Human Reproduction, Vol.35, No.6, pp. 1377–1390, 2020
Advance Access Publication on May 29, 2020 doi:10.1093/humrep/deaa062

human reproduction

ORIGINAL ARTICLE ***Reproductive biology***

TIPE2 inhibits the migration and invasion of endometrial cells by targeting β-catenin to reverse epithelial–mesenchymal transition

Yuqiu Liu[1,2,3,†], Xiaoyan Wang[1,†], Lu Wan[1], Xihong Liu[4], Huayun Yu[2], Derui Zhang[2], Yingshuo Sun[2], Yongyu Shi[1], Lining Zhang[1], Huaiyu Zhou[5], Jianing Wang[1], and Zengtao Wei[1,6,*]

[1]Department of Immunology, School of Basic Medical Sciences, Shandong University, Jinan, Shandong, P. R. China [2]Department of Gynecology and Obstetrics, Jinan Central Hospital affiliated to Shandong University, Jinan, Shandong, P. R. China [3]Department of Gynecology and Obstetrics, Weifang Medical College, Weifang, Shandong, P. R. China [4]Department of Pathology, The Fourth People's Hospital of Jinan, Jinan, Shandong, P. R. China [5]Department of Microbiology, School of Basic Medical Sciences, Shandong University, Jinan, Shandong, P. R. China [6]Department of Gynecology and Obstetrics, Clinical Medical School, Shandong University, Jinan, Shandong, P. R. China

*Correspondence address. Department of Immunology, School of Basic Medical Sciences, Shandong University, 44# Wenhua Xi Road, Jinan, Shandong, P. R. China. Department of Gynecology and Obstetrics, Clinical Medical School, Shandong University. 44# Wenhua Xi Road, Jinan, Shandong, 250012 P. R. China. Tel: +86-531-85695510 or +86-15318816286; E-mail: weizengtao1970@163.com or weizengtao@sdu.edu.cn

Submitted on September 14, 2019; resubmitted on February 26, 2020; editorial decision on March 9, 2020

STUDY QUESTION: Do changes in tumor necrosis factor-α-induced protein 8 (TNFAIP8)-like 2 (TIPE2) levels in endometrium of patients with adenomyosis alter the proliferation, migration and invasion ability of endometrial cells?

SUMMARY ANSWER: TIPE2 expression levels were low in eutopic and ectopic endometrium of adenomyosis patients, and TIPE2 inhibited the migration and invasion of endometrial cells, mainly by targeting β-catenin, to reverse the epithelial-mesenchymal transition (EMT).

WHAT IS KNOWN ALREADY: Adenomyosis is a benign disease, but it has some pathophysiological characteristics similar to the malignant tumor. TIPE2 is a novel negative immune regulatory molecule, and it also participates in the development of malignant tumors.

STUDY DESIGN, SIZE, DURATION: Control endometrium ($n = 48$ women with non-endometrial diseases) and eutopic/ectopic endometrium from patients with adenomyosis ($n = 50$), human endometrial cancer cell lines, and primary endometrial cells from the eutopic endometrium of adenomyosis patients were used in the study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The expression level of TIPE2 mRNA and protein in the eutopic/ectopic endometrial tissues of adenomyosis patients and control endometrium was determined by quantitative RT-PCR (qRT-PCR), western blot and immunohistochemistry. The effects of TIPE2 overexpression and knockdown on the proliferation, migration and invasion of endometrial cell lines and primary adenomyotic endometrial cells were determined using a cell counting kit-8, 5-ethynyl-2′-deoxyuridine assay, colony-forming assay, transwell migration assay and matrigel invasion assay. The expression of EMT-related markers and signal molecules was detected by western blot. The interaction between TIPE2 and β-catenin was detected by co-immunoprecipitation and laser confocal microscopy.

MAIN RESULTS AND THE ROLE OF CHANCE: The mRNA and protein expression levels of TIPE2 in the eutopic and ectopic endometrial tissues of adenomyosis patients were significantly downregulated compared with the control endometrium ($P < 0.01$). TIPE2 could bind to β-catenin and inhibit the nuclear translocation of β-catenin, downregulate the expression of stromal cell markers, upregulate the expression of glandular epithelial cell markers, decrease the occurrence of epithelial-mesenchymal transition (EMT) and suppress the migration and invasion of endometrial cells ($P < 0.01$).

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: In this study, the experiments were performed only in eutopic and ectopic endometrial tissues, endometrial cancer cell lines and primary adenomyotic endometrial cells. A mouse model of adenomyosis will be constructed to detect the effects of TIPE2 *in vivo*.

[‡]Contributed equally

WIDER IMPLICATIONS OF THE FINDINGS: These results suggest that TIPE2 is involved in the development of adenomyosis, which provides a potential new diagnostic and therapeutic strategy for the treatment of adenomyosis.

STUDY FUNDINGS/COMPETING INTEREST(S): This present study was supported by grants from the National Natural Science Foundation of China (81471437, 81771554), Natural Science Foundation of Shandong (ZR2018MH013), Science and technology development plan provided by Health and Family Planning Committee in Shandong (2014-25). The authors declare that they have no conflicts of interest.

Key words: TIPE2 / adenomyosis / migration / invasion / epithelial–mesenchymal transition / β-catenin

Introduction

Adenomyosis is an estrogen-dependent disease that occurs mostly in women of reproductive age. About 20% of reproductive age women have adenomyosis, and about 15% of adenomyosis patients simultaneously have endometriosis and about half have uterine fibroids (Canis *et al*., 2018). Adenomyosis is defined as the endometrial glands and stroma invading the myometrium, accompanied by hypertrophy and hyperplasia of the surrounding myometrial tissues (Bird *et al*., 1972; Benagiano and Brosens, 2006). The main symptoms include excessive menstruation, prolonged menstruation and progressive dysmenorrhea (Levgur *et al*., 2000). There are no curative drugs at present, and surgery is the primary treatment strategy (Tan *et al*., 2019). More importantly, adenomyosis can adversely affect a patient's pregnancy outcome. According to statistical analysis, among the women using ART, the pregnancy rate of adenomyosis patients is 28% lower than that of women without adenomyosis, and the miscarriage rate is also increased (Vercellini *et al*., 2014). At present, the pathogenesis of adenomyosis remains unclear. Adenomyosis is a benign disease, but it has some pathophysiological characteristics similar to the malignant tumor, such as abnormal proliferation, migration and invasion (Liu *et al*., 2018a). Therefore, the increase in proliferation, migration and invasion ability of the endometrium may be the critical factor in the occurrence of adenomyosis, but the exact mechanisms need further exploration.

Tumor necrosis factor-α-induced protein 8 (TNFAIP8)-like 2 (TIPE2) is a novel negative immune regulatory molecule that was first discovered in 2002 (Carmody *et al*., 2002; Zhang *et al*., 2009). It is a member of the TNFAIP8 family, and TNFAIP8 family members include TIPE, TIPE1, TIPE2 and TIPE3, which have high sequence homology (Carmody *et al*., 2002; Sun *et al*., 2008). TIPE2 is preferentially expressed in lymphoid and inflamed tissues and participates in negatively regulating innate and adaptive immune responses (Sun *et al*., 2008). TIPE2-deficient mice spontaneously develop fatal inflammatory diseases (Cao *et al*., 2013). Human TIPE2 expression levels are also associated with a variety of chronic inflammations. For example, TIPE2 expression in peripheral blood mononuclear cells of patients with systemic lupus erythematosus or children with asthma is significantly lower than those in healthy controls (Li *et al*., 2016a; Li *et al*., 2009). Currently, several research reports show that TIPE2 not only is a novel negative regulator of immune cell function but also affects many tumor development processes. Liu *et al*., reported that TIPE2 was downregulated in glioma cells, and TIPE2 overexpression inhibited hypoxia-induced Wnt/β-catenin pathway activation and epithelial-mesenchymal transition (EMT) in glioma cells (Liu *et al*., 2016). Another study showed that TIPE2 inhibited the metastasis of hepatocellular carcinoma cells (HCC) by inhibiting the activity of Rac1 and reducing the polymerization of F-actin, matrix metalloproteinase 9 (MMP9) and uridylyl phosphate adenosine (uPA), thereby inhibiting the growth, migration and invasion of tumor cells *in vitro* (Cao *et al*., 2013). Similarly, Li *et al*., reported that TIPE2 suppressed tumor invasiveness and angiogenesis via inhibiting the activation of Rac1 and subsequently weakening its downstream effects, including F-actin polymerization and vascular endothelial growth factor (VEGF) expression (Li *et al*., 2016b). These results indicate that TIPE2 plays an important inhibitory role in different malignant tumors.

Based on the similar characteristics of adenomyosis with malignant tumors, in the present study we analyzed the expression of TIPE2 in the eutopic and ectopic endometrium of adenomyosis patients and then investigated the effect and mechanism of TIPE2 overexpression and knockdown on the proliferation, migration and invasion ability of endometrial cells. The results showed that TIPE2 was decreased in the eutopic and ectopic endometrium of adenomyosis patients, and TIPE2 effectively inhibited the migration and invasion of endometrial cells mainly by targeting β-catenin to reverse EMT, which may provide a new strategy for the diagnosis and treatment of adenomyosis.

Materials and Methods

Patients and tissue samples

The eutopic and ectopic endometrial tissues of 50 patients with adenomyosis were from the Department of Obstetrics and Gynecology, Jinan Central Hospital Affiliated to Shandong University from 2013 to 2014. All women were confirmed to have adenomyosis by B-ultrasound or biopsy. The endometrium of the control group was from 48 women with non-endometrial diseases who were confirmed by pathological examination and had no adenomyosis. These patients did not receive any hormone or antibiotic treatment during the last 6 months before surgery. All samples were obtained during the proliferative or secretory phase based on the menstrual cycle and histological evaluation. There were no statistically significant differences in age between adenomyosis patients and controls. All participants provided written informed consent, which was approved by the Ethics Committee in the School of Basic Medical Sciences, Shandong University.

Antibodies and reagents

Rabbit-derived antibody specific for TIPE2 (ab110389) was purchased from Abcam (Cambridge, MA, USA). Rabbit-derived antibodies specific for phospho-AKT (Ser473), AKT, phospho-P65, P65, E-cadherin, N-cadherin, Vimentin, zonula occludens-1 (ZO-1), Claudin-1, Snail, Twist and β-catenin were purchased from Cell Signaling Technology (CST) (Boston, MA, USA). Rabbit-derived antibody specific for Cytokeratin 7 (CK7) was purchased from Bio-Techne (Minneapolis, MN, USA). Mouse-derived monoclonal antibody specific for Flag was purchased from Sigma-Aldrich (St Louis, MO, USA). Mouse-derived

monoclonal antibody specific for β-actin (ZSGB-BIO, Beijing, China) was used as an internal control. 17β-Estradiol (E_2) powder was purchased from Sigma-Aldrich and then diluted to 10 mM using sterile absolute ethanol and stored at −20°C.

