Tumor necrosis factor receptor type 1-associated death domain (TRADD) regulates epithelial-mesenchymal transition (EMT), M1/M2 macrophage polarization and ectopic endometrial cysts formation in endometriosis

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Background: Endometriosis is a gynecological non-malignant disease that is manifested by the presence of extrauterine ectopic endometrial cells and stroma. The aim of current study was to explore the role of tumor necrosis factor receptor type 1-associated death domain (TRADD) protein in endometriosis.

Methods: Cell migration, invasion, and the expression of epithelial-mesenchymal transition (EMT) inducers in TRADD silencing or overexpression in eutopic endometrial stromal cells (EuSCs) and ectopic endometrial stromal cells (EcSCs) were analyzed by wound healing assay, transwell assay, quantitative reverse transcription polymerase chain reaction (qRT-PCR), western blotting and rat endometriosis model. A cell line derived from THP-1 macrophages was used to measure M1/M2 polarization in endometriosis by flow cytometry and enzyme-linked immunosorbent assay (ELISA).

Results: The mRNA level and protein expression of TRADD, keratin, E-cadherin, N-cadherin, vascular endothelial growth factor, matrix metalloproteinase-9, CD40, and CD206 were abnormally expressed in ectopic endometrial tissues and EcSCs. TRADD silencing promoted migration, invasion, and EMT in EuSCs, while TRADD overexpression restrained migration, invasion, and EMT in EcSCs. TRADD knockdown prohibited M1/M2 polarization in normal endometrial homogenization-treated THP-1-derived macrophages, whereas TRADD upregulation facilitated M1/M2 polarization in patients with endometrial homogenization-treated THP-1-derived macrophages. In addition, TRADD overexpression suppressed ectopic endometrial cysts formation in a rat endometriosis model. TRADD overexpression activated NF-κB and MAPK signaling in EcSCs and rat models.

Conclusions: Our results indicated that the overexpression of TRADD prohibited migration, invasion, EMT, M1/M2 polarization and ectopic endometrial cysts formation in endometriosis, and this might due to regulating NF-κB and MAPK signaling.

Keywords: Endometriosis; tumor necrosis factor receptor type 1-associated death domain (TRADD); epithelial-mesenchymal transition (EMT); M1/M2 polarization

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Introduction

Endometriosis is a gynecological non-malignant disease that affects 10–15% of women of childbearing age (1). It is manifested by the presence of extrauterine ectopic endometrial cells and stroma, which mainly occur in the peritoneal cavity (2). Endometriosis can lead to abdominal pain, dysmenorrhea, difficulty with sexual intercourse, and infertility, resulting in severe physical and psychological distress (3). Changes in many aspects of humoral and cellular immunity promote the pathogenesis of endometriosis (4-6). Pain induced by endometriosis can be treated by removing peritoneal lesions and ovarian cysts, or utilizing hormonal preparations, such as progesterone, gonadotropin-releasing hormone agonists, and oral contraceptives. However, despite these therapies, there is still a high recurrence rate (7). Therefore, more efficient treatment approaches are required.

The abnormal distribution of immune cells in the abdominal cavity of patients with endometriosis has been confirmed (8,9). Accumulating research has demonstrated that macrophages in the peritoneum show high activity in patients with endometriosis, and are thought to regulate and facilitate inflammation and disease maintenance (10). In ectopic patients with endometriosis, macrophages exhibit elevated activation and secretion, and synthesize a variety of pro-inflammatory factors, such as tumor necrosis factor (TNF)-α, interleukins (ILs), and macrophage-derived growth factors (11,12).

Macrophages are phagocytic cells in the immune system that are distributed in different tissues, and play an essential part in numerous diseases, such as inflammation and tumors (1). In addition, macrophages are highly heterogeneous and moldable cells that undergo transition from the M1 to M2 phenotype and vice versa, and are induced by specific effectors (13). Although uterine macrophages play an essential role in the function of the endometrium, the effects of human uterine macrophage activation and polarization on endometrial function maintenance and disease state development are poorly understood. Only several previously published studies have discussed the polarization of the macrophage phenotypes M1 and M2 in the human endometrium (14). A previous study indicated that human endometrial macrophages are mainly M2 macrophages (15). Only a small number of studies have evaluated the M1/M2 polarization of macrophages in patients with endometriosis (16).

