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Original Article

High expression of ZEB1 in endometriosis and its role in 17β-estradiol-induced epithelial-mesenchymal transition

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Abstract: Endometriosis is an estrogen-dependent disease associated with pain and infertility. The objective of this study was to determine the expression of ZEB1 in endometriosis and its role in 17β-estradiol (E2)-induced epithelial-mesenchymal transition (EMT). 25 patients with endometriosis and 16 endometriosis-free patients were recruited for the study. Tissue expression of EMT makers was investigated by immunohistochemistry, then the expression of ZEB1 was quantified by qRT-PCR, immunohistochemistry, and western blot. The proliferation, DNA replication, and migration and invasion in ZEB1 knockdown Ishikawa cells were further respectively performed by MTS, Edu, wound healing and transwell assays. Luciferase assay was used to measure the ZEB1 promoter activity. Our results show that protein levels of E-cadherin and Keratin 18 decreased in endometriotic tissues. Meanwhile the expressions of ZEB1, Vimentin, and N-cadherin were significantly increased in endometriotic tissues. Down-regulation of ZEB1 inhibited Ishikawa cells proliferation, migration, invasion and EMT. E2 promoted the expression of ZEB1 through the ER genomic pathway, which contributed to the EMT process. The -1401 bp - -1901 bp region in the ZEB1 promoter was the main target of the E2 activity. The present results suggest that a high expression of ZEB1 plays an important role in the pathogenesis of endometriosis, and it may serve as a potential therapeutic target for endometriosis.

Keywords: Endometriosis, ZEB1, epithelial-mesenchymal transition, 17β-estradiol

Introduction

Endometriosis, defined as the presence of endometrial glands and stromal outside of their normal locations, is a common gynecological disorder which often causes pelvic pain, dysmenorrhea, and even infertility [1-3]. This disease affects approximately 10% of women of reproductive age, and brings about a $22 billion annual societal cost in the USA [4]. However, the underlying pathogenesis of endometriosis remains unclear. The most widely accepted hypothesis, proposed by Sampson, is that viable endometrial tissue sloughed through patent fallopian tubes into the peritoneal cavity during menstruation [5, 6]. Another interesting theory is that endometriosis is a result of a downward extension of the stromal endometrium from the uterine cavity [7]. Therefore, an increased invasiveness of the ectopic endometriotic cells is necessary for the development of endometriosis. It is noteworthy that endometriosis is a pathologically benign disease but displays an invasive phenotype [8].

Recently, growing evidence shows that the aberrant activation of epithelial-mesenchymal transition (EMT) plays an important role in the mechanisms of invasion in malignant tumors [9]. EMT is a process in which epithelial cells become elongated, lose their polarity, and begin to take on mesenchymal, fibroblast-like properties. EMT and its reverse process, mesenchymal to epithelial transition (MET), are essential for embryogenesis, fibrosis and tumor metastasis. EMT is induced by several tran-

scription factors, including Snail, Slug, Twist, ZEB1, and ZEB2 [10, 11]. Among these master EMT genes, Snail, Slug, and ZEB1 have been shown to induce EMT by suppressing E-cadherin expression in cancer cells, while Twist has been shown to promote breast cancer and colorectal metastasis. It is noteworthy that the poor prognosis of gastric cancers, breast, hepatocellular, and colorectal often correlate with the aberrant activation of Twist and Snail [12-14]. Another key transcription factor, ZEB1, binds E-box-like sequences (CACCTG) on target DNA by its two zinc-finger domains, containing one at the N-terminus and the other at the C-terminus. Recently, ZEB1 has been reported as an important player in the progression of various cancers (lung, prostate, and pancreatic cancers), and is associated with a poor prognosis and resistance to chemotherapy in pancreatic cancer [15-17].

Interestingly, the aberrant EMT phenomenon has been reported in endometriotic tissues. For example, the E-cadherin expression is reduced and vimentin expression is increased in the epithelial compartment of endometriotic lesions compared with normal endometrium lesions [18]. The results from immunohistochemistry show that EMT-like and MET-like processes may be involved in the pathogenesis of pelvic endometriosis [19]. Moreover, the E-cadherin gene polymorphism rs4783689 may be associated with endometriosis in the Japanese population [20]. Although a growing number of studies has demonstrated the role of ZEB1 in various kinds of carcinomas, no reports up till now have shown any data about the expression and function of ZEB1 in endometriosis. In the progress of EMT, estrogen signaling plays an important role. Some reports indicate that E2 induces EMT in cancer cells by increasing the expression of Snail or Slug [21, 22]. However, there is no study showing that the molecular mechanism of E2 induces EMT in endometriosis.

In the present study, we documented an elevated expression of ZEB1 and the related EMT process in the endometrial tissue of endometriosis. We further investigated the effects of ZEB1 knockdown by RNA interference on growth, mobility and invasion. The effects of E2 on the expression and promoter activity of ZEB1 were also studied.

Materials and methods

Reagents

The stock solutions of E2 (Sigma-Aldrich, Buchs, Switzerland) and ICI 182780 (Sigma-Aldrich, St. Louis, USA) were prepared in DMSO. The EGFP-tagged shRNA against ZEB1 (shZEB1) was purchased from GeneChem Corporation (Shanghai, China). The Lipofectamine 2000 was from Invitrogen (AG, Basel, Switzerland). The 5-ethynyl-20-deoxyuridine (Edu) was from Ruibobio (Guangzhou, China). The DAB kit and Ultra Sensitive TM SP kit were from Maixin Bio (Fuzhou, China). The SYBR PrimeScript RT-PCR Kit was from Takara Biotechnology Co. (Takara, Kyoto, Japan). The polyclonal antibody against Vimentin, the N-cadherin, the ZEB1, the GAPDH, and the monoclonal antibodies against E-cadherin and Keratin 18 were purchased from Cell Signaling Technology (CST, MA, USA).

Patients and sample collection

Endometrial tissue samples were obtained from 41 patients who were treated in the Department of Obstetrics and Gynecology at the First Affiliated Hospital of Xiamen University. To prevent the impact of tissue quality on PCR and staining results, four cases were removed from the study due to inadequate tissue quality as determined by the gynecological pathologist. Finally, 37 patients were enrolled in this study. Following confirmations using laparoscopy and histology, tissue samples from 21 patients with ovarian endometriosis cysts and 16 control patients who also underwent curettage due to tubal infertility were collected. From among the 21 endometriosis patients, we obtained paired eutopic and ectopic endometrial tissues. The stages of endometriosis were determined visually based on the revised American Society of Reproductive Medicine classification (1997). None of these patients had received hormone treatment within 6 months before the gynecological surgery. All patients were of reproductive age with regular menstrual cycles. The research protocol was approved by the Ethics Committee of the First Affiliated Hospital of Xiamen University and all tissue samples were obtained with full patient consent.

Cell culture and drug treatment

The Ishikawa human endometrial adenocarcinoma cell line (ER positive) was kindly provided by A.P. Qian-Sheng Huang (Institute of Urban Environment, Chinese Academy of Sciences, Xiamen). Ishikawa cells were placed in phenol red-free DMEM/F12 (Hyclone, Logan, USA) containing 10% charcoal-stripped FBS (CSFBS; Invitrogen) for 48 h before drug treatment to remove endogenous steroids. The cells were then incubated in a fresh medium with E2 (Sigma-Aldrich, St. Louis, USA), ICI 182780 (ER antagonist, Sigma-Aldrich, St. Louis, USA), SB203580 (p38 MAPK inhibitor), PD98059 (ERK inhibitor) or SP600125 (JNK inhibitor) alone or in combination for the indicated times. The Ishikawa cells were also used to carry out the ZEB1 knockdown and ZEB1 promoter experiments. All experiments were performed in triplicate.

