemistry; OS, osteosarcoma.

ZEB1 expression was significantly downregulated in the cells with Ovol2 expression (Figure 3C).

Ovol2 induces MET through direct transcriptional repression of ZEB1 in OS cells

ZEB1 is a zinc finger transcription factor located on human chromosome 10p11.22 and one of the most essential regulators during MET.²⁴ ZEB1 plays an important role in cancer metastasis.²⁵ ZEB1 overexpression induces the metastasis and invasion of OS.²⁶ By combining the results of previous and current studies,¹⁴ we proposed that ZEB1 may be a target gene of Ovol2 in OS. The interaction between

ZEB1 and Ovol2 was confirmed using reciprocal Co-IP analysis (Figure 4A). We constructed a ZEB1 luciferase reporter gene plasmid (inserted in pGL4.17) containing Ovol2-binding sites or mutated Ovol2-binding site (negative control) and used them to transfect the MG-63 and S1353 cells. We cotransfected the gene plasmid of Ovol2 into the MG-63 and SW1353 cells to detect the influence of Ovol2 on ZEB1 promoter. As shown in Figure 4B, Ovol2 inhibited the ZEB1 expression. Meanwhile, in ChIP assay, Ovol2 was found to binding to ZEB1 promoter (Figure 4C). Taken together, these findings demonstrate that the MET program induced by Ovol2 may be mediated by the direct repression of ZEB1.

Table 1 Correlation between *Ovol2* and clinicopathologic characteristics of osteosarcoma

Variable	Total	Ovol2 expression (%)		χ^2	P-value
		+	-		
Age				0.005	0.95
<14	10	3 (30.0)	7 (70.0)		
≥14	16	5 (31.3)	11 (68.7)		
Gender				0.07	0.79
Male	12	4 (33.3)	8 (66.7)		
Female	14	4 (28.6)	10 (71.4)		
Tumor size (cm)				2.50	0.11
≥5	9	1 (11.1)	8 (88.9)		
<5	17	7 (41.2)	10 (58.8)		
Tumor site				0.17	0.92
Femur	12	4 (33.3)	8 (66.7)		
Tibia	13	4 (30.8)	9 (69.2)		
Others	1	0 (0)	1 (100.0)		
Histological type				0.35	0.56
Conventional	24	8 (33.3)	16 (66.7)		
Special	2	0 (0)	2 (100.0)		
WHO grade				5.06	0.02*
TNM I + II	11	6 (54.5)	5 (45.5)		
TNM III + IV	15	2 (13.3)	13 (86.7)		
Recurrence and metastasis				5.27	0.02*
Present	12	1 (8.3)	11 (91.7)		
Absent	14	7 (50.0)	7 (50.0)		

Note: * $P < 0.05$, significantly different.

Ovol2 expression is concomitant with decreased ZEB1 expression in human OS tissues

To further validate the relationship between *Ovol2* and ZEB1, we investigated their expression in 26 human OS tissue specimens. As shown in Table 2 and Figure 5, the *Ovol2*-negative group exhibited higher ZEB1 expression than the *Ovol2*-positive group ($P < 0.05$). This result reinforces the finding that the MET-inducing effect of *Ovol2* on OS cells may be formed via the inhibition of ZEB1.

Discussion

Ovol2, a member of the *Ovo* family, encodes conserved zinc finger transcription factors.²⁷ Mouse *Ovol2* plays pivotal roles in hair follicle differentiation and kidneys and male germ cell development.^{14–16} In mice, *Ovol2* is required for embryonic angiogenesis and cardiac development.²⁸ *Ovol2* ablation can result in early embryonic lethality.²⁹ Recently, *Ovol2* was observed to be involved in the proper morphogenesis and regeneration of mammary epithelial cells and differentiation of corneal epithelial cells.^{30,31} In the present study, we initially investigated the *Ovol2* protein levels in the human OS tissue samples and observed that its expression

is inversely correlated with tumor stage and the presence of metastasis. *Ovol2* can also suppress the migration and invasion capabilities of OS cells in vitro. These results suggested that *Ovol2* may be an antitumor regulator and can provide important insight for future therapeutics. This finding is consistent with that of many previous studies on cancer tissues, including breast cancer, prostate cancer, colorectal cancer, hepatocellular carcinoma, and lung adenocarcinoma.^{17,19,21,22} To our knowledge, this study is the first to report the expression and function of *Ovol2* in sarcoma tissue.