Tumor necrosis factor receptor type 1-associated death domain (TRADD) protein is a TNF receptor-1 (TNFR1)-related signal transduction factor and a vital part of the TNFR1 compound. Both apoptotic and NF-κB pathways are activated by recruiting other constitutes of TNFR1 compounds to TRADD receptor (17). Studies on TRADD are generally focused on inflammatory responses and apoptosis (18,19). For instance, Ermolaeva *et al.* found that TRADD is a key part of TNFR1, toll-like receptor (TLR) 3, and TLR4 pathways and functions in TNF-induced apoptosis in a mice model (20). In the present study, we investigated the function of TRADD in endometriosis. We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-20-7866).

Methods

Clinical specimens

Clinical human normal adjacent tissues (normal group, n=20) and endometrial tissues (ectopic group, n=20) were attained from Beijing Obstetrics and Gynecology Hospital. Signed informed consent form was obtained from each patient prior to study participation. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The present study was approved by the Medical Ethics Committee of Beijing Obstetrics and Gynecology Hospital (No. SYXK (Jing) 2018-0003).

Cells and culture

Ectopic endometrial stromal cells (EcSCs) and eutopic endometrial stromal cells (EuSCs) were isolated from ectopic endometrial tissues with ovarian endometriosis and cultured in Dulbecco's modified Eagle's Media (DMEM; GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; GIBCO, USA). A human leukemia monocytic cell line THP-1 (ATCC, Manassas, VA, USA) was hatched in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, USA) with 10% fetal bovine serum (FBS) in an incubator containing 5% CO_2 at 37 ℃. To induce the differentiation of THP-1 cells into macrophages, cells were exposed to phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO, USA) for 72 h. After stimulation, non-adherent cells were discarded, and

adherent cells were used as M0 macrophages.

Cell treatment

THP-1 cell-derived macrophages were treated with eutopic or ectopic endometrial homogenate from patients with endometriosis or endometrial homogenate from normal patients for 72 h. Experimental cells were then treated by 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich, USA) for 24 h and collected.

Cell transfection

Short-hairpin RNAs (shRNAs) directed against TRADD were ligated into the U6/GFP/Neo plasmid (GenePharma, Shanghai, China) as shRNA-TRADD1, shRNA-TRADD2, and shRNA-TRADD3. The U6/GFP/Neo plasmid carrying non-targeting sequences was used as the control. The full length of the TRADD coding sequence was cloned into pc-DNA3.1 (GenePharma, China), which are referred to as pc-DNA-TRADD. The empty pc-DNA3.1 were used as the control. Transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the standard protocol. ShRNA sequences for TRADD were listed below: sh-TRADD1, 5'-GGGUC AGCCU GUAGU GAAUTT-3', sh-TRADD2, 5'-TGCAG CTGCG ATTCT GCGGGC-3', sh-TRADD3, 5'-AAGTA AAACA GGAAT CAATCTTG-3'.