Cell culture

The human endometrial cancer cell line (Ishikawa) was a gift from Qilu Hospital Laboratory of Shandong University and cultured in DMEM high glucose medium (HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and Penicillin/Streptomycin (HyClone, Logan, UT, USA). The human endometrial cancer cell line HEC-1-A was purchased from the China Center for Culture Collection (Wuhan, China) and cultured in McCoy's 5A medium (Gibco, Carlsbad, CA, USA) with 10% FBS and Penicillin/Streptomycin. All the cell lines were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Expression plasmid and lentivirus interference vector

The plasmid containing full-length TIPE2 cDNA was constructed by Shanghai GeneChem Co., Ltd (Shanghai, China). Three different lentiviral short hairpin RNAs (shRNAs) targeting TIPE2 and a non-specific negative control were synthesized by Hanbio Biotechnology Co., Ltd (Shanghai, China). The sequences are listed in Supplementary Table SI. Transfection was carried out using LipoFiter^{TM} liposome transfection reagent according to the manufacturer's protocol (Hanbio Biotechnology Co. Ltd, Shanghai, China). The multiplicity of infection (MOI) is 5, and transfection efficiency was confirmed by quantitative RT-PCR (qRT-PCR) and western blot.

RNA extraction and qRT-PCR

Total RNA was extracted using RNAfast 200 (Fastagen, Shanghai, China) according to the manufacturer's protocol, and then 1 μg of RNA was reverse-transcribed into cDNA (complementary DNA) using a Reverse Transcription System (Takara, Shiga, Japan). The expression of TIPE2 mRNA was assessed by qRT-PCR with an Applied Biosystems STEP One Plus real-time PCR System (Applied Biosystems, Foster City, CA, USA) using a 2 × Ultra SYBR Mixture and specific primers (CWBIO, Beijing, China). The sequences of primers are listed in Supplementary Table SII. The qRT-PCR reaction was performed according to the following conditions: predenaturation at 95°C for 10 min, followed by 39 cycles of amplification at 95°C for 15 s, 60°C for 1 min, 65°C for 5 s. Each sample was run in triplicate. The relative expression of TIPE2 mRNA was analyzed according to the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Western blot

The cells were washed with PBS (1×) and lysed with Radio Immunoprecipitation Assay (RIPA) Lysis Buffer (Beyotime, Beijing, China). After homogenization, the cells were centrifuged at 12 000*g* at 4°C for 30 min. The supernatant was quantified by bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, MA, USA) according to the protocol. Protein samples (40 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) for 1–2 h, the membranes were incubated with the corresponding primary antibody (1:1000) overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Jackson Immuno Research, West Grove, PA, USA) for 1 h. The membranes were finally analyzed using the enhanced chemiluminescence kit (Thermo Scientific, Waltham, MA, USA). β-actin was used as an internal reference. Each experiment was repeated three times.

Immunohistochemistry

The endometrial specimens collected from adenomyosis patients and controls were fixed in 4% paraformaldehyde and then sectioned after paraffin embedding. Paraffin sections were first placed in an oven at 75°C for 2 h, and followed by xylene dewaxing and gradient alcohol hydration. The slides underwent antigen microwave retrieval and blocking of endogenous peroxidase activity and then were blocked with 10% goat serum at 37°C for 10–15 min and incubated with the primary antibody (1:200) against TIPE2 overnight at 4°C. The next day, the slides were incubated with HRP-conjugated goat anti-rabbit IgG (Gene Tech., Shanghai, China) for 30 min at 37°C and visualized using the diaminobenzidine (DAB) substrate kit (Gene Tech., Shanghai, China) for 3 min. The nuclei were counterstained with hematoxylin.

Based on the staining intensity and positive staining area, the expression levels of TIPE2 were evaluated. The staining intensity was divided as follows: − (score 0), + (score 1), ++ (score 2) and +++ (score 3). The positive staining area, given as a percentage of the whole section, was classified as follows: − (<1%, score 0) + (1–33%, score 1), ++ (34%–66%, score 2), +++ (67–100%, score 3). The sum of staining intensity and positive staining area scores was used as the final score of TIPE2 expression (no expression, total score 0; weak expression, total score 1 and 2; moderate expression, total score 3 and 4; strong expression, total score 5 and 6). All of the IHC slides were evaluated by two pathologists who were blinded to the clinical data of patients.

Cell proliferation assay

After Ishikawa or HEC-1-A cells were transfected with the plasmid of TIPE2 or the lentiviral shRNAs targeting TIPE2 for 24 h, the cells were reseeded in 96-well plates at 3000 cells/well and cultured for 0, 24, 48 and 72 h. The cell viability was assessed using a cell counting kit-8 (CCK-8) Kit (Dojindo, Tokyo, Japan) according to the protocol. The absorbance for each well at a wavelength of 450 nm was determined using a Bio-Rad Microplate Reader.

The 5-ethynyl-2′-deoxyuridine (EDU) assay was used to detect the effect of TIPE2 overexpression on the proliferation of Ishikawa cells. After Ishikawa cells were transfected with the TIPE2 plasmid for 24 h, the cells were reseeded in 96-well plates at 3000 cells/well and cultured for 24 h. The proliferation of cells was detected using a Cell Light Edu Apollo 567 *In Vitro* Kit (RiboBio Co., Ltd, Guangzhou, China), according to the protocol.

Colony-formation assay

TIPE2-overexpressing/silenced cells were seeded in 6-well plates at a density of 1000 cells per well and cultured at 37°C for 2 weeks. Then, the cells were fixed in methanol for 10 min and stained with 1% crystal violet for 20 min. Colonies consisting of more than 50 cells were

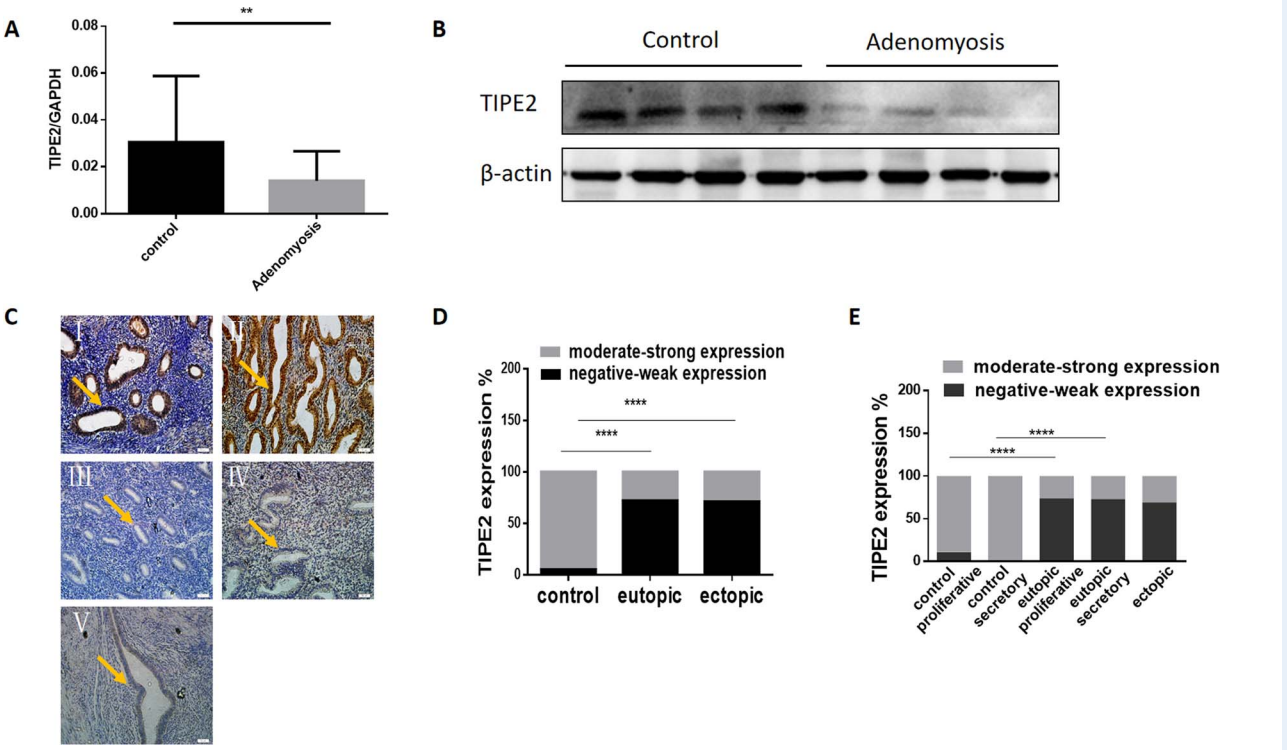

Figure 1 TIPE2 expression is downregulated in the eutopic and ectopic endometrium of adenomyosis patients compared with the control endometrium. (**A**) Tumor necrosis factor-α-induced protein 8 (TNFAIP8)-like 2 (TIPE2) mRNA levels in the eutopic endometrium of adenomyosis patients were lower than those in the control endometrium, as detected by quantitative RT-PCR (qRT-PCR) ($P < 0.01$). Data were normalized to GAPDH. Results are mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. (**B**) TIPE2 protein levels in the eutopic endometrium of adenomyosis were lower than those in the control endometrium, detected by western blot. β-actin was an internal control. (**C**) The expression of TIPE2 protein in the eutopic/ectopic endometrium of adenomyosis patients and the control endometrium was detected by immunohistochemistry (×200 magnification). (I) The proliferative phase of control endometrium; (II) the proliferative phase of eutopic endometrium from patients with adenomyosis; (III) the secretory phase of control endometrium; (IV) the secretory phase of eutopic endometrium from patients with adenomyosis; (V) the ectopic endometrium of adenomyosis patients. (**D**) The expression of TIPE2 protein in the eutopic and ectopic endometrium of adenomyosis patients was significantly lower than that in the control endometrium ($P < 0.0001$). Results are expressed as a percentage. Data were analyzed by chi-square test. (**E**) The expression of TIPE2 was significantly lower in the proliferative and secretory phases of the eutopic endometrium of adenomyosis patients than those of the control endometrium ($P < 0.0001$). Results are expressed as a percentage. Data were analyzed by chi-square test. However, there were no significant differences in TIPE2 expression between proliferative and secretory phases of the eutopic endometrium of adenomyosis patients or the control group ($P > 0.05$). Results are expressed as a percentage. Data were analyzed by chi-square test. $^{**}P < 0.01$, $^{****}P < 0.0001$.

counted. The colony-forming efficiency was evaluated by the formula: percentage of colonies = number of colonies formed/number of cells inoculated ×100%.