EMT endows tumor cells the capability to detach from primary tumors, invade surrounding tissues, enter circulation, and disseminate to distant organs. MET, the opposite program of EMT, helps tumor cells to adapt to new sites, proliferate, and establish metastatic colonies. Understanding the basic principles of phenotypic transitions between epithelial and mesenchymal cells is crucial in preventing OS metastasis. Accumulating evidence has suggested that multiple transcription factors, such as Twist, Snail, Slug, and ZEB family, are involved in the tumor formation, invasiveness, and lung metastasis in OS and are associated with unfavorable prognosis.^{8–12} As a transcription factor, *Ovol2* is implicated in the regulation of the MET program of tumor cells. Ye et al¹⁷ provided compelling evidence that *Ovol2* controls colorectal cancer progression by inhibiting EMT. Wu et al¹⁹ demonstrated that *Ovol2* antagonizes TGF- β signaling in mouse and human mammary tumor cells at multiple levels of the signaling cascade, resulting in EMT inhibition during mammary tumor metastasis. Wang et al²² discovered that *Ovol2* can suppress the distant metastasis and invasion capability of lung adenocarcinoma by repressing EMT. Fu et al²¹ reported that *Ovol2* expression is significantly lower in human hematocellular carcinoma than in adjacent noncancerous tissues and that patients with high *Ovol2* expression level showed a high expression level of E-cadherin and low expression levels of N-cadherin, vimentin, Snail, and Slug. Similarly, this study demonstrated that *Ovol2* upregulation correlates with high E-cadherin expression level and low expression levels of N-cadherin and ZEB1 and increases the migration and invasion capabilities of OS cells. This study presented novel evidence for understanding the correlations of *Ovol2*- and MET-associated markers in OS cells.

Ovols cause the induction of microRNAs, including miR-429, miR-208, and miR-200c, which are involved in the regulation of MET.¹⁸ EMT is regulated by reciprocal feedback loops between ZEB1/ZEB2 and members of the miR200 family.³² Watanabe et al³⁰ observed that *Ovol2*