Western blotting

Total protein was isolated from EcSCs and EuSCs and quantified utilizing a bicinchoninic acid kit (Thermo Scientific Pierce, Rockford, IL, USA). Extracted proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and further transferred to a Poly (vinylidene fluoride) (PVDF) membrane. Primary antibodies of keratin (ab8068), TRADD (ab110644), N-cadherin (ab76057), vascular endothelial growth factor (VEGF; ab2350), CD206 (ab64693), CD40 (ab22469), matrix metalloproteinase (MMP)-14 (ab3644), E-cadherin (ab15148), Twist1 (ab50581), Vimentin (ab137321), IKK alpha (ab32041), NF-κB p65 (ab32536), NF-κB p-p65 (S536) (ab76302), p-MAPK (T202) (ab194776), MAPK (ab264269) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab128915) were incubated with membranes at 4 ℃ overnight; all primary antibodies were purchased from Abcam (Cambridge, MA, USA). After washing, the membranes were hatched with appropriate secondary antibody at room temperature. Bands carrying protein blots were visualized using a Tanon-5200 Image Analyzer (Tanon, Shanghai, China).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from normal uterine tissues, endometrial tissues, EcSCs, and EuSCs using TRIzol reagent (Invitrogen, USA). Extracted RNA was reverse transcribed into complementary DNA using the SuperScript III First Stand Synthesis System (Invitrogen, USA). The relative amount of mRNA was evaluated utilizing the equation $2^{-\Delta\Delta CT}$ as previous reports (19). Primer sequences of indicated genes were listed below. TRADD, sense primer: 5'-ACCAG CCTGG CAGAG GACTT-3', antisense primer: 5'-AGCAA TAGCC GCAGA AGGAA CC-3'. Keratin, sense primer: 5'-TGGCA GTGGT GGTGG TGGTT-3', antisense primer: 5'-GGCTG AAGAA GGCTC TGGTTGA-3'. E-cadherin, sense primer: 5'-TGATT CTGCT GCTCT TGCTG TT-3', antisense primer: 5'-CCTCT TCTCC GCCTC CTTCT TC-3'. N-cadherin, sense primer: 5'-TCATC CTGCT TATCC TTGTG-3', antisense primer: 5'-AGTCA TAGTC CTGGT CTTCT-3'. VEGF, sense primer: 5'-CCCAG GTCAG ACGGA CAGAA AG-3', antisense primer: 5'-AGCAG GTGAG AGTAA GCGAA GG-3'. CD40, sense primer: 5'-TGCCT TCCTT GCGGT GAAAGC-3', antisense primer: 5'-ACTCG TACAG TGCCA GCCTT CT. CD206, sense primer: 5'-GGCGG TGACC TCACA AGTATCC-3', antisense primer: 5'-TCATG GCTTG GTTCT CCACG AA-3'.

Wound healing assay

EuSCs and EcSCs were seeded onto a 6-well plate. When cells reached 90% confluency, the medium was removed and the wells were scratched by pipette tip. Plates were rinsed with phosphate buffered saline (PBS) to remove loose cells. Cells were then cultured in DMEM without FBS for 24 h. Images of the cells were taken at 0 and 24 h post-incubation, and the wound closure rate was calculated as follows: (1-scratch width at 24 h/scratch width at 0 h) ×100.

Transwell assay

After transfection, cells were seeded on the upper chamber

of a 24-well plate. The lower chamber was filled with serum-free medium, and cells were incubated for 36 h. Unmigrated cells were discarded, and migrated cells were fixed and dyed with crystal violet (Beyotime Biotechnology, Shanghai, China). Stained cells were captured using an Olympus microscope (Olympus, Tokyo, Japan) at 5 random fields.

Interleukins (IL)-6 and IL-10 concentrations

Supernatants were collected, and IL-6 and IL-10 concentrations were determined utilizing an enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher Scientific, Waltham, MA, USA).

Flow cytometric assay

THP-1 cells were blocked with anti-immunoglobulin antibody, collected with trypsin, and rinsed with PBS. THP-1 cells were stained by Phycoerythrin (PE)-Cluster of Differentiation 40 (CD40) and PE-Cluster of Differentiation 206 (CD206) at 4 ℃ under dark conditions for 30 min. THP-1 cells were detected by FACSAria (BD Biosciences, San Jose, CA, USA) using FACSDiva software (BD Biosciences, USA).