Transwell migration assay

After Ishikawa or HEC-1-A cells were transfected with the TIPE2 plasmid or the lentiviral shRNAs targeting TIPE2 for 24 h, the cells were trypsinized, suspended in DMEM high glucose/McCoy's 5A medium containing 1% FBS, and counted. Then, 150 µl of cell suspension (5×10^4 cells) was added into the upper chamber of an 8 µm pore size membrane (Millipore, Billerica, MA, USA). The DMEM high glucose/McCoy's 5A medium containing 20% FBS (650 µl) was placed in the lower chamber. After incubation at 37°C for 24 h, the cells were fixed with 100% methanol for 10 min, stained with 0.5% crystal violet for 20 min and then observed under a microscope and photographed. Five fields were randomly selected from each chamber, and then the cells in each field of view were counted.

Matrigel invasion assay

The effect of TIPE2 on the invasive ability of endometrial cells was assessed using BD Biocoat Matrigel (BD Biosciences, Bedford, MA, USA). The experimental method was similar to the migration experiment. Fifty microliters of matrigel (1:4) was diluted using DMEM high glucose/McCoy's 5A medium without FBS and added into the chamber, and then the matrigel was quickly incubated at 37°C for 30 min to solidify. Six hundred and fifty microliters of DMEM high glucose/McCoy's 5A medium containing 20% FBS were added to the lower chamber, and then 150 µl of cell suspension (6×10^4 cells) was added to the upper chamber, and the chamber was incubated at 37°C for 48 h. The cells on the lower surface of the membrane

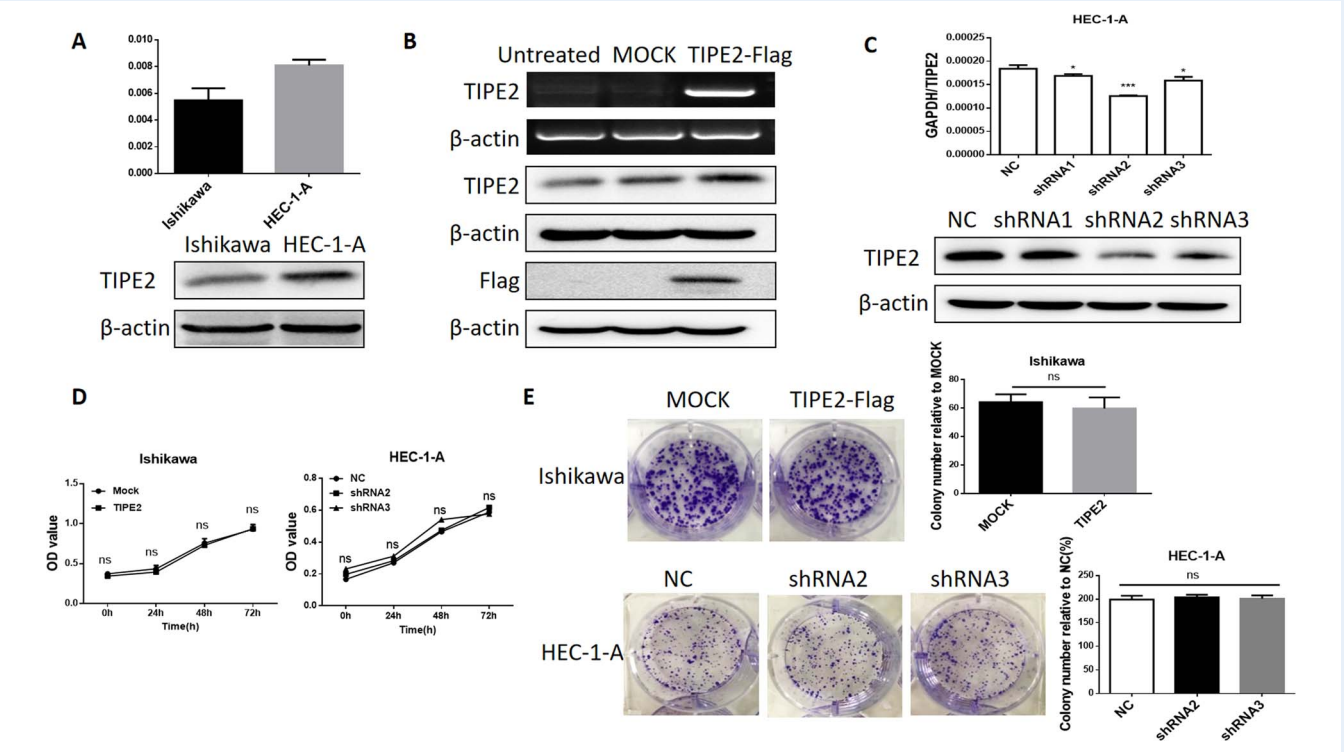

Figure 2 TIPE2 mRNA and protein expression after TIPE2 overexpression and TIPE2 knockdown in Ishikawa and HEC-1-A cells. (**A**) The expression of TIPE2 in Ishikawa cells was lower than that in human endometrial cancer cell line (HEC-1-A) cells detected by qRT-PCR and western blot. (**B**) Ishikawa cells transfected with the TIPE2 expression plasmid showed higher expression of TIPE2 than the control groups, detected by RT-PCR and western blot. (**C**) HEC-1-A cells transfected with short hairpin shRNA2 or shRNA3 showed lower expression of TIPE2 than the control group detected by qRT-PCR and western blot ($P < 0.05$). Results are expressed as the mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. (**D**) TIPE2 overexpression or knockdown had no effect on the proliferation of Ishikawa or HEC-1-A cells ($P > 0.05$). Results are expressed as the mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. (**E**) TIPE2 overexpression or knockdown had no effect on the colony number and size of Ishikawa or HEC-1-A cells ($P > 0.05$). Results are expressed as the mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. $^{*}P < 0.05$, $^{***}P < 0.001$. NC: negative control. MOCK: The cells transfected with an empty plasmid.

were fixed with methanol and stained with 0.1% crystal violet and then photographed by microscopy. The number of invading cells was counted in five microscope fields.

The isolation and identification of glandular epithelial cells and stromal cells derived from the eutopic endometrium of patients with adenomyosis

The eutopic endometrium of adenomyosis patients was put into a 50ml centrifuge tube. After red blood cells were removed with 2–3 ml of erythrocyte lysate, two volumes of collagenase type IA (Gibco, Carlsbad, CA, USA) were used to digest tissue for 50 min at 37°C. During this period, the tube was shaken up and down every 10 min, and then 15 ml of D-Hank's solution was added to stop collagenase activity. The tissue mixture was sequentially filtered through 100 molybdenum and 400 molybdenum copper mesh. Then, the copper mesh was repeatedly washed with D-Hank's solution, and the filtrate was centrifuged at 1000*g* for 5 min to obtain stromal cells. Next, the 400 molybdenum copper mesh was inverted and repeatedly washed, and then the filtrate was centrifuged at 1000*g* for 5 min to obtain glandular epithelial cells. The cells obtained were placed in a culture flask with 5 ml of DMEM/F12 medium (HyClone, Logan, UT, USA) containing 10% FBS, L-glutamine (2 mM; Sigma-Aldrich), and cultured in a humidified atmosphere with 5% CO_2 at 37°C.

After the primary endometrial glandular epithelial cells and stromal cells were cultured in 24-well plates with a coverslip at 37°C for 24 h, the cells on the coverslip were fixed in 4% paraformaldehyde for 30 min and incubated with 3% H_2O_2 for 15 min at room temperature, and then blocked with 10% goat serum at 37°C for 30 min. Next, the cells were incubated with rabbit-derived monoclonal antibodies against Vimentin or CK7 (1:1000) (ZSGB-BIO, Beijing, China) overnight at 4°C, followed by HRP-conjugated goat anti-rabbit IgG (Gene Tech., Shanghai, China) for 30 min at 37°C. The expression of Vimentin and CK7 was assessed using DAB (Gene Tech., Shanghai, China) and observed under a microscope, and each sample was photographed in duplicate.

F-actin detection

The TIPE2-overexpressing/silenced cells were fixed in freshly prepared 4% paraformaldehyde for 10 min and washed three times with PBS

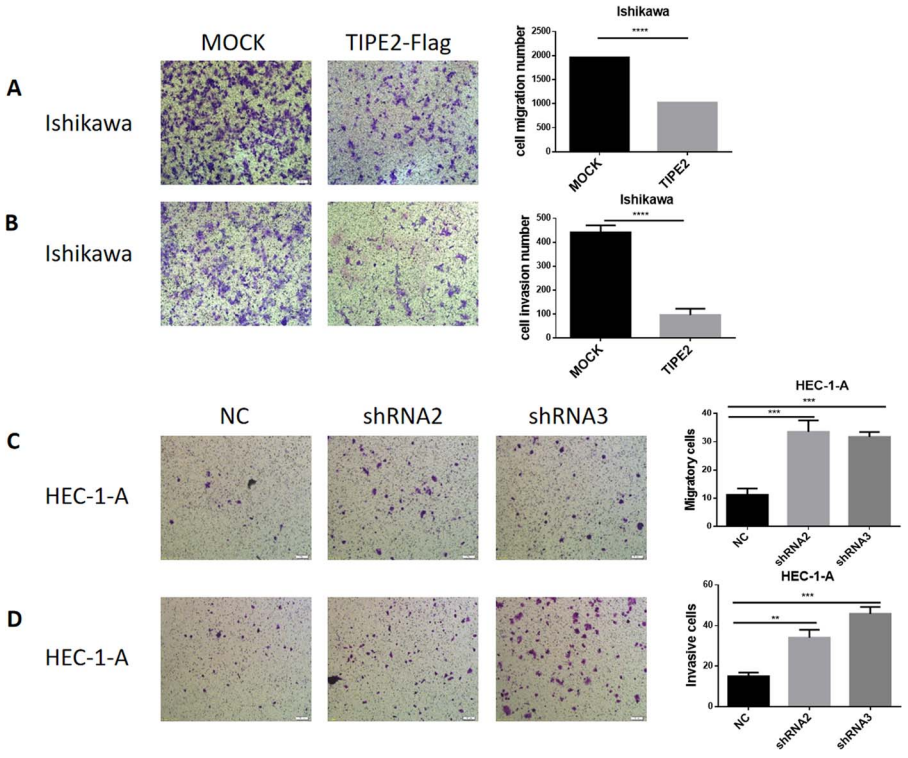

Figure 3 TIPE2 represses the migration and invasion of human endometrial cells. (**A**) TIPE2 overexpression in Ishikawa cells resulted in a significant reduction in the number of cells passing through the chambers compared with the control groups ($P < 0.0001$). Scale bar is 200 μm. Results are the mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. (**B**) TIPE2 overexpression in Ishikawa cells resulted in a significant decrease in the number of invasive cells compared with the controls ($P < 0.0001$). Scale bar is 200 μm. Results are mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. (**C**) TIPE2 knockdown in HEC-1-A cells increased the number of cells migrating into the lower surface of the transwell membrane ($P < 0.001$). Scale bar is 200 μm. Results are mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. (**D**) TIPE2 knockdown in HEC-1-A cells increased the number of cells invading to the lower surface of the transwell membrane ($P < 0.01$). Scale bar is 200 μm. Results are mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. MOCK: The cells transfected with an empty plasmid. NC: Negative control.

for 5 min/each time. The cells were permeabilized with 0.1% Triton X-100 for 10 min and washed three times with PBS for 5 min/each time. Next, the cells were blocked with 2% bovine serum albumin (BSA) for 1 h. The tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (P1951-1MG, Sigma-Aldrich, St Louis, MO, USA) stock solution was diluted to 1:100 with PBS and added to the cells. The cells were incubated for 1 h at 37°C in the dark and washed with PBS three times for 10 min/each time. DAPI was used to stain the nucleus at room temperature for 5 min. Then, 10 μl of fluorescence quenched agents was added. The cells were observed under a laser confocal microscope.