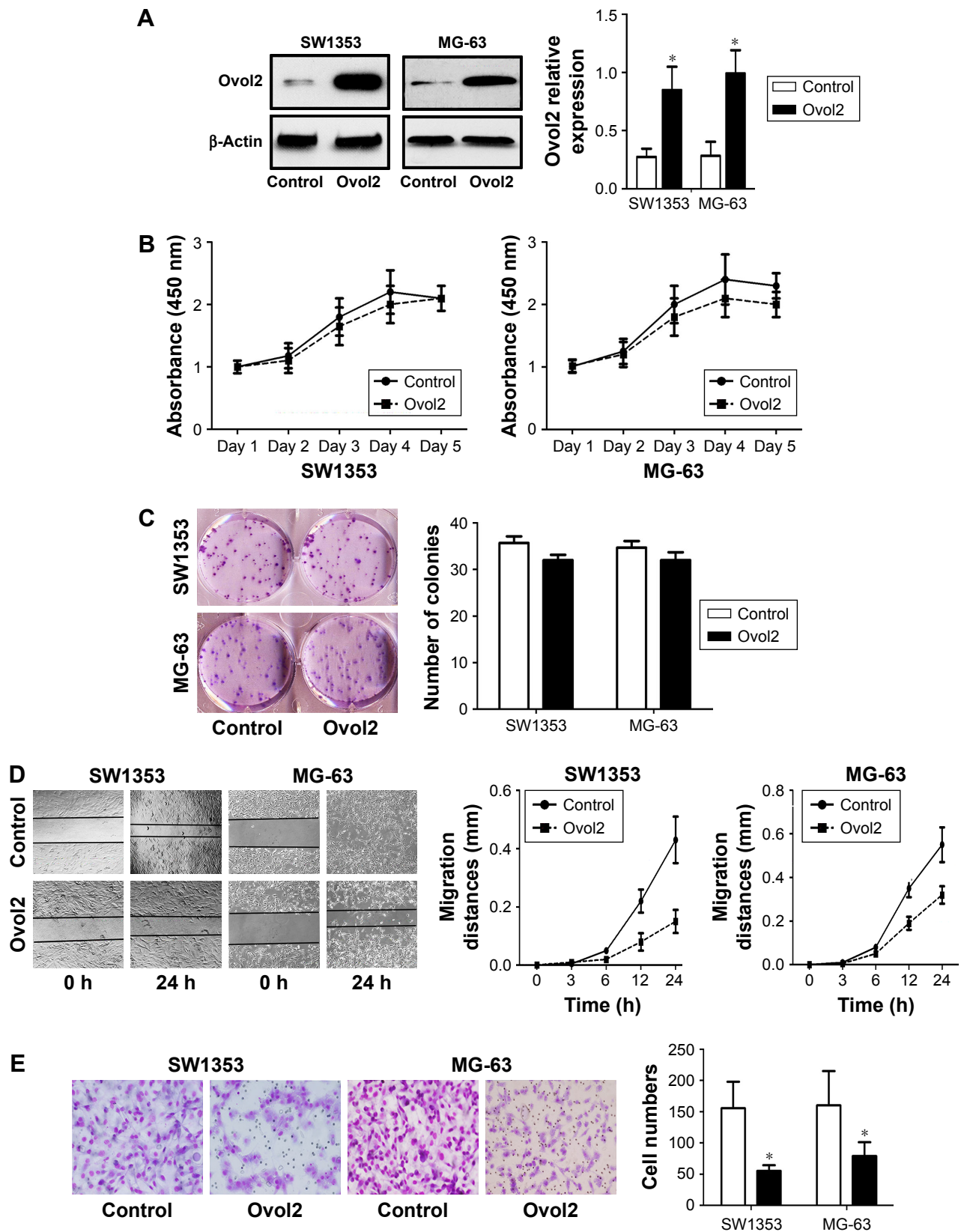


Figure 2 Overexpression of Ovol2 suppresses OS cells' migration and invasion and shows no effect on OS cell growth.

Notes: (A) Ovol2 protein levels significantly increased in SW1353 and MG-63 OS cells transfected with Ovol2 plasmid. (B and C) Cell proliferation ability was evaluated using the CCK8 assay and colony formation assay. (D) Cell migration ability was evaluated using the wound healing assay (magnification $\times 100$). (E) Cell invasion ability was evaluated using the transwell assay (magnification $\times 100$); * $P < 0.05$ vs control groups.

Abbreviations: CCK8, Cell Counting Kit-8; OS, osteosarcoma.