Rat endometriosis model

Twenty-four eight-week-old female Sprague-Dawley (SD) rats (240–260 g) were purchased from Lingchang Biotechnology (Shanghai, China). The present study was approved by the Medical Ethics Committee of Beijing Obstetrics and Gynecology Hospital (No. SYXK (Jing) 2018-0003). All animal handling and experimental procedures were in accordance with guideline of the Animal Experimental Ethnics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University for animal care and use. The rats were anesthetized with 4% isoflurane, then made a small incision at the center of abdomen. Excised the left uterine horn and placed it into saline solution. Dissected the endometrium from muscles, then cut into pieces (5 mm in each dimension). Made a subcutaneous pocket on the abdominal wall, then placed the uterine fragment in the pocket facing the abdominal muscle. Rats were then subcutaneously injected with 30 μg/kg benzoic estradiol immediately after surgery, then lasted for ten days. After successfully establishing of the rat endometriosis model, these rats were randomly divided into two groups (Endometriosis + TRADD and Endometriosis + EV). The rats of Endometriosis + TRADD group (n=8) received intraperitoneally injection with TRADD lentivirus for three times. The rats of Endometriosis + EV (empty vector) group (n=8) received intraperitoneally injection with EV lentivirus for three times. The sham group (n=8) underwent surgery without autotransplantation of endometrial tissues. The body weight and ectopic endometrial cyst volume were monitored every five days. The volume of ectopic endometrial cyst was calculated using the formula $V = 1/2 (L \times W^2)$.

Immunohistochemistry (IHC)

The paraffin-embedded tissue samples were cut into 20 μm thick sections for IHC staining following the standard protocol. Tissue sections were deparaffinized by xylene and rehydrated by immersing in ethanol gradients (100%, 95%, 70% and 50%). Then tissues were rinsed in deionized water, and stained with hematoxylin for 5 min at room temperature.

Statistical analysis

All experiments were repeated 3 times. Results were performed as the mean ± standard deviation (sd), and evaluated utilizing GraphPad Prism 6.0 statistical software (GraphPad Software, La Jolla, CA, USA). P values were determined by analysis of variance, and $P<0.05$ was considered statistically significant.

Results

Abnormal expression of TRADD, keratin, E-cadherin, N-cadherin, VEGF, CD40, and CD206 in ectopic endometrial tissues and EcSCs

The expression of TRADD, keratin, E-cadherin, N-cadherin, VEGF, CD40, and CD206 was detected by qRT-PCR and Western blotting. As shown in *Figure 1A,B*, mRNA levels of TRADD, keratin, E-cadherin, and CD206 were significantly reduced in the ectopic and EcSCs groups ($P<0.01$ for both groups). In addition, mRNA levels of N-cadherin, VEGF, and CD40 significantly increased in both the ectopic and EcSCs groups ($P<0.01$ for both groups). The protein expression of these parameters was analyzed. Consisted with the results in *Figure 1A,B*,