The generation of stable ZEB1-knockdown Ishikawa cells

The EGFP-tagged shRNA against ZEB1 (shZEB1) was commercially constructed by the GeneChem Company (Shanghai, China) and was used for stable transfection. The following sequences were used to target ZEB1: shZEB1-050, forward 5'-CCGGCCTCTCTGAAAGAACACATTATTTTTG-3' and reverse 5'-AATTCAAAAACCTCTCTGAAAGAACACATTATTTTTG-3'; shZEB1-051, forward 5'-CCGGGCAACAATACAAGAGGTTAAATTTTTG-3' and reverse 5'-AATTCAAAAAGCAACAATACAAG AGGTTAAATTTTTG-3'. The procedure for generating stable ZEB1-knockdown Ishikawa cells was performed as described previously [23]. Briefly, the Ishikawa cells were transfected with recombinant shZEB1 lentivirus or shCtrl lentivirus, then 400 ug/mL of G418 was added and single colonies were picked up on about the fifteenth day. The stable cell lines were identified, and qRT-PCR was used to quantify the expression levels of ZEB1.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was extracted from the endometriosis clinical samples using RNAiso Plus (Takara) following the manufacturer's protocol. The total intact RNA was confirmed by determining the appropriate sharp 28S and 18S rRNA bands using agarose gel electrophoresis, and the cDNA was synthesized using a Prime Script RT reagent Kit (Takara). Quantitative real-time PCR was performed using a Light Cycler 480 (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The primers used for real-time PCR were as follows: ZEB1 forward 5'-AAGTGGCGGTAGATGGTAATGT-3' and reverse 5'-AAGGAAGACTGATGGCTGAAAT-3'; Slug forward 5'-ACAGCGAACTGGACACACATAC-3' and reverse 5'-GGAATGGAGCAGCGGTAGT-3'; Vimentin forward 5'-GGAAGAGAACTTTGCCGTTG-3' and reverse 5'-TGGTATTCACGAAGGTGACG-3'; E-cadherin forward 5'-GGATGTGCTGGATGTGAATG-3' and reverse 5'-CTGGGCAGTGTAGGATGTGA-3'; GAPDH forward 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3'. All Primers were purchased from Sangon Biotech (Shanghai, China). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative mRNA level of each gene [24].

Western blotting

The western blotting procedures were performed as previously described [25, 26]. Briefly, the protein concentrations of the cells or tissues lysates were measured using a BCA Protein Assay Kit (TianGen, Beijing, China). The 25 μg sample extracts underwent 10% SDS-PAGE using a gel electrophoresis apparatus (Bio-Rad, CA, USA) and then were transferred onto PVDF membranes (Millipore, MA, USA). After blocking for 1 h with 5% nonfat dry milk in 0.05% TBST, the membranes were incubated overnight at 4°C with primary antibodies (anti-ZEB1, anti-Vimentin, anti-E-cadherin) (Cell Signaling, BOS, USA). After incubation with the secondary antibodies (1:10000, Pierce, USA), the proteins were analyzed using ECL chemiluminescence (Pierce, USA) and X-ray film. Quantity one software (Bio-Rad, CA, USA) was used to quantify the band intensities.

Immunohistochemistry (IHC) on tissue samples

The IHC procedures were performed as previously described [25]. The antibody characteristics are listed in **Table 1**. The slides incubated with normal rabbit IgG isotypes were applied as a negative control (data not shown). For a positive control, the cervical cancer samples were used as reported in our previous study [23]. The immunohistochemical staining results

Table 1. Summary antibody information tested in immunohistochemical and western blot protocols

Protein	Assay	Antibody	Origin	Dilution	Incubation period
ZEB1	IHC	05987, Novus Biologicals	rpab	1/250	Overnight, 4°C
ZEB1	WB	#3396, Cell Signaling Technology	rpab	1/1000	Overnight, 4°C
E-cadherin	IHC	#4065, Cell Signaling Technology	mmab	1/100	Overnight, 4°C
E-cadherin	WB	#4065, Cell Signaling Technology	mmab	1/1000	Overnight, 4°C
Vimentin	IHC	#45741, Cell Signaling Technology	rpab	1/100	Overnight, 4°C
Vimentin	WB	#45741, Cell Signaling Technology	rpab	1/2000	Overnight, 4°C
Keratin 18	IHC	#4548, Cell Signaling Technology	mmab	1/200	Overnight, 4°C
N-cadherin	IHC	#13116, Cell Signaling Technology	rpab	1/200	Overnight, 4°C
GAPDH	WB	#5174, Cell Signaling Technology	rpab	1/1000	Overnight, 4°C

Abbreviations: IHC, immunohistochemistry; rpab, rabbit polyclonal antibody; mmab, mouse monoclonal antibody; WB, Western blot.

were independently evaluated by two observers in an inverted microscope (Olympus Corporation, Tokyo, Japan). The immunohistochemical staining intensity of the objective proteins was carried out using a semiquantitative grading system (H-score). The H-score was calculated using the following equation: H-score $=\sum$ Pi (i + 1), where i represents the intensity scores with a value of 0, 1, 2, or 3 (no staining, weak, weak but detectable staining, moderate staining, intense staining, respectively) and Pi is the corresponding percentage of the cells, varying from 0 to 100% [27]. Scoring was determined at different times by two independent investigators blinded to the type of tissues.

Cell growth assay

A cell proliferation assay was analyzed by performing an MTS assay and a 5-ethynyl-20-deoxyuridine (EdU, RiboBio) incorporation assay as previously described [25]. For the MTS assay, a total of 2×10^3 shZEB1-050, shZEB1-051, and shCtrl Ishikawa cells were seeded in 96-well plates in a final volume of 200 μL. Then 20 μL per well of MTS (Promega, WI, USA) was directly added every 24 h. Following 2 h incubation at 37°C, the spectrometric absorbance at 490 nm was measured on a Microplate Reader (Bio-rad, Carlsbad, CA, USA). Each treatment was replicated three times on the plate.

For the EdU incorporation assay, the Ishikawa cells were cultured in triplicate in 96-well plates at a density of 1×10^3 cells for 24 h at 37°C, and then the cells were exposed to 25 μM EdU for 2 h at 37°C. Then the cells were fixed in 4% formaldehyde for 30 min, treated with 0.5% Triton X-100 for 20 min for permeabilization, and reacted with 100 μl of 1× Apollo® reaction cocktail for 30 min. Subsequently, the DNA contents of the cells were stained with Hoechst33342 for 30 min and visualized under a fluorescent microscope (Olympus Corporation, Tokyo, Japan). The EdU positive cells (red cells) were counted using Image-Pro Plus (IPP) 6.0 software (Media Cybernetics, Bethesda, MD, USA) according to the manufacturer's instructions. The ratio of EdU positive cells to total Hoechst33342 positive cells (blue cells) was used to express the EdU incorporation rate. All experiments were performed independently in triplicate.