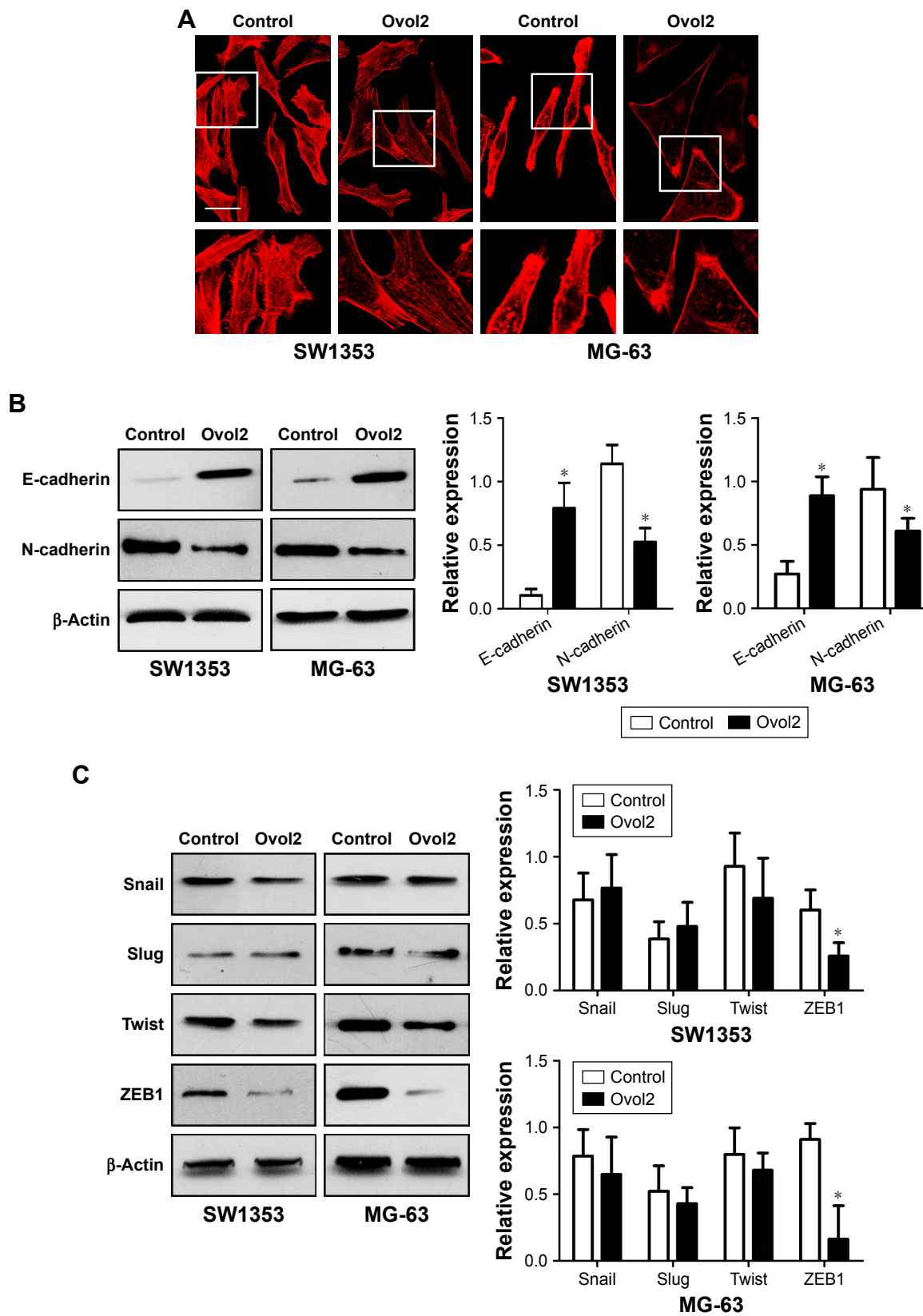


Figure 3 Overexpression of Ovo2-induced epithelial phenotype and decreased ZEB1 expression in OS cells.

Notes: (A) Phalloidin staining was used to dye F-actin cytoskeleton to display morphological changes in OS cells; scale bar: 10 μ m. (B and C) Western blot was used to detect the expression of MET-related markers (E-cadherin and N-cadherin) and MET-related transcription factors (Snail, Slug, Twist, and ZEB1) in SW1353 and MG-63 cells transfected with control or Ovo2 plasmid. A relative amount of protein expression was compared with β -actin (right); * P <0.05.