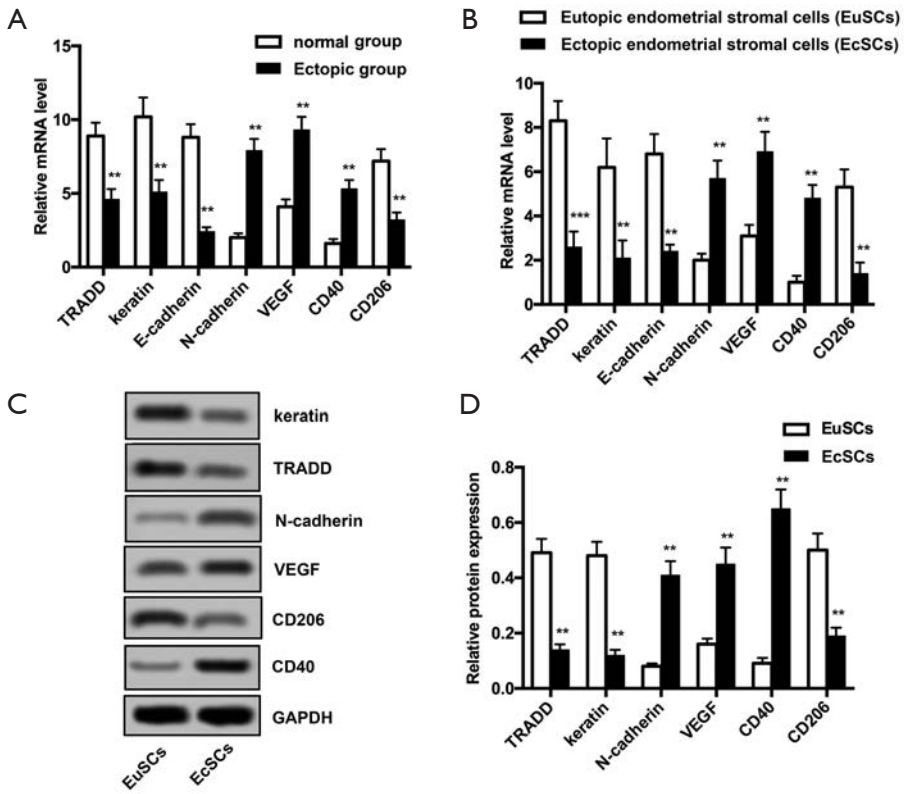

Figure 1 Tumor necrosis factor receptor type 1-associated death domain (TRADD), keratin, E-cadherin, N-cadherin, VEGF, CD40, and CD206 were abnormally expressed in ectopic endometrial tissues and EcSCs. (A,B) Experimental cells were divided into the EuSC and EcSC groups. Moreover, quantitative reverse transcription polymerase chain reaction was used to measure mRNA levels of the parameters in the normal and ectopic groups as follows; TRADD, keratin, E-cadherin, and CD206 were collectively reduced, while mRNA levels of N-cadherin and vascular endothelial growth factor (VEGF) were clearly increased in both ectopic endometrial tissues and EcSCs. (C,D) Western blotting results showed that protein expression of TRADD, keratin, and CD206 reduced, while the protein expression of N-cadherin, VEGF, and CD40 was increased in EcSCs. **P<0.05, ***P<0.001.

the protein expression of TRADD, keratin, and CD206 was significantly lower in the EcSCs group than that in the EuSCs group, but the protein expression of N-cadherin, VEGF, and CD40 was significantly increased in the EcSCs group compared with the EuSCs group (P<0.01 for both) (*Figure 1C*,*D*).

TRADD silencing in EuSCs or overexpression in EcSCs

To explore the function of TRADD in EuSCs and EcSCs, TRADD was silenced in EuSCs or overexpressed in EcSCs. qRT-PCR was performed to evaluate the efficiency of transfection in both cells. The mRNA level of TRADD1 was significantly reduced when TRADD was silenced in EuSCs (P<0.001), and the mRNA level of TRADD significantly increased with its overexpression in EcSCs (P<0.01) (*Figure 2A*). The protein expression of TRADD was detected using western blotting. TRADD downregulation could significantly decrease its protein expression in EuSCs (P<0.01), and TRADD upregulation resulted in increased expression in EcSCs (P<0.01) (*Figure 2B*).

Overexpression of TRADD prohibits invasion and migration in EcSCs

The effects of TRADD on migration and invasion were determined *in vitro*, and the results of the transwell and wound healing assays are shown in *Figure 3A*,*B*,*C*,*D*. Cell invasion was clearly increased by silencing TRADD in EuSCs, but decreased by overexpression of TRADD in EcSCs (P<0.01). Similarly, the wound closure rate was