Cell migration and invasion assays

Scratch wound healing assays and transwell assays were performed to evaluate the cell migration assay, and the invasion assay was measured using a matrigel invasion chamber. For the wound healing assay, the cells were cultured in a six-well plate and grown overnight to 100% confluence. A micro-pipette tip was used to scratch in the monolayer to create a wound, and then the cells were washed twice with PBS. After being stained with 0.05% crystal violet, a photograph was taken at 0 and 48 h incubation. For the transwell assay, cells (1×10^4 or 3×10^4) were harvested and seeded in the upper chambers of the transwell chambers with polycarbonate membranes (8 μm pore size, Corning) coated without/with Matrigel (BD Biosciences) for the migration or invasion assay. A RPMI1640 medium containing 10% FBS was added into the lower chamber. After 24 h, the migrated or invaded cells were fixed, stained, and counted by averaging ten fields with a Nikon Eclipse 50i fluorescent micro-

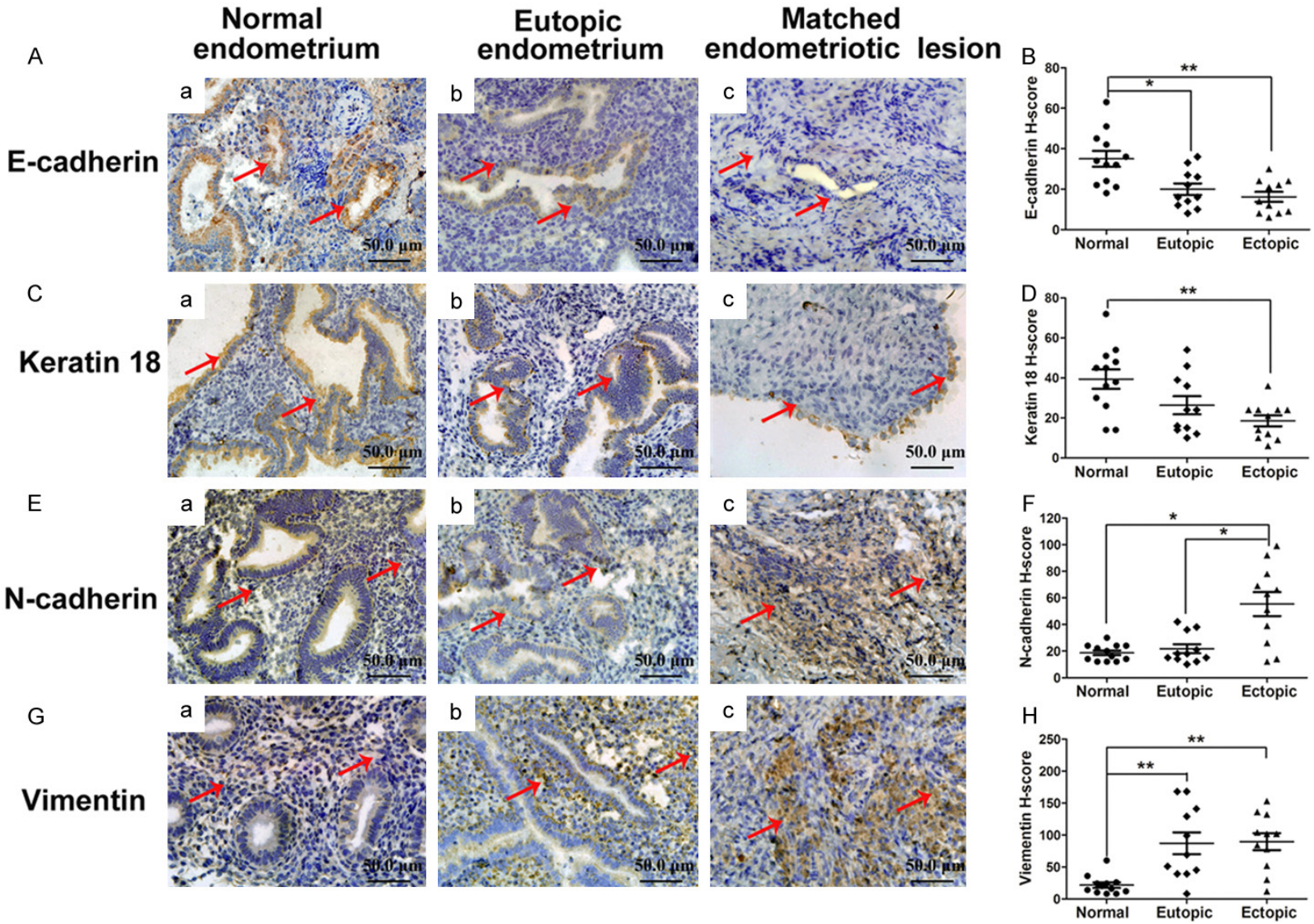

Figure 1. Immunohistochemical staining of EMT markers. Representative photomicrographs and histological score (H-score) of E-cadherin (A, B), Keratin 18 (C, D), Vimentin (E, F), and N-cadherin (G, H) in normal endometria from control patients (n = 12), and eutopic endometria and matched endometriotic lesions from endometriosis patients (n = 11). Red arrows indicated glandular epithelium or stromal cell immunostaining. Scale bars = 50 μm. Data were presented as means ± SEM, $*P < 0.05$, $**P < 0.01$.

scope (Olympus Corporation, Tokyo, Japan). The results were drawn from three independent experiments.

ZEB1 promoter plasmid constructs and luciferase assay

The human ZEB1 promoter sequence was analyzed u a bioinformatic website (http://asia.ensembl.org/index.html). Five potential promoter regions (P2160, -20 to -2180 bp; P1561, -20 to -1581 bp; P1316, -20 to -1336 bp; P1102, -301 to -1401 bp; P886, -1016 to -1901 bp) of ZEB1 were cloned into the pGL3-basic vector. The primers of the five potential promoter regions were as follows: P2160, forward 5'-CGGGGTACCGCATCTACTATTGGCTACG-3' and reverse 5'-CCGCTCGAGCGACATCACCTTCCTTAC-3'; P1561, forward 5'-CGGGGTACCGGTATCAAGGAAAGCGAGGAC-3' and reverse 5'-CCGCTCGAGAGGGGCGAGGGAAAAGT-3'; P1316, forward 5'-CGGGGTACCTATGAGGTGAGGATGAAC-3' and reverse 5'-CCGCTCGAGTTAGTAGAGCGGAATGAG-3'; P886, 5'-CGGGGTACCGTAGGCGACTGAAAAACA-3' and reverse 5'-CCGCTCGAGCAGGACCTTA AGGCAAGA-3'; P1102, forward 5'-CGGGGTACCGTAGGCGACTGAAAAACA-3' and reverse 5'-CCGCTCGAGCCTTAGTAGAGCGGAATG-3'. The Ishikawa or 293T cells were plated in 24-well plates at a density of 2×10^4 cells per well in phenol red-free DMEM media supplemented with 10% CSFBS (Invitrogen) for the luciferase assays. Cells were transfected with 1 μg of ZEB1 promoter reporter construct together with 0.5 μg pGL3-βGal plasmid as a normalization reference using lipofectamine 2000 for 24 h. The β-galactosidase enzyme activity and luciferase activity were performed with a β-galactosidase enzyme assay system and a luciferase reagent according to the manufacturer's instructions (Promega). Luciferase units were presented as luciferase activity/β-galactosidase activity. The fold change was calculated by comparison with the pGL3-Basic. Each luciferase assay was performed five times.

Statistics

All the experiments were repeated at least three times, and the data were presented as the mean ± SEM. Statistical analyses were performed using Student's unpaired t-test, a Mann-Whitney *U*-test, or one-way ANOVA. The analysis was performed with Graphpad Prism (San Diego, CA, USA). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

The EMT phenomenon exists in endometriosis tissue samples

To study the EMT maker protein expression in the endometriosis tissues, an IHC analyses of E-cadherin, Keratin 18, N-cadherin, and Vimentin in normal endometria and eutopic and matched endometriotic lesions were performed in samples from 12 normal control cases and 11 patients with endometriosis. As shown in **Figure 1**, positive E-cadherin and Keratin 18 expressions were present in the majority of normal endometrium glandular epitheliums (**Figure 1A**, **1C**). And the E-cadherin and Keratin 18 protein expressions in the endometrial epithelial cells from eutopic endometria and endometriotic lesions were markedly decreased (**Figure 1B**, **1D**, $P < 0.05$, $P < 0.01$), when compared with the normal endometrium cells. On the other hand, positive N-cadherin and Vimentin expressions were present in the majority of the ectopic endometrial stromal cells (**Figure 1E**, **1G**), and significantly increased proportions of positive N-cadherin (**Figure 1F**, $P < 0.05$) and Vimentin (**Figure 1H**, $P < 0.01$) were detected in the endometriotic lesions compared with the normal endometria. In conclusion, these results indicated that the EMT phenomenon exists in the progression of endometriosis.