Abbreviations: F-actin, fibrous actin; MET, mesenchymal-epithelial transition; OS, osteosarcoma; ZEB1, zinc finger E-box-binding homeobox 1.

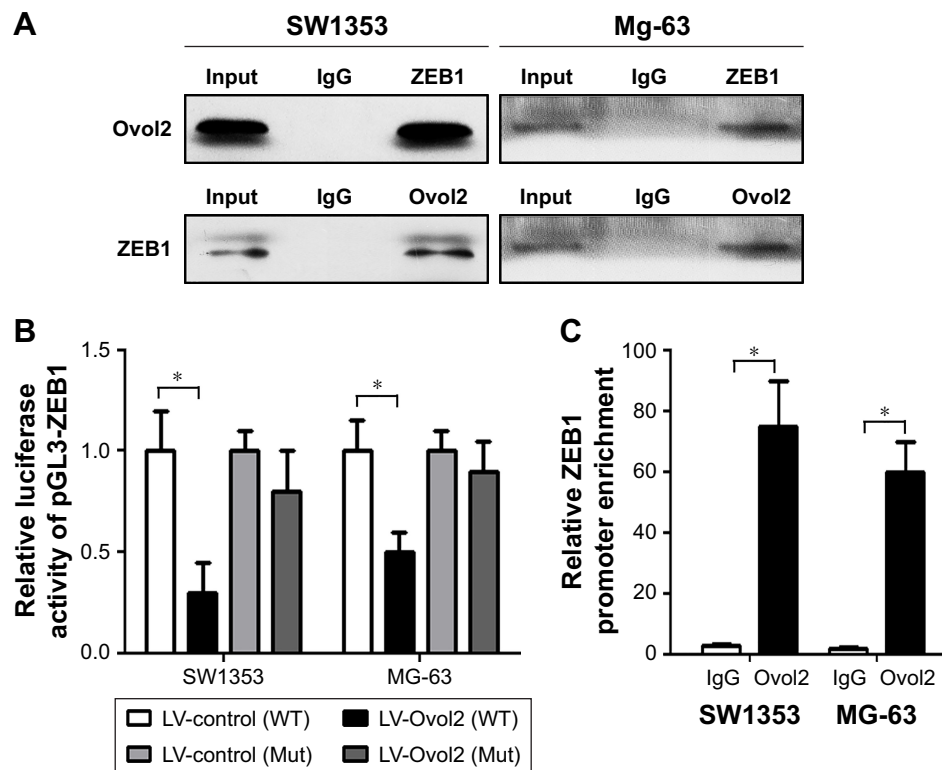


Figure 4 Ovov2 induces MET through direct transcriptional repression of ZEB1 in OS cells.

Notes: (A) Reciprocal Co-IP of endogenous Ovov2 and ZEB1 in SW1353 and MG-63 cells. (B) Effect of Ovov2 overexpression on WT and Mut ZEB1 promoter activities was determined by using the pGL3 basic luciferase reporter cassette in SW1353 and MG-63 cells and lentiviruses expressing a vector control (LV-control) and Ovov2 (LV-Ovov2); $*P < 0.05$. (C) ChIP assay of endogenous Ovov2 for the ZEB1 promoter using quantitative real-time PCR analyses. The primer amplifying ORF region was used as negative controls; $*P < 0.05$.

Abbreviations: ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; MET, mesenchymal–epithelial transition; Mut, mutated; ORF, open reading frame; OS, osteosarcoma; WT, wild type; ZEB1, zinc finger E-box-binding homeobox 1.

deletion can induce epithelial cells to undergo EMT, become non-epithelial cell types, and block mammary ductal morphogenesis, whereas reduction of ZEB1 expression can partially rescue Ovov2 loss-induced mammary defects. Lee et al¹⁴ provided compelling evidence that Ovov1/2 promotes the differentiation of epidermal progenitor cells in part by inhibiting ZEB1, which in turn represses α -catenin transcription. In this study, we demonstrated that Ovov2 decreased the ZEB1 expression by direct interaction with the ZEB1 promoter region in the OS cells. Lower levels of ZEB1 expression were also observed in the Ovov2-positive expression group than

in the negative group of human OS tissue samples. These results suggested that Ovov2-mediated MET may involve the regulation of ZEB1 in mesenchymal tumors, similar to epithelial tumors.