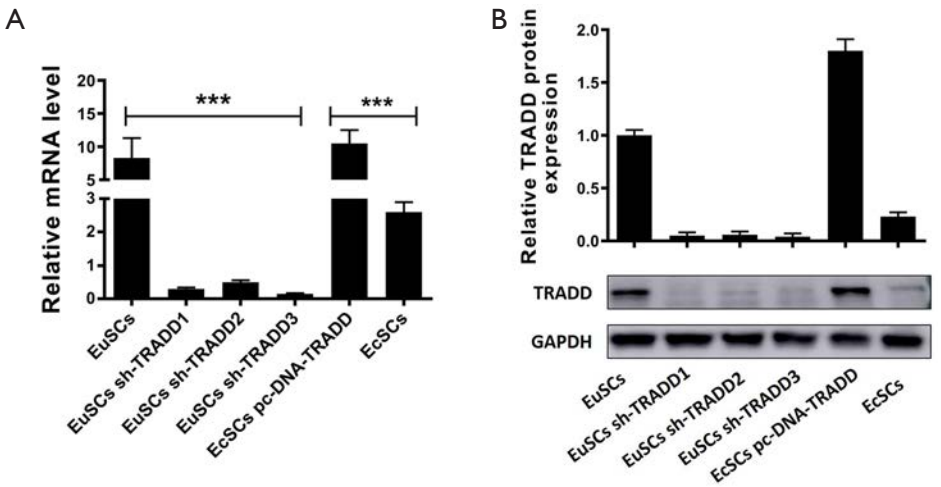

Figure 2 Tumor necrosis factor receptor type 1-associated death domain (TRADD) expression was silenced in eutopic endometrial stromal cells (EuSCs) and overexpressed in ectopic endometrial stromal cells (EcSCs). Cells were divided into the EuSCs, EcSCs, EuSCs sh-TRADD1, EuSCs sh-TRADD2, EuSCs sh-TRADD3 and EcSCs pc-DNA-TRADD group. (A) TRADD mRNA level was determined by qRT-PCR. (B) Protein expression of TRADD in each group was detected by Western blotting. Protein expression was reduced by TRADD silencing in EuSCs, and increased by TRADD overexpression in EcSCs. ***P<0.001.

significantly increased by TRADD knockdown in EuSCs, but was reduced by overexpression of TRADD in EcSCs (P<0.05). *Figure 3E,F* shows that TRADD silencing in EuSCs significantly increased the protein expression of MMP-14 and VEGF, and the high expression of TRADD in EcSCs significantly reduced these indexes (P<0.01). These data imply that TRADD silencing promotes migration and invasion in EuSCs, and TRADD overexpression reduced migration and invasion in EcSCs.

Overexpression of TRADD prohibits epithelial-mesenchymal transition (EMT) in EcSCs

EMT has been proven to be a key factor in migration and drug resistance (14). As shown in *Figure 4A*, morphological changes were observed; EuSCs were usually round; however, after transfection, most cells tended to be spindle shaped following TRADD silencing. In contrast, EcSCs were usually spindle shaped, and most cells tended to be round after TRADD overexpression. As shown in *Figure 4B,C,* TRADD3 silencing significantly increased the protein expression of N-cadherin, Twist1, and Vimentin in EuSCs (P<0.01). Conversely, TRADD3 knockdown clearly reduced E-cadherin expression in EuSCs (P<0.01). The expression of N-cadherin, Twist1, and Vimentin was significantly reduced by TRADD overexpression in EcSCs (P<0.05), and overexpression of TRADD significantly increased E-cadherin expression in EcSCs (P<0.05).

Upregulation of TRADD facilitated M1/M2 polarization in macrophages

THP-1 cell-derived macrophages were exposed to LPS for 24 h, followed by treatment with serum from patients with endometriosis or a normal endometrium for 72 days. After treatment, F4/80$^+$CD40 positive cells of normal endometrial homogenization-treated THP-1-derived macrophages were notably elevated by TRADD silencing, whereas F4/80$^+$CD40 positive cells of patients with endometrial homogenization-treated THP-1-derived macrophages were significantly reduced with TRADD overexpression (P<0.01 and P<0.001 for both). Additionally, TRADD inhibition clearly reduced the F4/80$^+$CD206 positive cells of normal endometrial homogenization-treated THP-1-derived macrophages; however, F4/80$^+$CD40 positive cells of patients with endometriosis and with endometrial homogenization-treated THP-1-derived macrophages were significantly increased with TRADD overexpression (P<0.01) (*Figure 5A,B,C*). In addition, the production of IL-6 and IL-10 was evaluated by ELISA. TRADD silencing clearly increased IL-6 production in normal endometrial homogenization-treated THP-1-derived macrophages, and