Co-immunoprecipitation

For co-immunoprecipitation (co-IP), after Ishikawa cells were transfected with the TIPE2 expression vector with Flag-tag for 24 h, the cells were washed twice with PBS and re-suspended with 500 μl of IP buffer and then lysed on ice for 2 min. Next, the cells were scraped off and centrifuged at 14 000*g* for 10 min at 4°C to collect the supernatant. The supernatant was divided into three groups: IP group for Flag (200 μl), IP group for IgG (200 μl) and Input group (40 μl). The supernatant of the IP group for Flag or the IP group for IgG was, respectively, incubated with 1.5 μl of mouse-derived monoclonal antibody against Flag (Sigma-Aldrich, Shanghai, China) or IgG (CST, Boston, MA, USA) at 4°C in a chromatography cabinet for 1 h. Then, 40 μl of Protein A/G Plus-Agarose (CST, Boston, MA, USA) was added to this supernatant and incubated at 4°C in a chromatography cabinet overnight. The next day, the beads were washed five times with IP buffer (IP buffer includes 4.4 g NaCl, 5 ml NP-40, 50 ml PH7.4 Tris–Cl (1 M), 50 ml PH8.0 EDTA (0.5 M), finally made up to 500 ml with double-distilled water) and dissolved in 40 μl 2 × loading buffer. For the Input group, 8 μl of 6 × loading buffer was added to the supernatant and immediately centrifuged for 1 min and placed at 100°C for 5 min. The proteins were detected by western blot.

Co-localization analysis of TIPE2 and β-catenin

TIPE2-overexpressing cells were fixed in freshly prepared 4% paraformaldehyde for 10 min and washed three times with PBS for

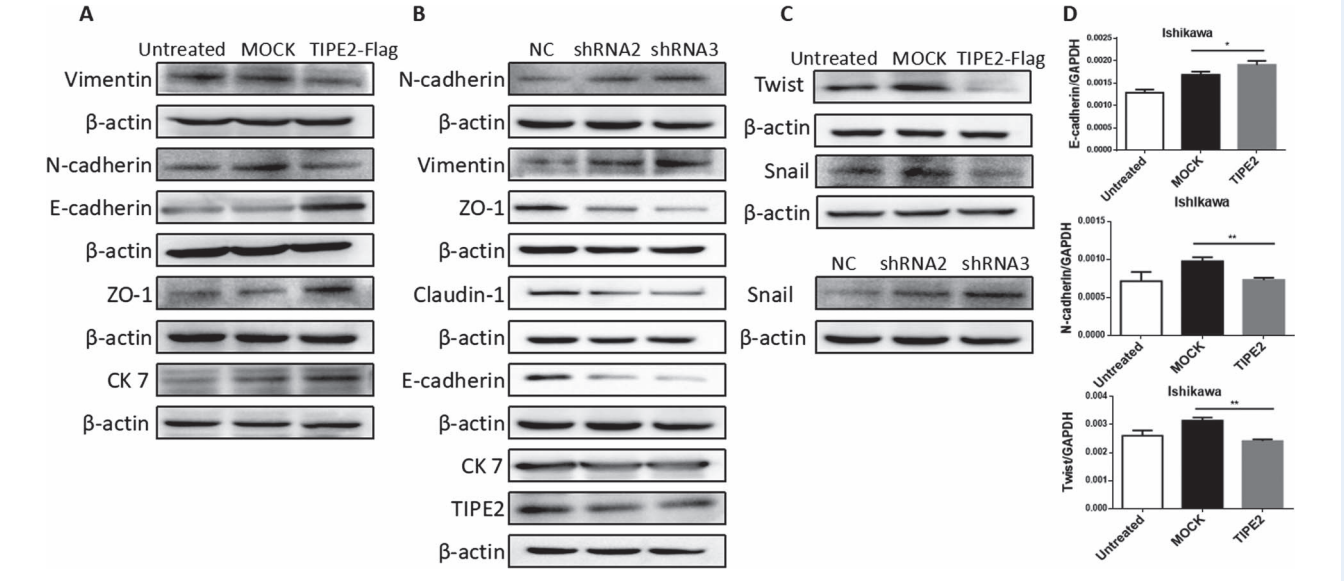

Figure 4 TIPE2 inhibits the epithelial-mesenchymal transition of human endometrial cells. (**A**) TIPE2 overexpression in Ishikawa cells upregulated the levels of E-cadherin, cytokeratin 7 (CK7) and zonula occludens-1 (ZO-1) and downregulated the protein levels of Vimentin and N-cadherin. (**B**) TIPE2 knockdown in HEC-1-A cells decreased the expression of E-cadherin, CK7, Claudin-1 and ZO-1 and increased the expression of Vimentin and N-cadherin. (**C**) The protein levels of Twist and Snail were downregulated in TIPE2-overexpressed Ishikawa cells, but the levels of Snail were upregulated after TIPE2 knockdown in HEC-1-A cells. (**D**) TIPE2 overexpression upregulated the levels of E-cadherin mRNA and downregulated the levels of N-cadherin and Twist mRNA ($P < 0.05$). Results are mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$. MOCK: The cells transfected with an empty plasmid. NC: Negative control.

5 min/each time. The cells were permeabilized with 1% Triton X-100 for 15 min and washed three times with PBS for 5 min/each time. Next, the cells were blocked with 2% BSA for 1 h. Then, the cells were incubated with primary antibody against TIPE2 (1:200) overnight at 4°C and washed three times with PBS for 5 min/each time and then incubated with Alexa Fluor 488-conjugated secondary antibody (red light, 1:500) (Absin Bioscience Co., Ltd, Shanghai, China) in the dark at room temperature for 1 h and washed three times with PBS for 5 min/each time. Next, the cells were incubated with primary antibody against β-catenin (1:200) overnight at 4°C and washed three times with PBS for 5 min/each time. The next day, the cells were incubated with TRITC-conjugated secondary antibody (green light, 1:500) (Proteintech Group, Inc., Wuhan, China) in the dark for 1 h at room temperature, and washed three times with PBS for 5 min/each time. DAPI staining for 10 min followed, and then 10 μl of fluorescence quenching agents was added. Finally, the cells were observed under a laser confocal microscope and photographed.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad, La Jolla, CA, USA). All data are reported as mean ± SD. A chi-square test was performed to assess the results of IHC staining; Student's *t* test was used to determine the statistical significance between the test group and the control group. $P < 0.05$ was considered statistically significant.

Results

Expression of TIPE2 in eutopic endometrium of adenomyosis patients is downregulated

To explore the expression levels of TIPE2 in adenomyosis, we collected the eutopic endometrium of adenomyosis patients and control endometrium and determined the expression levels of TIPE2 mRNA and protein using qRT-PCR and western blot. The results showed that TIPE2 mRNA and protein levels were downregulated in the eutopic endometrium of adenomyosis patients compared with the control endometrium (Fig. 1A and B).

The expression of TIPE2 in the eutopic/ectopic endometrium of adenomyosis patients is decreased detected by IHC

To further determine the relationship between TIPE2 and adenomyosis, we investigated TIPE2 protein in the eutopic or ectopic endometrium of adenomyosis patients and control endometrium using IHC staining. As shown (Fig. 1C, I II, III, IV and V), TIPE2 protein was observed in the cytoplasm of endometrial glandular epithelial cells, while TIPE2 staining was negative or relatively weak in endometrial stromal cells. TIPE2 protein levels in the eutopic or ectopic endometrium of adenomyosis patients were much lower

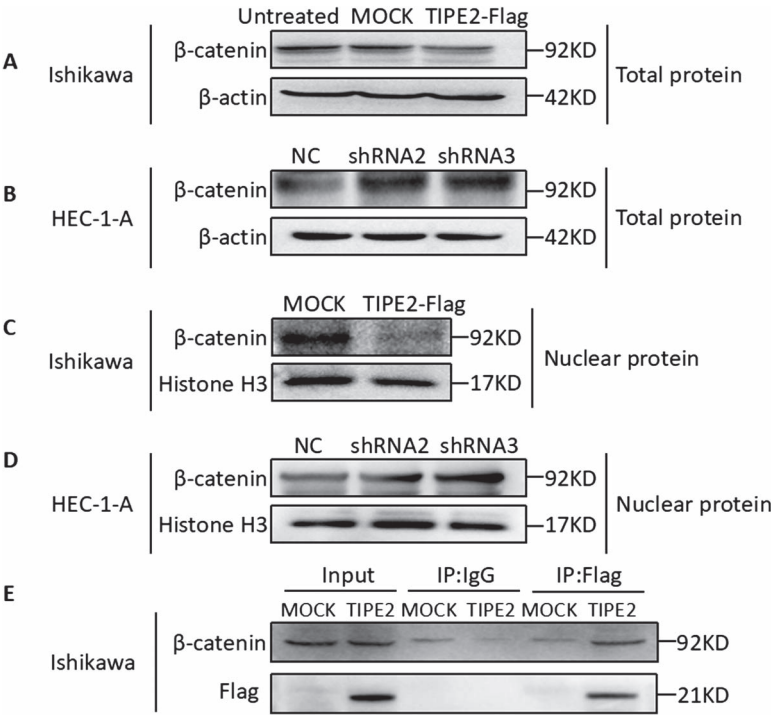

Figure 5 TIPE2 inhibits the nuclear translocation of β-catenin in human endometrial cells. (**A**, **B**) Total β-catenin protein was downregulated after TIPE2 overexpression in Ishikawa cells and upregulated after TIPE2 silence in HEC-1-A cells. (**C**, **D**) TIPE2 overexpression reduced the level of nuclear β-catenin in Ishikawa cells, but TIPE2 knockdown increased the level of nuclear β-catenin in HEC-1-A cells. (**E**) Co-immunoprecipitation confirmed that TIPE2 could bind with β-catenin. MOCK: The cells transfected with an empty plasmid. NC: Negative control.

than those in the control endometrium (Fig. 1D). Adenomyosis is an estrogen-dependent disease (Chen *et al.*, 2010); therefore, we analyzed TIPE2 levels in the proliferative and secretory phases of the eutopic endometrium of adenomyosis patients and control endometrium. We found that TIPE2 staining was significantly lower in the proliferative and secretory phases of the eutopic endometrium of adenomyosis patients than in the control endometrium (Fig. 1E). However, there were no significant differences in staining of TIPE2 among proliferative and secretory phases of the eutopic endometrium of adenomyosis patients or the control subjects (Fig. 1E). The results indicate that TIPE2 may be involved in the progression of adenomyosis and exerts an inhibitory effect.