However, inconsistent results have been reported. Ye et al¹⁷ reported that Ovov2 inhibited the Slug expression in colorectal cancer cells. Wang et al²² showed that Ovov2 can repress the transcription of Twist1 by directly binding to its promoter in lung adenocarcinoma cells. Therefore, Ovov2 may exert different actions depending on the type and origin of the cells. Ovov2 may also induce MET via additional mechanisms, including inhibiting TGF- β /smad4 signaling, suppressing Wnt/ β -catenin signaling, and activating nuclear factor- κ B signaling.^{17,19} The current study can serve as basis for the future assessment of the precise function of Ovov2 in epithelial–mesenchymal plasticity and its incorporation with different signaling pathways in OS cells. A better understanding and control of Ovov2 may provide valuable information for the development of novel therapies targeting OS metastasis in the future.

Table 2 Correlation between expression of Ovov2 and ZEB1 in human osteosarcoma tissues

Variable	Total	Ovov2 expression (%)		χ^2	P-value
		+	-		
ZEB1 expression				5.060	0.024*
+	15	2 (13.3)	13 (86.7)		
-	11	6 (54.5)	5 (45.5)		

Note: $*P < 0.05$, significantly different.

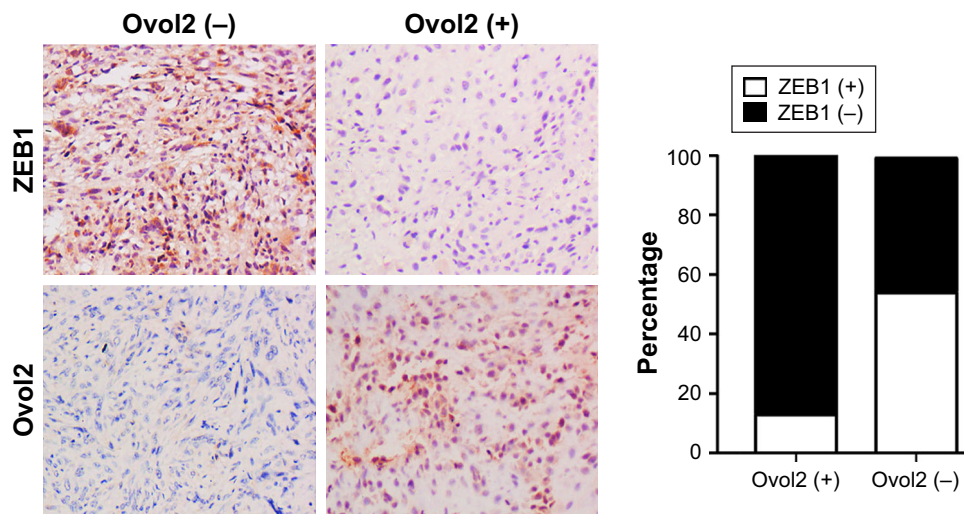


Figure 5 Expression of Ovov2 is concomitant with decreased ZEB1 expression in human OS tissues.

Notes: Representative images of the expression of ZEB1 in Ovov2-negative and Ovov2-positive groups (left). Percentages of ZEB1-negative and ZEB1-positive expressions in the Ovov2-negative and Ovov2-positive groups (right) and IHC staining (magnification $\times 200$).

Abbreviations: IHC, immunohistochemistry; OS, osteosarcoma; ZEB1, zinc finger E-box-binding homeobox 1.

Conclusion

Ovov2 expression is associated with MET and suppresses OS progression.