ZEB1 expression was aberrantly increased in human endometriotic tissues

ZEB1 is a known target of E-cadherin and a key mediator of angiogenesis, proliferation, and extracellular invasion. Therefore, we next investigated the expression profile of ZEB1 in normal endometrial and endometriotic tissues. As shown in **Figure 2A**, the mRNA expression of ZEB1 was significantly up-regulated in both eutopic endometria ($P < 0.05$,) and matched endometriotic lesions ($P < 0.01$) compared with the normal endometria. Interestingly, among the 21 paired endometrial tissues, the levels of ZEB1 in 7 paired tissues were greater in eutopic endometria compared with ectopic endometriotic tissues (**Figure 2B**), but no difference was found between eutopic endometria and matched endometriotic lesions (n = 21, **Figure 2A**). We also analyzed the protein expression of

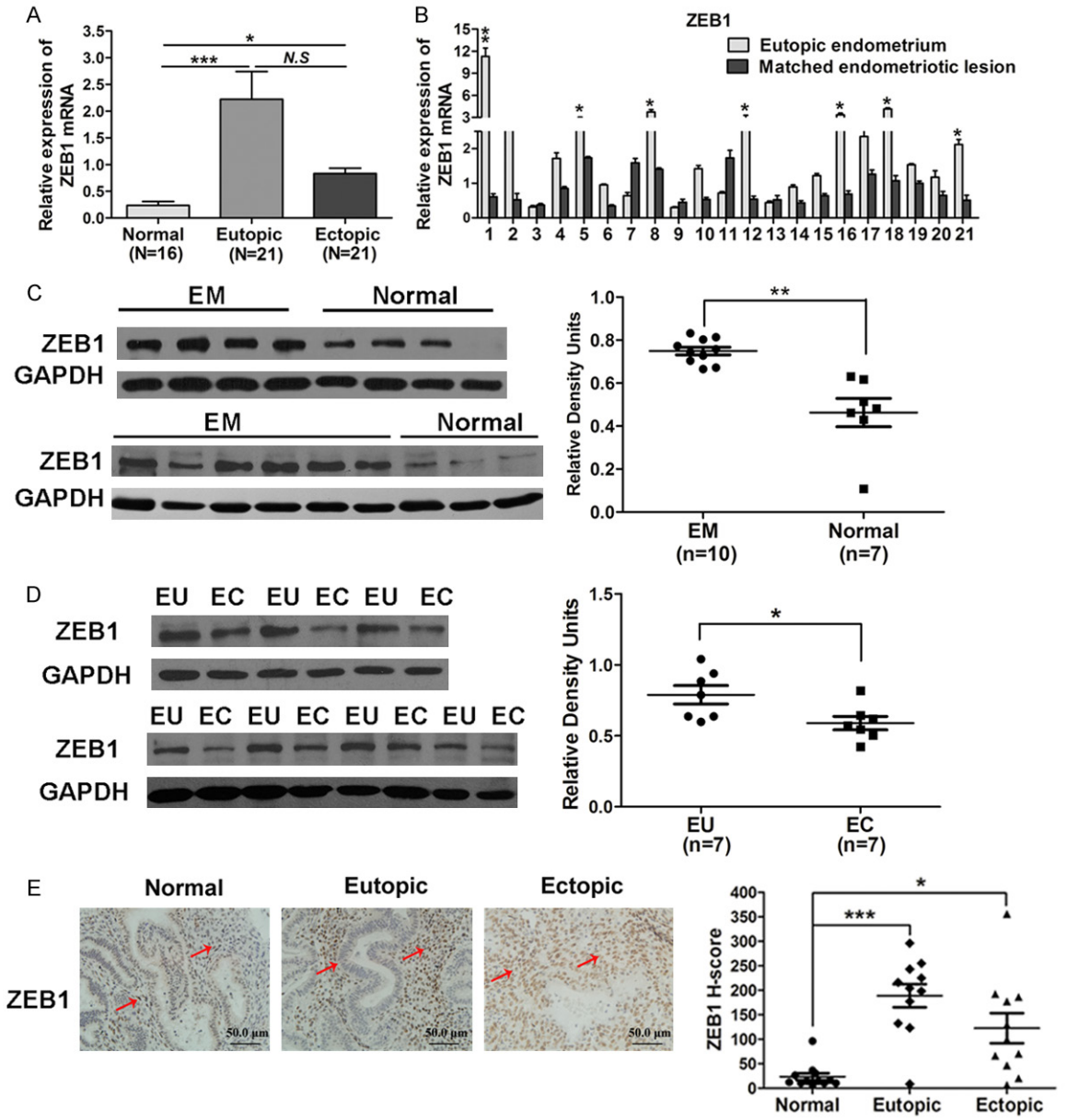

Figure 2. Both the mRNA and protein levels of ZEB1 were increased in human endometriotic tissues. A, B. Expression of ZEB1 was examined on mRNA level by qRT-PCR in normal endometrium from control patients (n = 16), and eutopic endometrium and matched endometriotic lesions from endometriosis patients (n=21). C. Western blot analysis of ZEB1 expression in normal endometrial (Normal, n = 7) and endometriotic tissues (EM, n = 10) (left panel). Ratios of ZEB1 to GAPDH were determined following densitometry measurements of the specific protein bands (right panel). D. Western blot analysis of ZEB1 protein expression in eutopic endometrium and matched endometriotic lesions from endometriosis patients (n = 7) (left panel). Ratios of ZEB1 to GAPDH were determined following densitometry measurements of the specific protein bands (right panel). E. Immunohistochemical staining and histological score (H-score) for ZEB1 in normal endometrium from control patients (n = 12), and eutopic endometrium and matched endometriotic lesions from endometriosis patients (n = 11). Scale bar = 50 μm. Data were presented as means ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

ZEB1 in endometria from patients with and without endometriosis by western blot. As shown in **Figure 2C**, the protein expression of ZEB1 was significantly greater in the EM group (n = 10) compared with the normal group (n = 7, $P < 0.05$). Meanwhile, the protein expression of ZEB1 was significantly increased in eutopic endometria compared with matched endome-

Table 2. Relationships between ZEB1 and the clinical features of endometriosis

Features	Cases (N)	ZEB1 mRNA expression in eutopic endometrium of endometriosis patients#	*P*
Age (years)			
<30	12	1.937	
≥30	9	2.435	0.859
Infertility			
Negative	4	2.234	
Positive	17	2.168	0.687
Dysmenorrhea			
Negative	12	1.772	
Positive	9	2.822	0.915
Menstrual phase			
Proliferative	6	0.683	
Secretory	15	2.884	0.003**
CA125 (U/mL)			
<37	9	1.011	
≥37	12	3.101	0.002**
CA199 (U/mL)			
<37	11	1.812	
≥37	10	2.672	0.546

#The data of ZEB1 mRNA expression in eutopic endometrium of endometriosis patients was from **Figure 2A**. ***P* value of less than 0.01 was considered statistically significant.

triotic lesions (n = 7, **Figure 2D**, $P < 0.05$). To examine the cell-specific expression of ZEB1, we next performed an immunohistochemical analysis in the normal endometria and in the eutopic and matched endometriotic lesions. ZEB1 expression was strongly stained in the stromal cell nuclei of endometria in both eutopic (**Figure 2E**, $P < 0.001$) and matched endometriotic lesions (**Figure 2E**, $P < 0.05$), but a negative staining was found in the normal endometria. Thus, these data clearly show that ZEB1 is markedly overexpressed in endometriosis patients.