TIPE2 overexpression/knockdown does not affect the proliferation and colony-forming ability of endometrial cells

We assessed TIPE2 mRNA and protein in two kinds of endometrial cancer cell lines (Ishikawa and HEC-1-A). As shown in Fig. 2A, the expression of TIPE2 mRNA and protein was lower in Ishikawa cells compared with HEC-1-A cells. To elucidate the role of TIPE2 overexpression in Ishikawa cells, we constructed the overexpression plasmid targeting TIPE2, and then analyzed the overexpression efficiency of TIPE2 at the mRNA and protein levels. The results from RT-PCR and western blot revealed that the TIPE2 plasmid was successfully overexpressed in Ishikawa cells (Fig. 2B). To elucidate the effect of TIPE2 knockdown on HEC-1-A cells, we constructed three lentiviral interfering vectors targeting TIPE2 and used them to transfect HEC-1-A cells. Next, we detected the interfering efficiency by qRT-PCR and western blot, and the results revealed that shRNA2 and shRNA3 were both efficient for knockdown of the expression of TIPE2 (Fig. 2C).

To explore the effect of TIPE2 on the growth of endometrial cells, we further overexpressed TIPE2 in Ishikawa cells using the TIPE2 expression plasmid or interfered with the expression of TIPE2 in HEC-1-A cells using the specific shRNAs targeting TIPE2. The results from the CCK-8, EDU assay, and colony-forming assay showed that TIPE2 overexpression and knockdown had no significant effects on the proliferation and colony-forming ability of endometrial cells (Fig. 2D and E, Supplementary Fig. S1).

TIPE2 inhibits the migration and invasion ability of endometrial cells

The increase in migration and invasion ability is one of the most significant biological features of adenomyosis (Liu *et al.*, 2018a). Here, we overexpressed TIPE2 in Ishikawa cells and found that the number of cells passing through the chamber was significantly reduced in the TIPE2 overexpression group compared with the MOCK group (Fig. 3A). In contrast, TIPE2 knockdown in HEC-1-A cells significantly increased the number of cells that migrated into the lower surface of the transwell membrane (Fig. 3C). We used the matrigel invasion assay to determine the effects of TIPE2 overexpression or knockdown on the invasive ability of Ishikawa or HEC-1-A cells. As shown in Fig. 3B and D, the number of cells passing through the chamber in the TIPE2 plasmid

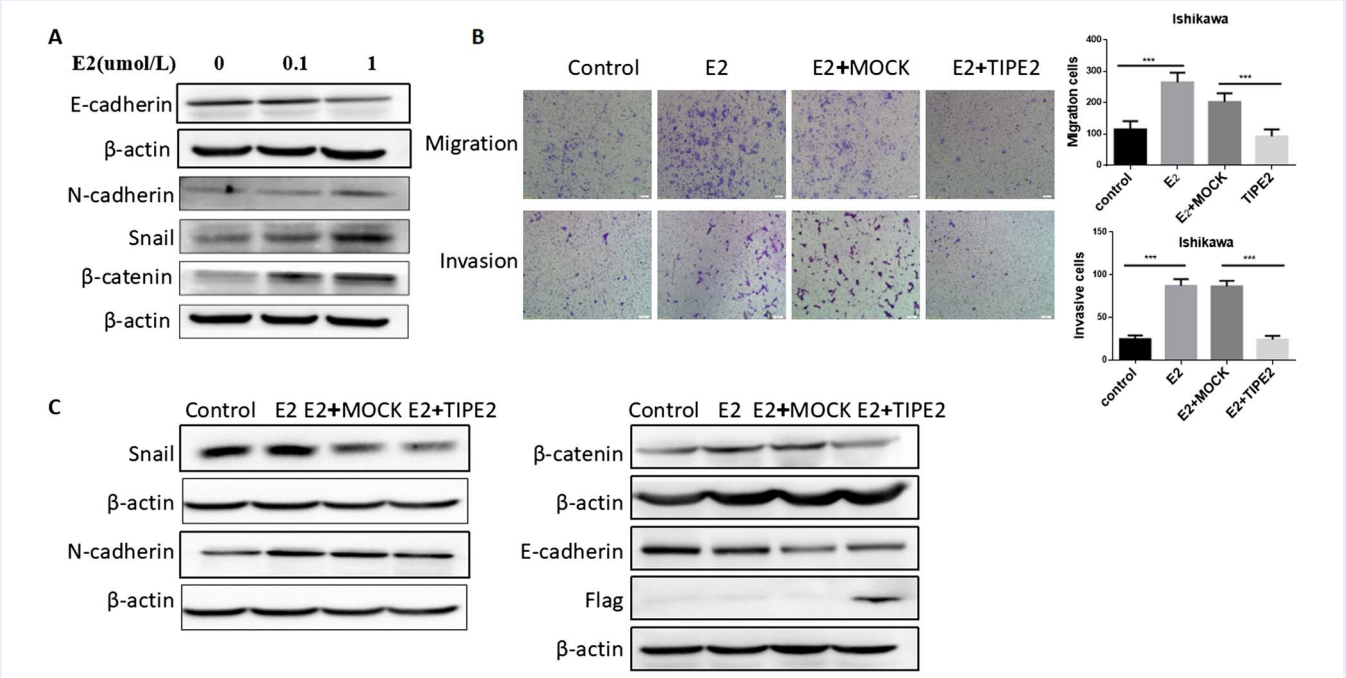

Figure 6 TIPE2 reverses estrogen-induced migration, invasion and epithelial-mesenchymal transition of endometrial cells. (**A**) Ishikawa cells were treated with different concentrations (0, 0.1, 1 μmol) of estrogen (E_2) for 24 h. The results show that 1 μmol estrogen could downregulate the expression of E-cadherin and upregulate the expression of N-cadherin, Snail and β-catenin. (**B**) TIPE2 overexpression inhibited the estrogen-induced migration and invasion ($P < 0.001$). Scale bar is 200 μm. Results are mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. (**C**) TIPE2 overexpression reversed the estrogen-induced decrease in the expression of E-cadherin and estrogen-induced increase in the expression of N-cadherin, Snail and β-catenin. MOCK: The cells transfected with an empty plasmid.

transfection group was significantly lower than that in the MOCK group, and the number of cells invading the lower surface of transwell membrane was significantly increased after TIPE2 knockdown. The results reveal that TIPE2 can inhibit the migration and invasion of endometrial cells in this system.

TIPE2 inhibits the EMT of endometrial cells

EMT plays an important role in cell migration and invasion (Acloque *et al*., 2009), as well as the pathogenesis of adenomyosis. To investigate the mechanism by which TIPE2 inhibited endometrial cell migration and invasion ability, we examined the expression of EMT-related markers, including epithelial cell markers (E-cadherin, CK7, Claudin-1 and ZO-1) and mesenchymal cell markers (Vimentin and N-cadherin) after TIPE2 overexpression or knockdown. As shown in Fig. 4A, TIPE2 overexpression in Ishikawa cells upregulated the levels of E-cadherin, CK7 and ZO-1 and downregulated the levels of Vimentin and N-cadherin. On the contrary, TIPE2 knockdown in HEC-1-A cells decreased the expression of E-cadherin, CK7, Claudin-1 and ZO-1 and increased the expression of Vimentin and N-cadherin (Fig. 4B). EMT-related markers are regulated by corresponding transcription factors such as Twist and Snail. Therefore, we further detected the expression of Twist and Snail after overexpressing or interfering with TIPE2, and the results showed that the levels of Twist and Snail were downregulated in TIPE2-overexpressing Ishikawa cells, but the levels of Snail were upregulated after TIPE2 knockdown in HEC-1-A cells (Fig. 4C). We overexpressed TIPE2 in the Ishikawa cells and detected the change of mRNA levels for EMT-related markers by qRT-PCR. The results were consistent with the above results (Fig. 4D).

TIPE2 inhibits the nuclear translocation of β-catenin in endometrial cells

The Wnt/β-catenin signaling pathway is involved in cell migration and invasion (Jiang *et al*., 2007); therefore, we examined the changes of total β-catenin and found that total β-catenin was downregulated after TIPE2 overexpression in Ishikawa cells, and upregulated after TIPE2 knockdown in HEC-1-A cells (Fig. 5A and B). The nuclear translocation of β-catenin could activate EMT-related transcription factors and mediate the occurrence of EMT. We also detected the expression of nuclear β-catenin, and the results showed that TIPE2 overexpression reduced the level of nuclear β-catenin in Ishikawa cells, but TIPE2 knockdown increased the level of nuclear β-catenin in HEC-1-A cells (Fig. 5C and D). In order to investigate whether TIPE2 could bind to β-catenin, we performed co-IP after TIPE2 overexpression in Ishikawa cells and found that TIPE2 could bind with β-catenin (Fig. 5E). The interaction between TIPE2 and β-catenin was further confirmed by co-localization analysis of TIPE2 and β-catenin (Supplementary Fig. S2).