Acknowledgment

This study was funded by the Science and Technology Project of Bayannaer City (K201526).

Disclosure

The authors report no conflicts of interest in this work.

References

- Morrow JJ, Khanna C. Osteosarcoma genetics and epigenetics: emerging biology and candidate therapies. *Crit Rev Oncog*. 2015;20(3–4):173–197.
- Moriarty BS, Otto GM, Rahrman EP, et al. A sleeping beauty forward genetic screen identifies new genes and pathways driving osteosarcoma development and metastasis. *Nat Genet*. 2015;47(6):615–624.
- Lamplot JD, Denduluri S, Qin J, et al. The current and future therapies for human osteosarcoma. *Curr Cancer Ther Rev*. 2013;9(1):55–77.
- Jolly MK, Boareto M, Huang B, et al. Implications of the hybrid epithelial/mesenchymal phenotype in metastasis. *Front Oncol*. 2015;5:155.
- Kahlert UD, Joseph JV, Kruyt FAE. EMT and MET-related processes in non-epithelial tumors: importance for disease progression, prognosis and therapeutic opportunities. *Mol Oncol*. 2017;11(7):860–877.
- Stone RC, Pastar I, Ojeh N, et al. Epithelial–mesenchymal transition in tissue repair and fibrosis. *Cell Tissue Res*. 2016;365(3):495–506.
- Pradella D, Naro C, Sette C, Ghigna C. EMT and stemness: flexible processes tuned by alternative splicing in development and cancer progression. *Mol Cancer*. 2017;16(1):8.
- Yang H, Zhang Y, Zhou Z, Jiang X, Shen A. Snail-1 regulates VDR signaling and inhibits 1,25(OH)-D(3) action in osteosarcoma. *Eur J Pharmacol*. 2011;670(2–3):341–346.
- Sharili AS, Allen S, Smith K, Price J, McGonnell IM. Snail2 promotes osteosarcoma cell motility through remodelling of the actin cytoskeleton and regulates tumor development. *Cancer Lett*. 2013;333(2):170–179.
- Okamura H, Yoshida K, Haneji T. Negative regulation of TIMP1 is mediated by transcription factor TWIST1. *Int J Oncol*. 2009;35(1):181–186.
- Zhou Y, Huang Z, Wu S, Zang X, Liu M, Shi J. MiR-33a is up-regulated in chemoresistant osteosarcoma and promotes osteosarcoma cell resistance to cisplatin by down-regulating TWIST. *J Exp Clin Cancer Res*. 2014;33:12.
- 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(8):830–834.
- Fei D, Zhao K, Yuan H, Xing J, Zhao D. MicroRNA-187 exerts tumor-suppressing functions in osteosarcoma by targeting ZEB2. *Am J Cancer Res*. 2016;6(12):2859–2868.
- Lee B, Villarreal-Ponce A, Fallahi M, et al. Transcriptional mechanisms link epithelial plasticity to adhesion and differentiation of epidermal progenitor cells. *Dev Cell*. 2014;29(1):47–58.
- Ito T, Tsuji G, Ohno F, et al. Activation of the OVOL1-OVOL2 axis in the hair bulb and in pilomatricoma. *Am J Pathol*. 2016;186(4):1036–1043.
- Aue A, Hinze C, Walentin K, et al. A grainy head-like 2/Ovo-like 2 pathway regulates renal epithelial barrier function and lumen expansion. *J Am Soc Nephrol*. 2015;26(11):2704–2715.
- Ye GD, Sun GB, Jiao P, et al. OVOL2, an inhibitor of WNT signaling, reduces invasive activities of human and mouse cancer cells and is down-regulated in human colorectal tumors. *Gastroenterology*. 2016;150(3):659–671.e616.
- Jolly MK, Jia D, Boareto M, et al. Coupling the modules of EMT and stemness: a tunable ‘stemness window’ model. *Oncotarget*. 2015;6(28):25161–25174.
- Wu RS, Hong JJ, Wu JF, et al. OVOL2 antagonizes TGF-beta signaling to regulate epithelial to mesenchymal transition during mammary tumor metastasis. *Oncotarget*. 2017;8(24):39401–39416.
- Mooney SM, Parsana P, Hernandez JR, et al. The presence of androgen receptor elements regulates ZEB1 expression in the absence of androgen receptor. *J Cell Biochem*. 2015;116(1):115–123.
- Fu H, Qi L, Chen L, He Y, Zhang N, Guo H. Expression of Ovov2 is related to epithelial characteristics and shows a favorable clinical outcome in hepatocellular carcinoma. *Onco Targets Ther*. 2016;9:5963–5973.
- Wang ZH, Li Z, Hu M, et al. Ovov2 gene inhibits the epithelial-to-mesenchymal transition in lung adenocarcinoma by transcriptionally repressing Twist1. *Gene*. 2017;600:1–8.