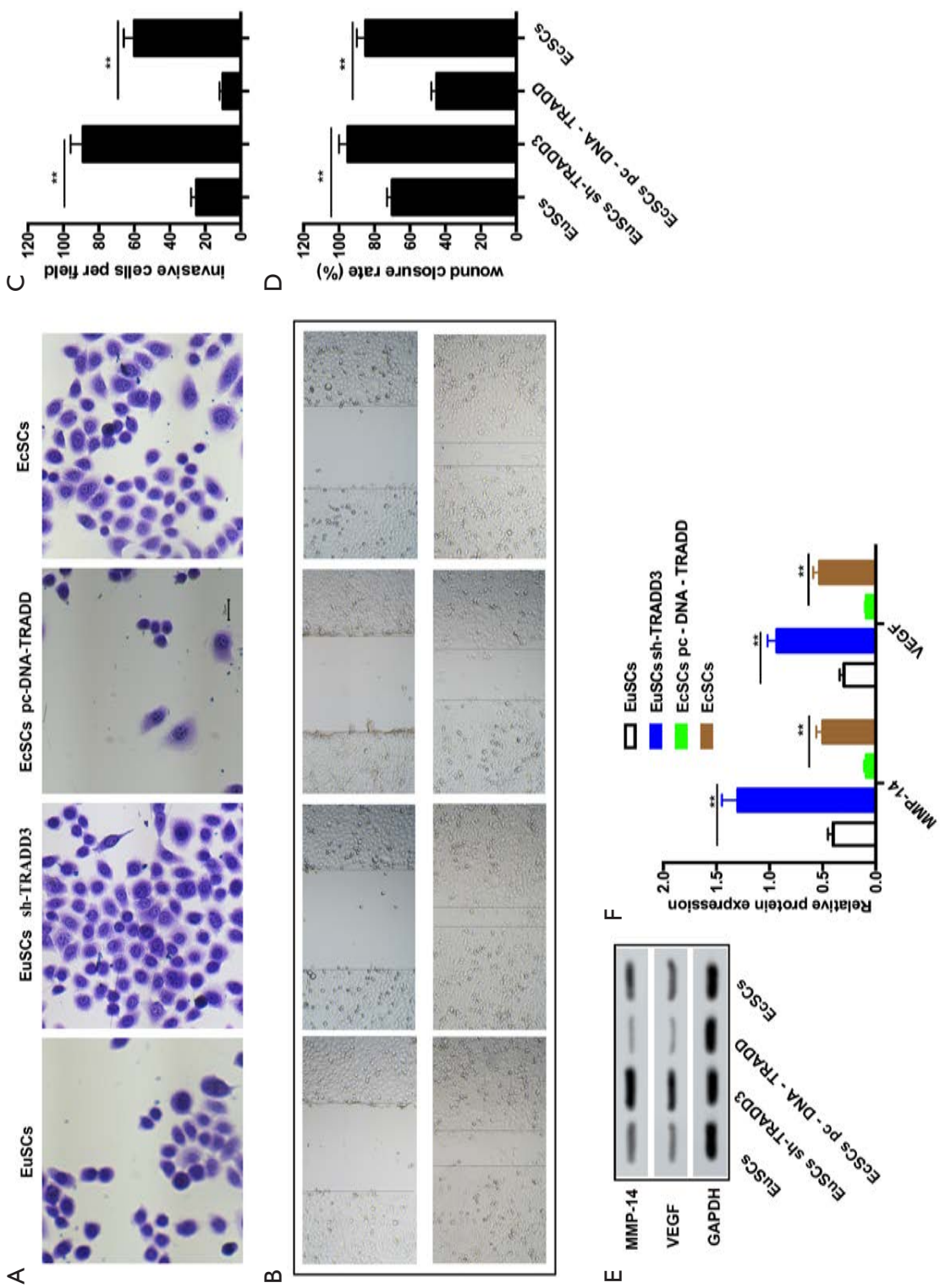

Figure 3 Tumor necrosis factor receptor type 1-associated death domain (TRADD) overexpression prohibited migration and invasion in ectopic endometrial stromal cells (EcSCs). Cells were divided into the eutopic endometrial stromal cell (EuSC) group, EcSC group, EuSC sh-TRADD3 group, and EcSC pc-DNA-TRADD group. (A,B,C,D) Transwell and wound healing assays were used to estimate migration and invasion abilities. Migrated and invasive cells were clearly increased by TRADD silencing in EuSCs and reduced by TRADD overexpression in EcSCs. Scale bar =20 μm. Cells for transwell invasion assay were stained with crystal violet. White field images for wound scratch assay were obtained by a microscope. Magnification =400× for would healing assay. (E,F) Protein