We also performed a comprehensive analysis of the general clinical and pathologic examinations of the patients in the experiment. As shown in **Table 2**, ZEB1 mRNA expression in eutopic endometria of endometriosis patients was not associated with age, infertility, dysmenorrhea, or CA199 ($P > 0.05$). However, we found that ZEB1 mRNA expression was significantly correlated with menstrual phase and CA125 level ($P < 0.01$). The ZEB1 mRNA expression was increased in the secretory phase compared with the proliferative phase ($P < 0.01$). These results indicated that an abnormal activation of ZEB1 may play an important role in the pathogenesis of endometriosis.

Down-regulation of ZEB1 inhibited Ishikawa cell proliferation, migration and invasion

Based on our study of the clinical samples, we established a ZEB1 stable knockdown of Ishikawa cells by transfecting the with recombinant shZEB1 lentivirus or shCtrl lentivirus. As shown in Supplementary Figure 1A, 1B, the mRNA of ZEB1 was significantly lower in the shZEB1-050 and shZEB1-051 Ishikawa cells when compared with shCtrl Ishikawa cells. Therefore, we used the shZEB1-050 and shZEB1-051 Ishikawa cells as the objects of our study in the subsequent experiments.

We first investigated the role of ZEB1 in the Ishikawa cells = growth by MTS assay from 24-120 h. As shown in **Figure 3B**, the ZEB1 knockdown resulted in a remarkable suppression of cell proliferation at 96 h and 120 h ($P < 0.01$). Furthermore, the Edu incorporation assay showed that the percentage of Edu-positive cells was reduced in shZEB1-050 and shZEB1-051 Ishikawa cells compared with the shCtrl Ishikawa cells (**Figure 3A**, **3C**, $P < 0.01$). To further study the effect of ZEB1 knockdown on Ishikawa cells migratory and invasive ability, we performed wound healing and transwell assays. As shown in Supplementary Figure 3, the ZEB1 knockdown inhibited the migration of Ishikawa cells compared with the shCtrl group. Moreover, data from the transwell assays also demonstrated that the number of invaded cells is significantly lower in the shZEB1-050 and shZEB1-051 Ishikawa cells compared with the shCtrl Ishikawa cells. (**Figure 3D**, **3E**, $P < 0.05$, $P < 0.01$).

Effects of ZEB1 knockdown on E-cadherin and Vimentin expression

To further confirm the role of ZEB1 in the EMT phenomenon, we assessed the effect of ZEB1 knockdown on the expression of epithelial and mesenchymal markers (E-cadherin and Vim-

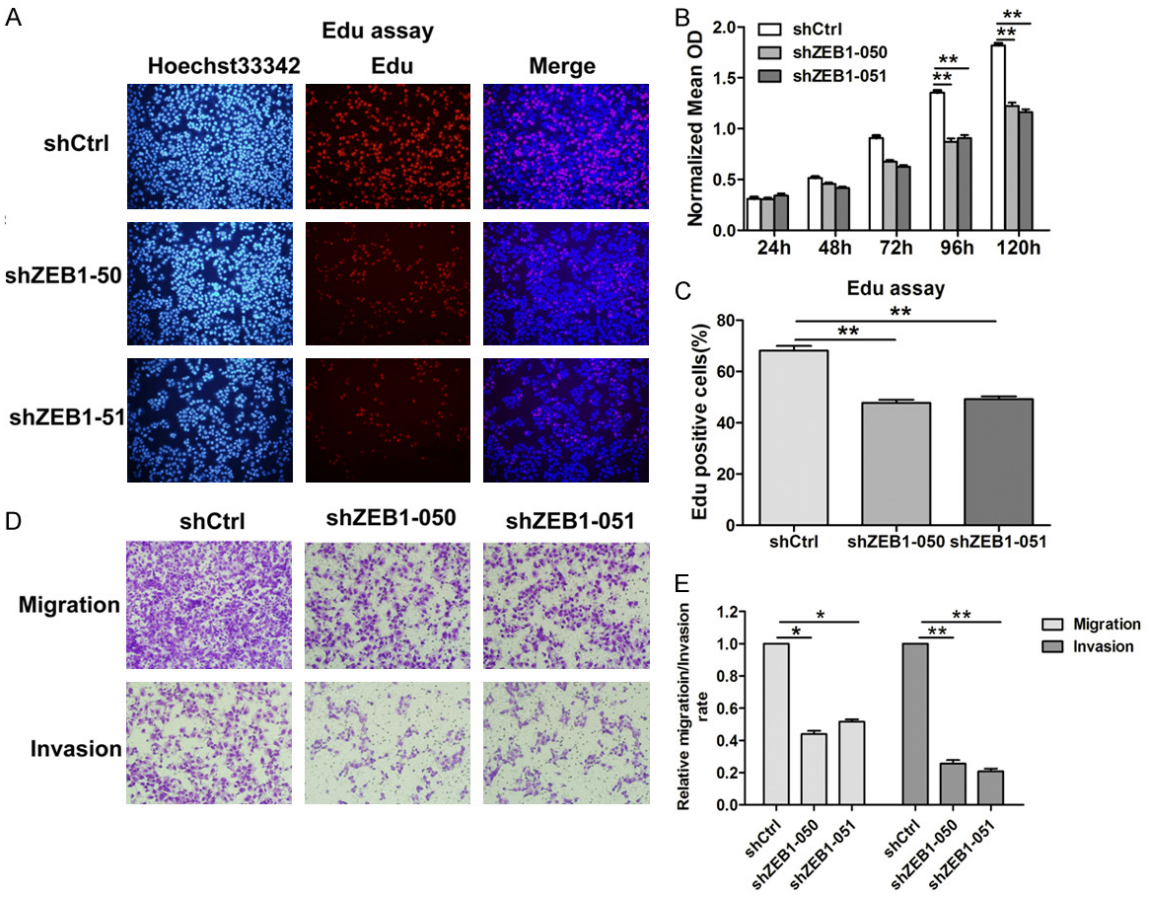

Figure 3. ZEB1 knockdown suppressed Ishikawa cells proliferation, migration and invasion. (A, C) The DNA replication analysis of shZEB1-050, shZEB1-051 and shCtrl Ishikawa cells by the EdU incorporation assay (A). Original magnifications: ×200. EdU-positive cell counts were analyzed by using a software program and a microscope (C). (B) The growth of Ishikawa cells stably transfected with shZEB1-050, shZEB1-051, or shCtrl were measured by MTS assay from 24-120 h. (D, E) A representative cell migration and invasion assay from transwell analysis (D), and the calculated number from triplicates (E). Results were from three independent experiments and shown as mean ± SEM, $*P < 0.05$, $**P < 0.01$.

entin) by western blot. As shown in **Figure 4A**, **4B**, ZEB1 knockdown induced E-cadherin protein (epithelial marker) expression, and inhibited Vimentin protein (mesenchymal markers) expression, which suggested that ZEB1 knockdown induces the reversal of the EMT phenomenon.