TIPE2 reverses estrogen-induced migration, invasion and EMT of endometrial cells

Adenomyosis is an estrogen-dependent disease, and estrogen promotes migration, invasion, and EMT of endometrial cells (Chen *et al*.,

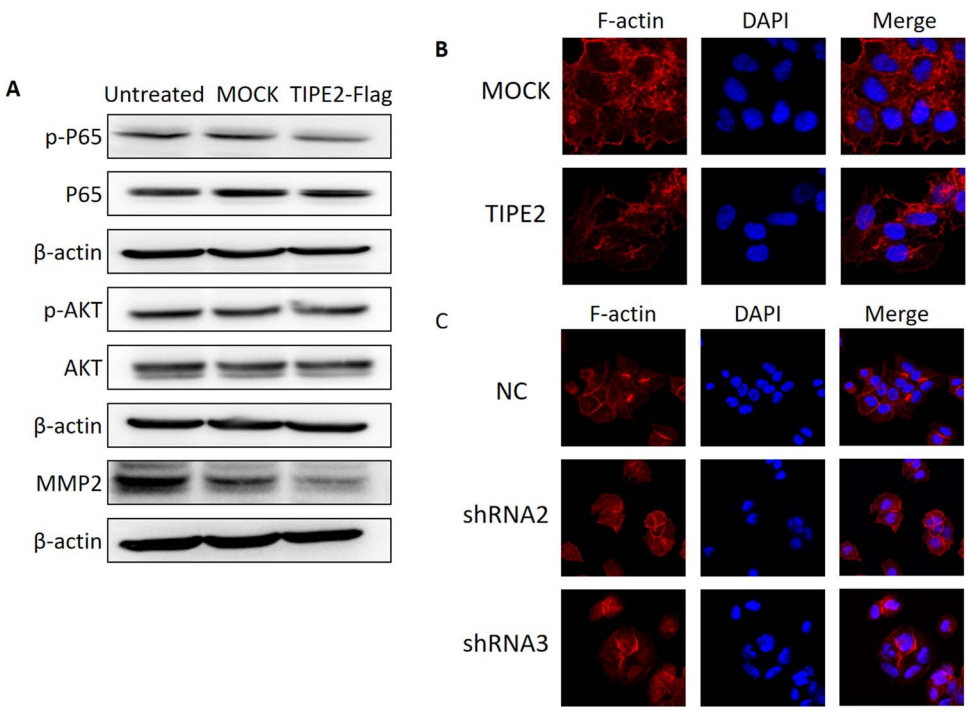

Figure 7 TIPE2 inhibits matrix metalloproteinase 2 and p-P65 expression but does not affect the expression of F-actin in human endometrial cells. (**A**) After Ishikawa cells were transfected with the TIPE2 expression vector for 48 h, the results of western blot analysis showed no significant change in p-AKT level, while the levels of p-P65 and MMP2 showed a slight downregulation after the overexpression of TIPE2. (**B**, **C**) After Ishikawa cells were transfected with the TIPE2 expression vector or the TIPE2 interference vector for 24 h, the results of immunofluorescence showed TIPE2 overexpression or knockdown had no effect on the expression of F-actin in endometrial cells. MOCK: The cells transfected with an empty plasmid. NC: Negative control.

2010). In order to explore whether TIPE2 could reverse estrogen-induced migration, invasion and EMT, we used different concentrations (0, 0.1, 1 μmol) of estrogen to treat Ishikawa cells for 24 h. The results showed that 1 μmol estrogen downregulated the expression of the epithelial marker (E-cadherin) and upregulated the expression of the mesenchymal marker (N-cadherin) and the transcription factor (Snail), as well as β-catenin (Fig. 6A). Next, we overexpressed TIPE2 in Ishikawa cells and added 1 μmol estrogen to stimulate for 24 h. It was found that estrogen promoted the migration, invasion and EMT compared with the control group. However, TIPE2 overexpression not only inhibited the estrogen-induced migration and invasion (Fig. 6B) but also increased the levels of E-cadherin and decreased the levels of N-cadherin, Snail and β-catenin (Fig. 6C). The results indicate that estrogen can promote the migration, invasion and EMT of endometrial cells, and this effect can be reversed by TIPE2.

TIPE2 slightly inhibits MMP2 and p-P65 expression on protein levels but does not affect the expression of F-actin in endometrial cells

Besides EMT, the changes in basement membrane degradation and cell movement ability also play a vital role in the migration and invasion of endometrial cells (Shan *et al.*, 2013). Here, we detected the expression of MMP2 and MMP2-related phosphoinositide 3-kinase (PI3K)/AKT/nuclear factor (NF)-κB signaling pathway molecules (AKT, p-AKT, P65, p-P65) after TIPE2 overexpression and found no significant change in p-AKT level, while the levels of p-P65 and MMP2 showed a slight downregulation after overexpression of TIPE2 (Fig. 7A). However, the results of immunofluorescence showed that TIPE2 overexpression or knockdown did not affect the expression of F-actin in endometrial cells (Fig. 7B and C).

TIPE2 inhibits the migration, invasion and EMT of primary endometrial cells

To further determine the effect of TIPE2 on migration, invasion and EMT of endometrial cells, we isolated glandular epithelial cells and stromal cells from the eutopic endometrium of adenomyosis patients and performed immunocytochemical staining. The results showed that CK7 was strongly expressed in glandular epithelial cells, and Vimentin was strongly expressed in stromal cells. However, glandular epithelial cells had weak Vimentin expression, and stromal cells had weak CK7 expression (Fig. 8A). Furthermore, we overexpressed TIPE2 using the TIPE2-Flag plasmid and interfered with the expression of TIPE2 using two kinds of lentiviral interfering vectors targeting TIPE2 (Fig. 8B and C). As shown in Fig. 8D and E, TIPE2 overexpression obviously inhibited the migration and invasion of primary endometrial cells; on the contrary, the downregulation of TIPE2 promoted the migration and invasion of primary endometrial cells. The results from western blot showed that TIPE2 overexpression upregulated the levels

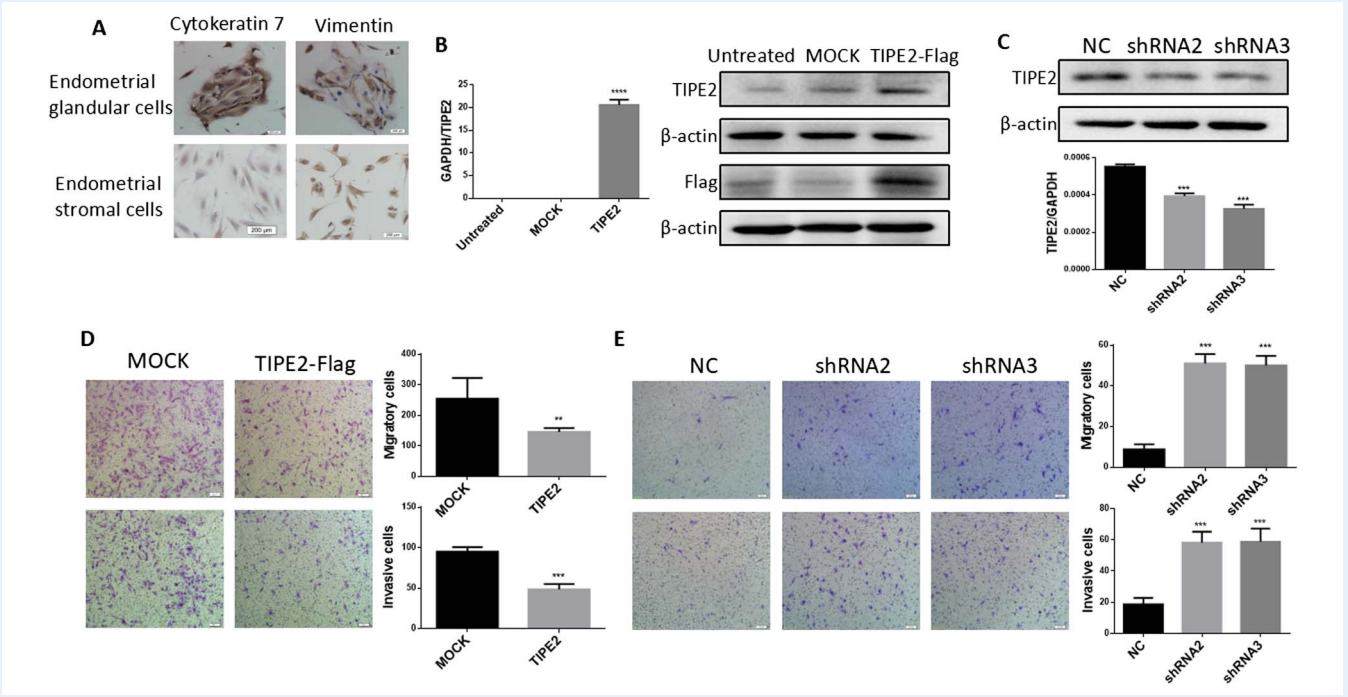

Figure 8 TIPE2 inhibits the migration, invasion of primary human endometrial cells. (**A**) Immunocytochemistry staining showed CK7 was strongly expressed in glandular epithelial cells, and Vimentin was strongly expressed in stromal cells. However, glandular epithelial cells had weak Vimentin expression, and stromal cells had weak CK7 expression. (**B**) The primary endometrial cells transfected with the TIPE2 expression plasmid showed higher expression of TIPE2 than the control group, as detected by qRT-PCR and western blot ($P < 0.0001$). Results are mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. (**C**) The primary endometrial cells transfected with shRNA2 or shRNA3 showed lower expression of TIPE2 than the control group, as detected by qRT-PCR and western blot ($P < 0.001$). Results are mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. (**D**) TIPE2 overexpression inhibited the migration and invasion of primary endometrial cells ($P < 0.01$). Scale bar is 200 μm. Results are mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. (**E**) The downregulation of TIPE2 promoted the migration and invasion of primary endometrial cells ($P < 0.001$). Scale bar is 200 μm. Results are mean ± SD of three independent experiments. Data were analyzed by Student's *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$. MOCK: The cells transfected with an empty plasmid. NC: negative control.

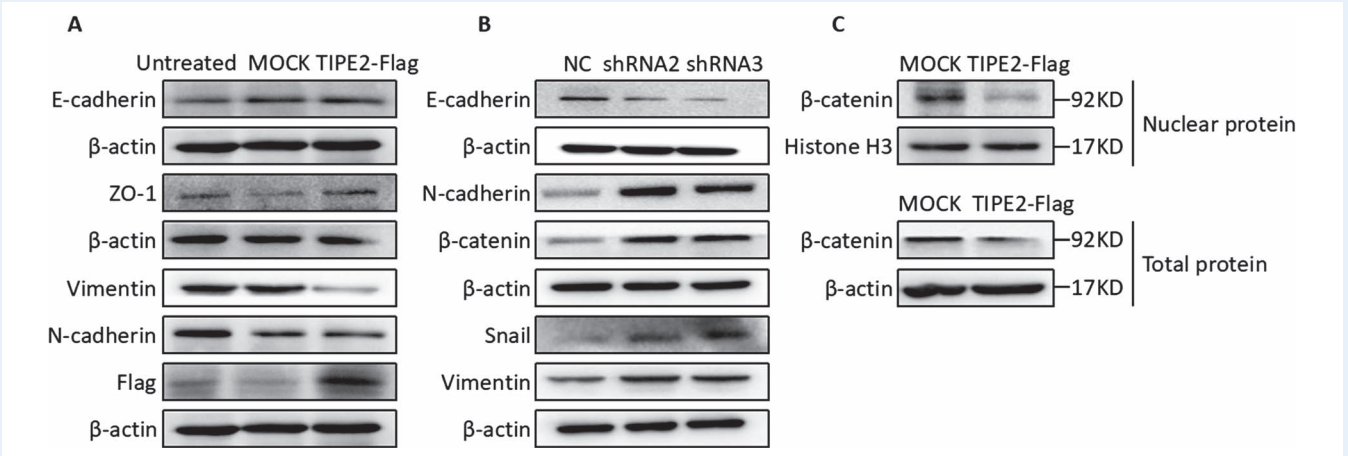

Figure 9 TIPE2 inhibits the epithelial–mesenchymal transition of human primary endometrial cells. (**A**) TIPE2 overexpression upregulated the levels of E-cadherin and ZO-1 and downregulated the levels of Vimentin and N-cadherin. (**B**) TIPE2 knockdown decreased the expression of E-cadherin, increased the expression of Vimentin, N-cadherin, Snail and total β-catenin. (**C**) TIPE2 overexpression decreased the expression of nuclear β-catenin and total β-catenin. MOCK: The cells transfected with an empty plasmid. NC: Negative control.