23. Tania M, Khan MA, Fu J. Epithelial to mesenchymal transition inducing transcription factors and metastatic cancer. *Tumour Biol.* 2014;35(8): 7335–7342.
24. Zhang P, Sun Y, Ma L. ZEB1: at the crossroads of epithelial–mesenchymal transition, metastasis and therapy resistance. *Cell Cycle.* 2015;14(4): 481–487.
25. Liu Z, Sun B, Qi L, Li H, Gao J, Leng X. Zinc finger E-box binding homeobox 1 promotes vasculogenic mimicry in colorectal cancer through induction of epithelial-to-mesenchymal transition. *Cancer Sci.* 2012;103(4):813–820.
26. Liu C, Pan C, Cai Y, Wang H. Interplay between long noncoding RNA ZEB1-AS1 and miR-200s regulates osteosarcoma cell proliferation and migration. *J Cell Biochem.* 2017;118(8):2250–2260.
27. Ito T, Tsuji G, Ohno F, Nakahara T, Uchi H, Furue M. Potential role of the OVOL1-OVOL2 axis and c-Myc in the progression of cutaneous squamous cell carcinoma. *Mod Pathol.* 2017;30(7):919–927.
28. Unezaki S, Horai R, Sudo K, Iwakura Y, Ito S. Ovol2/Movo, a homologue of *Drosophila ovo*, is required for angiogenesis, heart formation and placental development in mice. *Genes Cells.* 2007;12(6):773–785.
29. Mackay DR, Hu M, Li B, Rheaume C, Dai X. The mouse Ovol2 gene is required for cranial neural tube development. *Dev Biol.* 2006; 291(1):38–52.
30. Watanabe K, Villarreal-Ponce A, Sun P, et al. Mammary morphogenesis and regeneration require the inhibition of EMT at terminal end buds by Ovol2 transcriptional repressor. *Dev Cell.* 2014;29(1):59–74.
31. Kitazawa K, Hikichi T, Nakamura T, et al. OVOL2 maintains the transcriptional program of human corneal epithelium by suppressing epithelial-to-mesenchymal transition. *Cell Rep.* 2016;15(6): 1359–1368.
32. Chen J, Wang L, Matyunina LV, Hill CG, McDonald JF. Overexpression of miR-429 induces mesenchymal-to-epithelial transition (MET) in metastatic ovarian cancer cells. *Gynecol Oncol.* 2011;121(1):200–205.

OncoTargets and Therapy

Publish your work in this journal

OncoTargets and Therapy is an international, peer-reviewed, open access journal focusing on the pathological basis of all cancers, potential targets for therapy and treatment protocols employed to improve the management of cancer patients. The journal also focuses on the impact of management programs and new therapeutic agents and protocols on

Submit your manuscript here: <http://www.dovepress.com/oncotargets-and-therapy-journal>

patient perspectives such as quality of life, adherence and satisfaction. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Dovepress