E2 enhanced the expression of ZEB1 by genomic ER activity

As endometriosis has been widely treated as an estrogen-dependent and inflammatory disease [5, 28], we next investigated whether EMT in endometrial epithelial cells is induced by E2 or IL-1β. Firstly, qRT-PCR was performed to assess the expression of E-cadherin, Vimentin and ZEB1 mRNA in Ishikawa cells after treatment with various concentrations of E2 (1 to 200 nM) or IL-1β (0.1 to 10 ng/mL) for 48 h. As shown in **Figure 5A**, E-cadherin mRNA expression was significantly decreased after E2 administration, along with an increase in Vimentin and ZEB1 mRNA expressions. Similarly, E2 reduced the protein expression of E-cadherin, but at the same time it increased the protein expression of Vimentin and ZEB1 in a dose-dependent manner (**Figure 5B**, **5C**). However, after stimulation with IL-1β for 48 h, the mRNA expression of E-cadherin, Vimentin, and ZEB1 in Ishikawa cells did not significantly alter (Data not shown). Dose-response experiments revealed maximal ZEB1 mRNA induction with 200 nM after 48 h of treatment in the Ishikawa cells about 2.4-fold over the control group (**Figure**

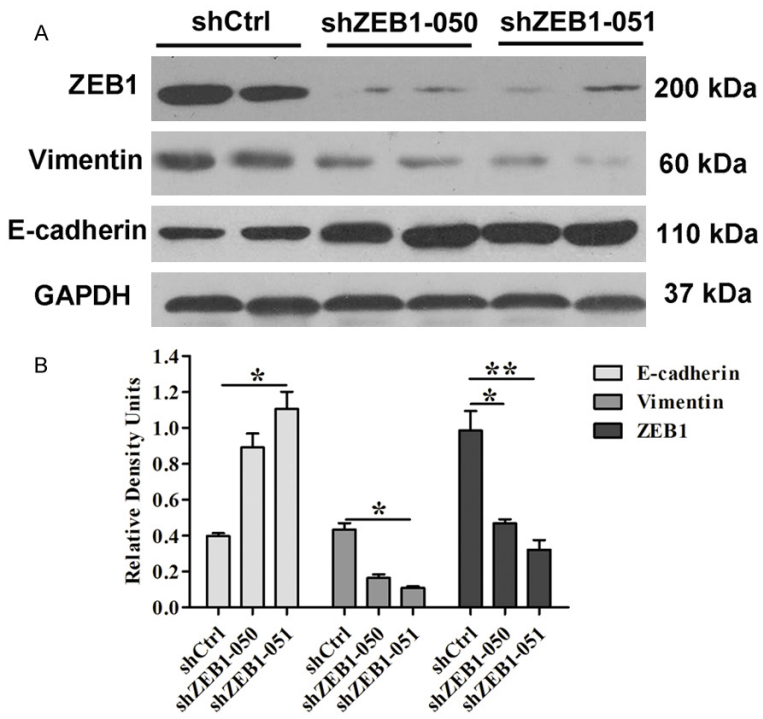

Figure 4. ZEB1 knockdown suppressed Ishikawa cells EMT phenomenon. A. Western blot analyzed effects of ZEB1 silencing on E-cadherin and Vimentin expression in shZEB1 Ishikawa cells and shCtrl Ishikawa cells. B. Ratios of E-cadherin, Vimentin and ZEB1 to GAPDH were determined following densitometry measurements of the specific protein bands. Results were from three independent experiments and shown as mean ± SEM, $*P < 0.05$.

5D). These results suggested that E2 indeed up-regulates ZEB1 expression. To determine whether the E2-induced increase in ZEB1 was mediated directly by the estrogen receptor (ER), the Ishikawa cells were pretreated with ICI 182780 for 6 h before the E2 treatment (**Figure 5E**). ICI 182780 blocked the E2-induced increase in ZEB1 mRNA, indicating that ER mediated this response. Then, to determine whether the E2-induced increase in ZEB1 was mediated by nongenomic ER activity, the Ishikawa cells were pretreated for 1 h with the p38 MAPK inhibitor (SB203580, 10 μM), ERK inhibitor (PD98059, 50 μM) or JNK inhibitor (SP600125, 10 μM) respectively before incubation with 200 nM E2 for 24 h. Among the three inhibitors, none could alter the E2-induced increase in ZEB1 (**Figure 5F**), indicating that the E2 response is mediated by genomic ER activity.

E2 activated ZEB1 promoter activity

To investigate the effect of E2 on the ZEB1 promoter, we used luciferase assays. Promoter gene assay procedures were performed as previously described [29]. Firstly, five potential promoter regions of ZEB1 were cloned into the promoterless luciferase pGL3 vector. Luciferase activity was performed to measure the transcriptional activity of the ZEB1 promoter reporter gene in the 293T and Ishikawa cells. As shown in **Figure 6A**, the four recombinant vectors included P2160, P1561, P1316, and P886 and had a greater transcriptional activity when compared with the empty pGL3 vector. We further found that ZEB1 had two mainly transcriptional activity areas, which included -20 bp - -301 bp (Area 1), and -1401 bp - -1901 bp (Area 2). Furthermore, to investigate the effect of E2 on the ZEB1 promoter, 293T or Ishikawa cells were transfected with ZEB1 reporter gene recombinant vectors (P2160, P1316, P886) and treated with E2 (200 nM) for periods up to 24 h. Interestingly, both the P2160 and P886 reporter gene recombinant vectors responded to E2 (200 nM) treatment except the P1316 reporter gene recombinant vectors (**Figure 6B**, **6C**). Concurrent with the increase of ZEB1 mRNA expression, treatment with E2 for 24 h strongly activated ZEB1 promoter activity both in the 293T and Ishikawa cells ($P < 0.01$). These results suggest that in the area of -1401 bp- -1901 bp there may exist an E2 responsive element (ERE).

Discussion

Although the pathogenesis of endometriosis remains largely unclear, growing evidence suggests that the dysregulation of the EMT phenomenon may play an important role in the pathological process of endometriosis [30-32]. In the present study, we focused on ZEB1, the expression of which correlated with the mesenchymal phenotype in endometriosis. Meanwhile, ZEB1 knockdown suppressed the growth, migration and invasion of Ishikawa cells. We also demonstrated that E2 induced the expres-