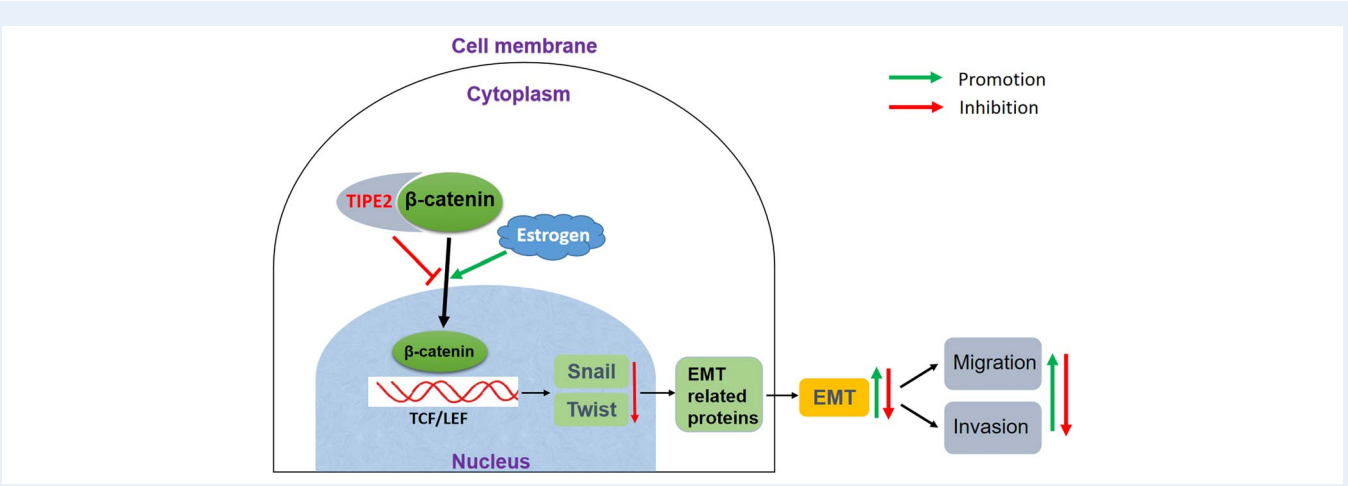

Figure 10 TIPE2 reverses estrogen-induced migration, invasion and EMT in endometrial cells by targeting β-catenin. Estrogen could promote the EMT by regulating β-catenin. TIPE2 could interact with β-catenin and inhibit the translocation of β-catenin, then downregulate the expression of stromal cell markers, upregulate the expression of glandular epithelial cell markers, decrease the occurrence of epithelial–mesenchymal transition (EMT) and suppress the migration and invasion of endometrial cells. TCF: T-cell factor; LEF: lymphoid enhancer-binding factor.

of E-cadherin and ZO-1 and downregulated the levels of Vimentin and N-cadherin. However, after TIPE2 knockdown, the expression of Vimentin, N-cadherin, Snail and total β-catenin was increased, and the expression of E-cadherin was decreased (Fig. 9A and B). TIPE2 overexpression similarly decreased the expression of nuclear β-catenin and total β-catenin (Fig. 9C). These results confirm that TIPE2 can inhibit the migration, invasion and EMT of primary endometrial cells.

Discussion

Adenomyosis is a kind of benign disease, but it exhibits many characteristics that are similar to malignant tumors. Many tumor oncogenes and suppressor genes also participate in the development of adenomyosis. TIPE2 is a negative immune regulatory molecule that could be involved in regulating innate and adaptive immune responses and maintaining immune homeostasis (Sun *et al.*, 2008). Current studies show that TIPE2 acts as a tumor suppressor gene to inhibit the growth, migration and invasion of tumor cells (Cao *et al.*, 2013). However, the possible role(s) of TIPE2 in adenomyosis remain unclear. In the present study, the effects of TIPE2 on human endometrial cells and underlying mechanisms were investigated.

At present, there are conflicting results about the expression of TIPE2 in malignant tumors. For example, TIPE2 is downregulated in human primary HCC (Cao *et al.*, 2013) and gastric cancer (Wu *et al.*, 2016; Liu *et al.*, 2018b; Yin *et al.*, 2017) compared with the paired adjacent non-tumor tissues. However, TIPE2 protein expression was upregulated in colon cancer (Li *et al.*, 2014), renal cell carcinoma (Zhang *et al.*, 2013) and non-small cell lung cancer (NSCLC) tissues (Li *et al.*, 2015; Li *et al.*, 2016b) compared with the adjacent normal tissues. These results indicate that TIPE2 expression in tumors may be tissue-specific. Here, we found that the expression of TIPE2 mRNA and protein was downregulated in eutopic/ectopic endometrium compared with the control endometrium. Adenomyosis is an estrogen-dependent disease (Chen *et al.*, 2010), and the proliferative and secretory phases of the endometrium are greatly affected (Liu *et al.*, 2018a) by hormones. Therefore, we analyzed the expression levels of TIPE2 in the proliferative phase and the secretory phase and found that the level of TIPE2 protein in proliferative and secretory phases of adenomyosis patients was significantly lower than that in the corresponding phases of the control group. Our results suggest that downregulation of TIPE2 may also be involved in the pathogenesis of adenomyosis.

TIPE2 expression is higher in some tumor tissues than in adjacent non-tumor tissues; however, TIPE2 serves as a tumor suppressor and has inhibitory effects on the growth, migration and invasion of tumor cells, such as NSCLC (Li *et al.*, 2015; Li *et al.*, 2016b). The development of adenomyosis is mainly determined by the increase in growth, migration and invasive ability of endometrial cells. For example, PAK4 is a serine/threonine-protein kinase that plays a role in a variety of different signaling pathways, including cytoskeleton regulation, cell migration, growth proliferation or cell survival (Dummler *et al.*, 2009). It is reported that the expression level of PAK4 in adenomyosis is significantly increased: it can regulate the invasion, survival and proliferation of endometrial cells to promote the development of adenomyosis (Yi *et al.*, 2015; Zayratyants *et al.*, 2018). We evaluated the roles of TIPE2 in the growth, migration and invasion of endometrial cells *in vitro* by TIPE2 overexpression and knockdown. We found that TIPE2 significantly inhibited the migration and invasion of endometrial cell lines and primary endometrial cells from adenomyosis patients, but there was no significant effect on the growth of the endometrial cells. These results suggest that TIPE2 may work through different mechanisms in the development of tumors and adenomyosis.

Next, we further investigated the potential mechanism by which TIPE2 inhibits the migration and invasion of endometrial cells. EMT refers to the biological process by which epithelial cells are transformed into mesenchymal phenotype cells by a specific procedure (Das *et al.*, 2019). It plays a crucial role in the invasion, metastasis and development of cancers and adenomyosis (Liu *et al.*, 2016). During the EMT process,

the cells lose intercellular connections, degrade the extracellular matrix and eventually transform into mesenchymal cells, which increases the migration and invasion ability of the cells (Yang *et al*., 2017). This process is precisely regulated by corresponding transcription factors, such as Snail (Shi *et al*., 2019), Slug and Twist (Mäder *et al*., 2018). These transcription factors could suppress the expression of epithelial cell markers (E-cadherin, CK7 and ZO-1) and maintain the phenotype of mesenchymal cells that express Vimentin and N-cadherin. It has been reported that TIPE2 inhibits gastric cancer cell migration, invasion and metastasis, very probably via a reversal of EMT (Wu *et al*., 2016; Yin *et al*., 2017; Liu *et al*., 2018b). In this study, we found that TIPE2 can significantly increase the expression of epithelial cell markers and decrease the levels of mesenchymal cell markers and EMT-related transcription factors. Adenomyosis is a kind of estrogen-related disease, and a document published by Chen *et al*., showed that estrogen-induced EMT of endometrial epithelial cells contributed to the development of adenomyosis (Chen *et al*., 2010). In the present study, our results showed that TIPE2 could effectively reverse the estrogen-induced migration, invasion and EMT of endometrial cells. These results indicate that TIPE2 can inhibit the migration/invasion of endometrial cells by inhibiting the EMT phenotype.

Many studies have found that the Wnt/β-catenin signaling pathway participates in the process of EMT (Jiang *et al*., 2007). β-Catenin is the core factor of this pathway. Activation of the Wnt pathway or estradiol stimulation (Ali *et al*., 2019) leads to an increase in β-catenin nuclear translocation, upregulates the expression of Twist, Snail and other transcription factors and inhibits the transcription of epithelial cell markers, such as E-cadherin. β-Catenin can also promote fibronectin and vimentin gene transcription, induce cell epithelial–mesenchymal transition (Gradl *et al*., 1999; Gilles *et al*., 2003). Wu *et al*., reported that TIPE2 inhibited the metastasis of gastric cancer by inhibiting the nuclear translocation of β-catenin (Wu *et al*., 2016). Here, we found that TIPE2 overexpression significantly downregulated the nuclear protein level of β-catenin; on the contrary, the nuclear protein level of β-catenin was upregulated after TIPE2 knockdown. Further investigation showed that TIPE2 could bind to β-catenin and inhibit its nuclear translocation. These results confirm that TIPE2 may reverse EMT by inhibiting the Wnt/β-catenin signaling pathway.

Besides EMT, the changes in cell motility and the degradation of the basement membrane are also involved in cell migration and invasion (Shan *et al*., 2013). Cao *et al*., reported that TIPE2 inhibited migration and invasion through targeting Rac1 and then reduced F-actin polymerization, as well as the expression of MMP9 and uPA (Cao *et al*., 2013). In NSCLC, TIPE2 suppressed tumor invasiveness and angiogenesis by inhibiting the activation of Rac1 and subsequently weakening its downstream effects, including F-actin polymerization and VEGF expression (Li *et al*., 2016b). The study of Zhang *et al*., showed that TIPE2 overexpression efficiently abolished the effects of TNF-α on MMP-13/MMP-3 upregulation, cell migration and ERK1/2-NF-κB activation (Zhang *et al*., 2015). Here, we found that TIPE2 could slightly decrease the expression of p-P65 and MMP2 with no effect on the expression of F-actin, which indicates that TIPE2 also inhibits the migration and invasion of endometrial cells by affecting degradation of the basement membrane.