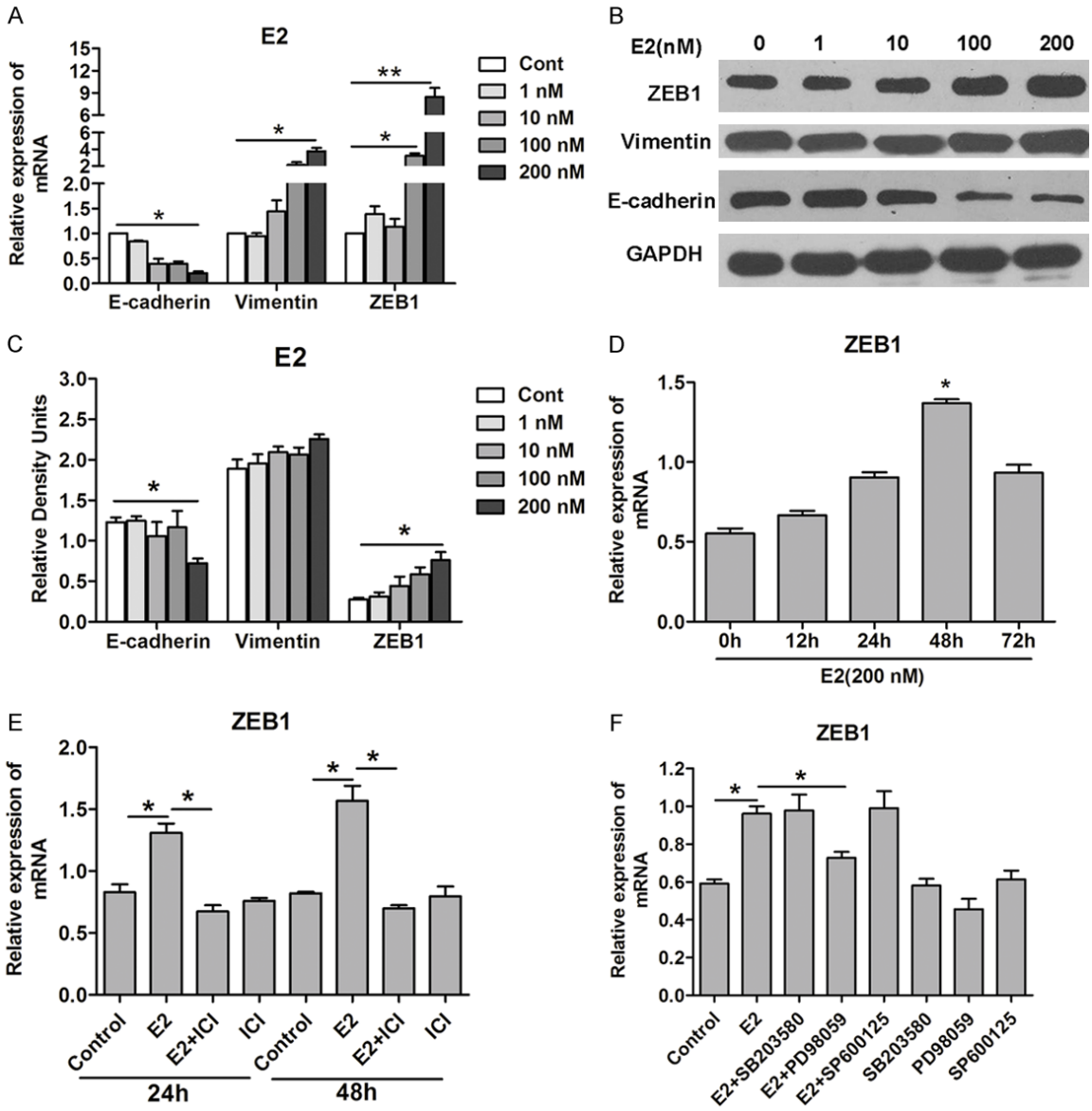

Figure 5. E2 induced the expression of ZEB1 by an ER genomic manner in Ishikawa cells. A, B. Ishikawa cells were treated with vehicle (Cont) or different concentrations of E2 (1, 10, 100, 200 nM) for 48 h. Then, whole-cell lysates were extracted, and ZEB1, E-cadherin, Vimentin were detected by qRT-PCR and western blot. C. Ratios of E-cadherin, Vimentin and ZEB1 to GAPDH were determined following densitometry measurements of the specific protein bands. D-F. ZEB1 expression was investigated by qRT-PCR after different treatments. D. Ishikawa cells were treated with vehicle (Cont) or 200 nM E2 for the time indicated. E. Ishikawa cells were pretreated with 1 μM ICI 182780 for 6 h before the addition of 200 nM E2 for 48 h. F. Ishikawa cells were pretreated with 10 μM SB203580, 50 μM PD98059 or 10 μM SP600125 for 1 h before incubation with 200 nM E2 for 24 h. The data were shown as mean ± SEM of three repeated experiments. $*P < 0.05$, $**P < 0.01$.

sion and promoter activity of ZEB1, which contributed to the EMT of endometriosis. To the best of our knowledge, this is the first study to show the involvement of E2-induced ZEB1 in the development of endometriosis. These results may provide a rationale for developing therapeutics targeting ZEB1 function in endometriosis.

Aberrant activation of the EMT phenomenon has been identified as having an important role in tumor cell invasion and metastasis [9, 33, 34]. During EMT, the expression of epithelial markers (e.g., E-cadherin, keratin 18) in epithelial cells is reduced, while the expression of mesenchymal markers is increased (e.g., vimentin, N-cadherin). At the same time, EMT

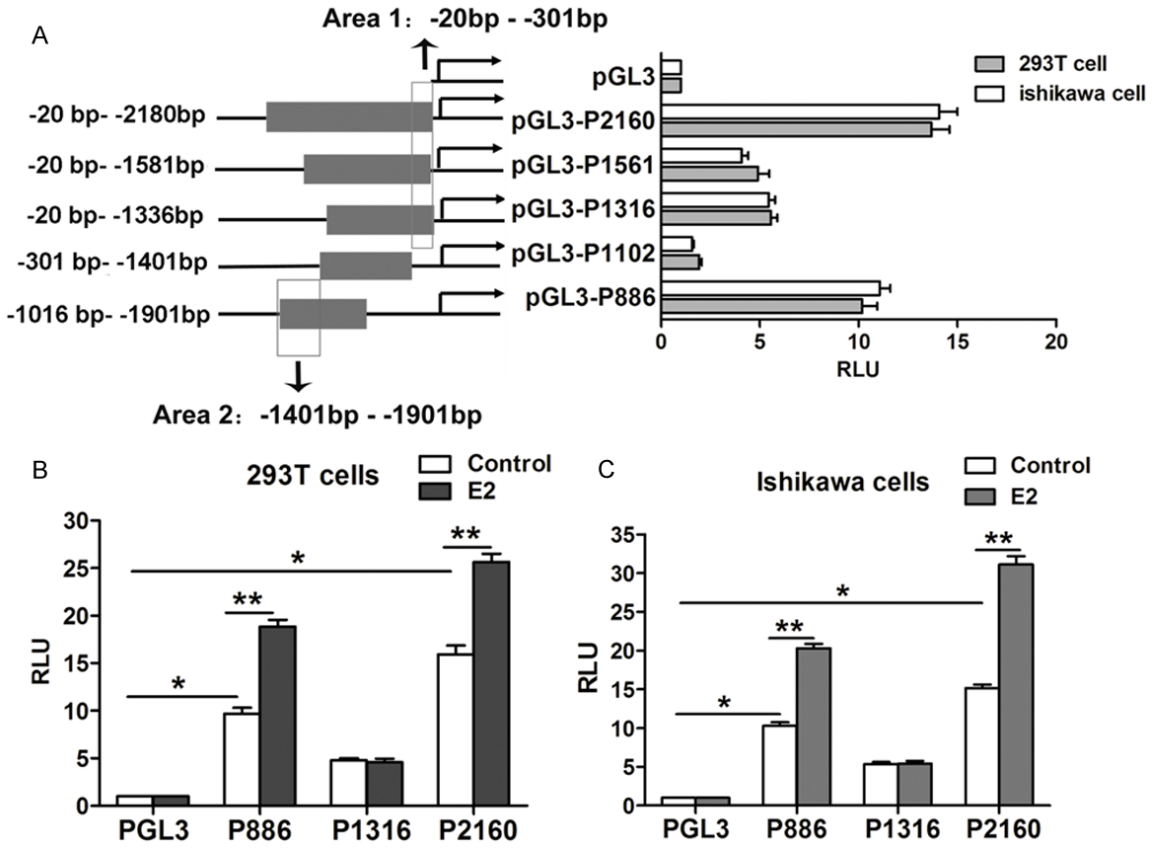

Figure 6. E2 stimulated ZEB1 promoter luciferase reporter activity in 293T and Ishikawa cells. A. Schematic drawing of the different positions of the promoter of human ZEB1 (left panel) and data of promoter activity from three independent experiments using different batches of cells (right panel). B, C. 293T or Ishikawa cells were transfected with either human ZEB1 promoter-reporter gene construct (P886, P1316, P2160) and pSV-βgal for normalization. The luciferase activities were detected in cell lysates 24 h after initiation of E2 (200 nM) exposure. The luciferase activities were calculated relative to the pGL3-Basic vector (promoterless vector) and expressed as fold change relative to vehicle control. The data were shown as the mean ± SEM of three repeated experiments. $*P < 0.05$, $**P < 0.01$.

endows cells with migratory and invasive properties. Consistent with these reports [18, 31], we found that the expression of vimentin and N-cadherin were higher in endometriotic lesions compared with normal endometrium tissues, which indicated that the EMT phenomenon was an aberrant activation in endometriosis. In EMT progression, transcriptional factors including Slug and ZEB1 have been reported to play a crucial role. In our study, the mRNA and protein expressions of ZEB1 were an aberrant activation, but Slug did not show a difference between the normal endometrium and endometriotic lesions (Supplementary Figure 2). The results implied that the EMT transcriptional factors may have a tissue-specific expression in the development of endometriosis.

Previous data indicated that ZEB1 plays a key role in cancer progression by suppressing E-cadherin or other epithelial markers. The aberrant expression of ZEB1 in cervical cancer, lung cancer, and colorectal cancer has been associated with aggressive disease and poor clinical prognosis [23, 35, 36]. However, the ZEB1 expression in endometriosis tissues and its correlation with the clinical pathology of endometriosis have not been reported. In our study, we confirmed that ZEB1 mRNA expression was higher in endometriosis tissues than in the normal endometrium tissues, consistent with the results of the protein levels. Interestingly, ZEB1 protein expression was upregulated in eutopic endometria compared with the matched endometriotic lesions, but the mRNA levels had no statistical significance. The results seem to be contradictory to the EMT maker expression data in endometriotic lesions. One possible explanation is that the number of cases was limited in our study.

Another possible explanation is that the high expression of ZEB1 in the eutopic endometria of endometriosis patients induces the EMT phenomenon and then further endows cells with stronger migration and invasion to grow outside the uterus.

It has been shown that endometriotic cells need to undergo an attachment-aggression-angiogenesis (3A) procedure to develop into endometriosis [2, 37]. The ZEB1 knockdown experiment further supported the hypothesis. We found that ZEB1 knockdown dramatically suppressed Ishikawa cell proliferation, migration and invasion. Meanwhile, we demonstrated that ZEB1 knockdown increased E-cadherin protein expression and decreased vimentin protein expression. The results were consistent with our previous findings on caski cells [23]. Although previous studies have reported that ZEB1 mostly directly inhibits the transcription of E-cadherin in several types of cancer cells [38, 39], they could not deny the possibility that ZEB1 up-regulates Vimentin expression. The results suggest that ZEB1 may indirectly induce vimentin expression through its other downstream genes. Collectively, our data, along with those of other teams, imply a key role of ZEB1 in the development of endometriosis.

Although it is reported that estrogen induces EMT in the development of endometriosis [18], the mechanism remains unclear. Previous studies have demonstrated that estrogen enhances ovarian cancer or the non-small-cell lung cancer cell EMT phenomenon by regulating Snail, Slug or midkine expression [22, 40], suggesting that the mechanism of estrogen-induced EMT may involve a cell-specific response. In the present study, our results showed that E2-induced ZEB1 expression was suppressed mainly by the ER antagonist ICI 182780 in Ishikawa cells. We further found that E2 induced ZEB1 expression through activating ZEB1 promoter activity in 293T and Ishikawa cells. The ERs execute gene transcription mainly by binding the estrogen response element (ERE) or non-ERE elements including cAMP-response elements [41], activator protein 1 sites [42], and Sp1 sites [43]. By a sequence homology search, we found one potential ERE elements site within the ZEB1 promoter region (Area 2: -1401 bp - -1901 bp), which is a possible explanation why E2 activates ZEB1 promoter activity. To determine whether E2 induces ER recruitment to this ERE in Ishikawa cells, further study involving chromatin immunoprecipitation assays is needed.

In conclusion, the present work not only demonstrated the EMT phenomenon and ZEB1 are abnormal activations in endometriosis, and also found that E2-induced ZEB1 expression contributes to the EMT of endometriosis. Our findings suggested that the down-regulation of ZEB1 may serve as a potential therapeutic target to inhibit the development of endometriosis.

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Disclosure of conflict of interest

None.

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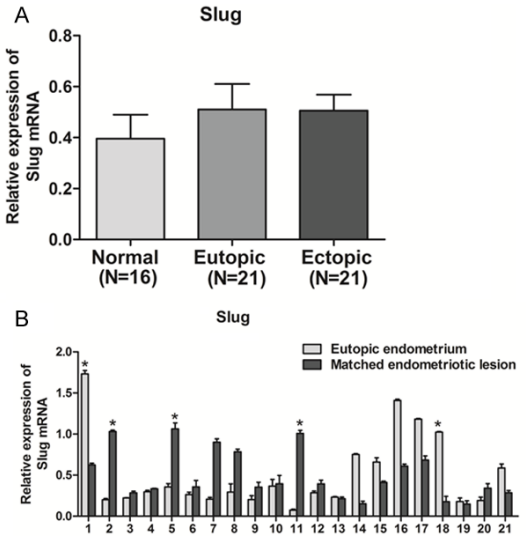
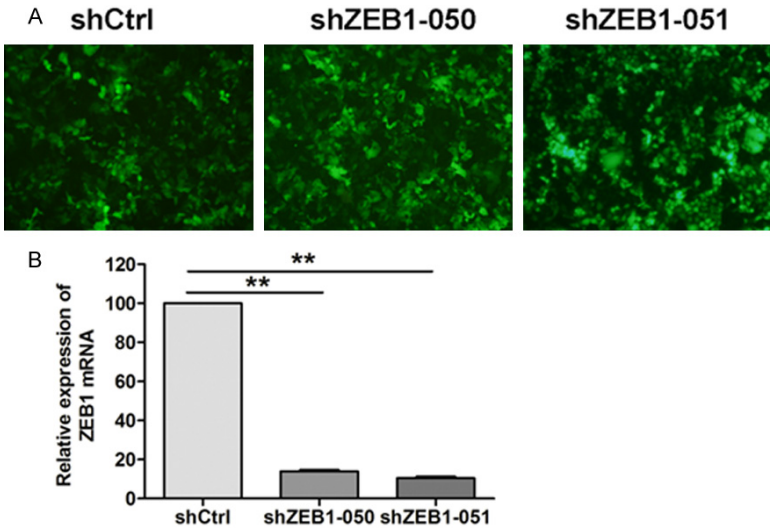
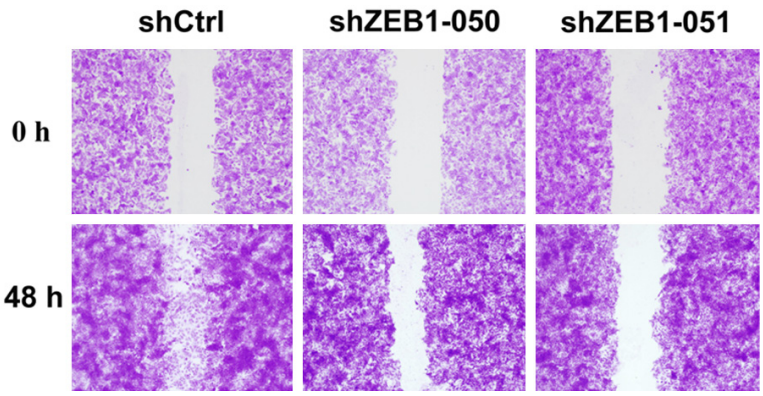


Supplementary Figure 1. Expression of ZEB1 mRNA in shZEB1 and shCtrl Ishikawa cells. A. A stable ZEB1 knockdown Ishikawa cells were screened after incubating with 200 μg/mL of G418 for 10 days. The green fluorescence protein was detected in the Nikon Eclipse 50i fluorescent microscope (Tokyo, Japan). B. Relative mRNA expression of ZEB1 in shZEB1-050, shZEB1-051 and shCtrl Ishikawa cells were detected by real-time RT-PCR and normalized to GAPDH expression. Data were presented as means ± SEM, $*P < 0.05$.



Supplementary Figure 2. The mRNA expression of Slug did not show any difference between normal endometria and endometriotic lesions. The expression of slug was examined on the mRNA level by qRT-PCR in normal endometria from the control patients (n = 16), and eutopic endometria and matched endometriotic lesions from the endometriosis patients (n = 21). Data were presented as the mean ± SEM, $**P < 0.01$.



Supplementary Figure 3. ZEB1 knockdown suppressed Ishikawa cell migration. The migrations of shZEB1-050, shZEB1-051 and shCtrl Ishikawa cells were detected by wound healing assays at 0 h and 48 h. (One representative experiment is shown, scale bar = 50 μm).