In conclusion, we demonstrate that TIPE2 is downregulated in adenomyosis, and we propose that TIPE2 may play an important inhibitory role in the migration and invasion of endometrial cells, mainly by targeting β-catenin to reverse EMT (Fig. 10). TIPE2 may be a potential molecular target for the diagnosis and treatment of adenomyosis.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

Dr Z.T.W. designed the project and conducted an experimental study. Y.Q.L. collected the samples, performed the experiments and wrote the manuscript. X.Y.W. participated in designing the experiments, writing and reviewing the manuscript. X.H.L. participated in sample detection. L.W. and H.Y.Y. were involved in performing some experiments. D.R.Z. and Y.S.S. were involved in data collection and statistical analysis. L.N.Z. and Y.Y.S. were involved in reviewing the manuscript. H.Y.Z. and J.N.W. participated in sample collection. All authors read and approved the final manuscript.

Funding

National Natural Science Foundation of China (81471437, 81771554); Natural Science Foundation of Shandong (ZR2018MH013); science and technology development plan provided by Health and Family Planning Committee in Shandong (2014-25).

Conflict of interest

The authors declare that they have no conflicts of interest.

References

Acloque H, Adams MS, Fishwick K. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest* 2009;**119**:1438–1449.

Ali M, Shahin SM, Ali Sabri N, Al-Hendy A, Yang Q. Activation of β-catenin signaling and its cross talk with estrogen and histone deacetylases in human uterine fibroids. *J Clin Endocrinol Metab* 2020;**105**:dgz227.

Bird CC, McElin TW, Manalo-Estrella P. The elusive adenomyosis of the uterus–revisited. *Am J Obstet Gynecol* 1972;**112**:583–593.

Benagiano G, Brosens I. History of adenomyosis. *Obstet Gynaecol* 2006;**20**:449–463.

Canis M, Gremeau AS, Bourdel N. Elusive adenomyosis: a plea for an international classification system to allow artificial intelligence approaches to reset our clinical management. *Fertil Steril* 2018;**110**:1039–1040.

Cao X, Zhang L, Shi Y, Sun Y, Dai S, Guo C, Zhu F, Wang Q, Wang J, Wang X *et al*. Human tumor necrosis factor (TNF)-alpha-induced protein 8-like 2 suppresses hepatocellular carcinoma metastasis through inhibiting Rac1. *Mol Cancer* 2013;**12**:149–158.

Carmody RJ, Hilliard B, Maguschak K, Chodosh LA, Chen YH. Genomic scale profiling of autoimmune inflammation in the central nervous system: the nervous response to inflammation. *J Neuroimmunol* 2002;**133**:95–107.

Chen YJ, Li HY, Huang CH, Twu NF, Yen MS, Wang PH, Chou TY, Liu YN, Chao KC, Yang MH. Oestrogen-induced epithelial-mesenchymal transition of endometrial epithelial cells contributes to the development of adenomyosis. *J Pathol* 2010;**222**:261–270.

Das V, Bhattacharya S, Chikkaputtaiah C, Hazra S, Pal M. The basics of epithelial-mesenchymal transition (EMT): a study from a structure, dynamics, and functional perspective. *J Cell Physiol* 2019; doi: 10.1002/jcp.28160.

Dummler B, Ohshiro K, Kumar R, Field J. Pak protein kinases and their role in cancer. *Cancer Metastasis Rev* 2009;**28**:51–63.

Gilles C, Polette M, Mestdagt M, Nawrocki-Raby B, Ruggeri P, Birembaut P, Foidart JM. Transactivation of vimentin by beta-catenin in human breast cancer cells. *Cancer Res* 2003;**63**:2658–2664.

Gradl D, Kühl M, Wedlich D. The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. *Mol Cell Biol* 1999;**19**: 5576–5587.

Jiang YG, Luo Y, He DL, Li X, Zhang LL, Peng T, Li MC, Lin YH. Role of Wnt/beta-catenin signaling pathway in epithelial-mesenchymal transition of human prostate cancer induced by hypoxia-inducible factor-1alpha. *Int J Urol* 2007;**14**:1034–1039.

Li D, Song L, Fan Y, Li X, Li Y, Chen J, Zhu F, Guo C, Shi Y, Zhang L. Down-regulation of TIPE2 mRNA expression in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Clin Immunol* 2009;**133**:422–427.

Li F, Zhu X, Yang Y, Huang L, Xu J. TIPE2 alleviates systemic lupus erythematosus through regulating macrophage polarization. *Cell Physiol Biochem* 2016a;**38**:330–339.

Li XM, Su JR, Yan SP, Cheng ZL, Yang TT, Zhu Q. A novel inflammatory regulator TIPE2 inhibits TLR4-mediated development of colon cancer via caspase-8. *Cancer Biomark* 2014;**14**:233–240.

Li Y, Li X, Liu G, Sun R, Wang L, Wang J, Wang H. Downregulated TIPE2 is associated with poor prognosis and promotes cell proliferation in non-small cell lung cancer. *Biochem Biophys Res Commun* 2015;**457**:43–49.

Li Z, Guo C, Liu X, Zhou C, Zhu F, Wang X, Wang Q, Shi Y, Wang J, Zhao W *et al*. TIPE2 suppresses angiogenesis and non-small cell lung cancer (NSCLC) invasiveness via inhibiting Rac1 activation and VEGF expression. *Oncotarget* 2016b;**7**:62224–62239.

Liu L, Luo N, Guo J, Xie Y, Chen L, Cheng Z. Berberine inhibits growth and inflammatory invasive phenotypes of ectopic stromal cells: imply the possible treatment of adenomyosis. *J Pharmacol Sci* 2018a;**137**:5–11.

Levgur M, Abadi MA, Tucker A. Adenomyosis: symptoms, histology, and pregnancy terminations. *Obstet Gynecol* 2000;**95**:688–691.

Liu W, Fan Y, Shi Y, Lin Z, Huang X, Huang W, Shen D, Qi Z. Knockdown of TIPE2 increases the proliferation in lipopolysaccharide-stimulated gastric cancer cells. *BMC Cancer* 2018b;**18**:857.

Liu ZJ, Liu HL, Zhou HC, Wang GC. TIPE2 inhibits hypoxia-induced Wnt/β-catenin pathway activation and EMT in glioma cells. *Oncol Res* 2016;**24**:255–261.

Mäder L, Blank AE, Capper D, Jansong J, Baumgarten P, Wirsik NM, Zachskorn C, Ehlers J, Seifert M, Klink B *et al*. Pericytes/vessel-associated mural cells (VAMCs) are the major source of key epithelial-mesenchymal transition (EMT) factors SLUG and TWIST in human glioma. *Oncotarget* 2018;**9**:24041–24053.

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 2008;**3**:1101–1108.

Shi Y, Li T, Zhou J, Li Y, Chen L, Shang H, Guo Y, Sun Y, Zhao J, Bao C *et al*. Herbs-partitioned moxibustion combined with acupuncture inhibits TGF-1-Smad-snail-induced intestinal epithelial mesenchymal transition in Crohn's disease model rats. *Evid Based Complement Alternat Med* 2019;**2019**:8320250.

Shan Y, Zhang L, Bao Y, Li B, He C, Gao M, Feng X, Xu W, Zhang X, Wang S. Epithelial-mesenchymal transition, a novel target of sulforaphane via COX-2/MMP2, 9/Snail, ZEB1 and miR-200c/ZEB1 pathways in human bladder cancer cells. *J Nutritional Biochem* 2013;**24**:1062–1069.

Sun H, Gong S, Carmody RJ, Hilliard A, Li L, Sun J, Kong L, Xu L, Hilliard B, Hu S *et al*. TIPE2, a negative regulator of innate and adaptive immunity that maintains immune homeostasis. *Cell* 2008;**133**:415–426.

Tan J, Yong P, Bedaiwy MA. A critical review of recent advances in the diagnosis, classification, and management of uterine adenomyosis. *Curr Opin Obstet Gynecol* 2019;**31**:212–221.

Vercellini P, Consonni D, Dridi D, Bracco B, Frattaruolo MP, Somigliana E. Uterine adenomyosis and in vitro fertilization outcome: a systematic review and meta-analysis. *Hum Reprod* 2014;**29**:964–977.

Wu J, Zhang H, Xu C, Xu H, Zhou X, Xie Y, Tao M. TIPE2 functions as a metastasis suppressor via negatively regulating β-catenin through activating GSK3β in gastric cancer. *Int J Oncol* 2016;**48**: 199–206.

Yang YM, Yang WX. Epithelial-to-mesenchymal transition in the development of endometriosis. *Oncotarget* 2017;**8**:41679–41689.

Yi KW, Kim SH, Ihm HJ, Oh YS, Chae HD, Kim CH, Kang BM. Increased expression of p21-activated kinase 4 in adenomyosis and its regulation of matrix metalloproteinase-2 and-9 in endometrial cells. *Fertile Steril* 2015;**103**:1089–1097.

Yin H, Huang X, Tao M, Hu Q, Qiu J, Chen W, Wu J, Xie Y. Adenovirus-mediated TIPE2 overexpression inhibits gastric cancer metastasis via reversal of epithelial-mesenchymal transition. *Cancer Gene Ther* 2017;**24**:180–188.

Zayratyants OV, Adamyan LV, Manukyan LM, Kalinin DV, Arslanyan KN. The expression of moesin, p21-activated kinase 4 (PAK 4), matrix metalloproteinases (MMP 2, MMP 9), and CD34 in the eutopic and ectopic endometrium in adenomyosis. *Arkh Patol* 2018; **80**:14–21.

Zhang X, Wang J, Fan C, Li H, Sun H, Gong S, Chen YH, Shi Y. Structural and m. Crystal structure of TIPE2 provides insights into immune homeostasis. *Nat Struct Mol Biol* 2009;**16**:89–90.

Zhang YH, Yan HQ, Wang F, Wang YY, Jiang YN, Wang YN, Gao FG. TIPE2 inhibits TNF-α-induced hepatocellular carcinoma cell metastasis via Erk1/2 downregulation and NF-κB activation. *Int J Oncol* 2015;**46**:254–264.

Zhang Z, Qi H, Hou S, Jin X. TIPE2 mRNA overexpression correlates with TNM staging in renal cell carcinoma tissues. *Oncol Lett* 2013;**6**:571–575.