expression was determined by Western blotting. Results implied that matrix metalloproteinase-14 (MMP-14) and vascular endothelial growth factor (VEGF) were elevated by TRADD silencing in EuSCs and reduced by TRADD overexpression in EcSCs. **P<0.05.

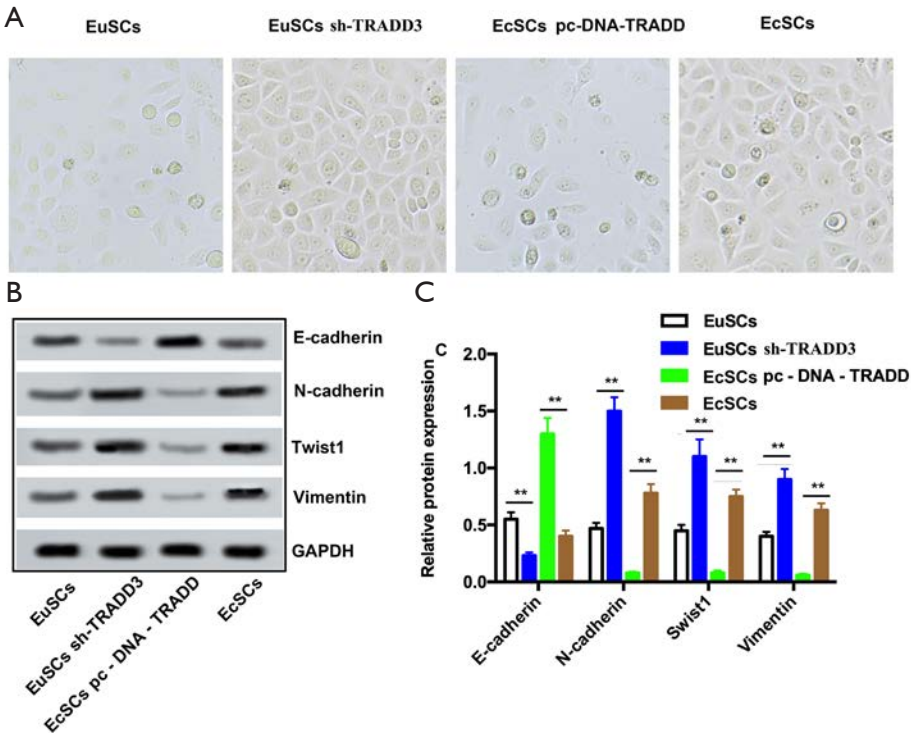

Figure 4 Tumor necrosis factor receptor type 1-associated death domain (TRADD) overexpression prohibited epithelial–mesenchymal transition (EMT) in ectopic endometrial stromal cells (EcSCs). Cells were divided into the eutopic endometrial stromal cell (EuSC) group, EuSC sh-TRADD3 group, and EcSC pc-DNA-TRADD group. (A) Morphology of EuSCs and EcSCs changed into spindle type from round. White field images were obtained by a microscope. Magnification =800×. (B,C) Western blotting was performed to evaluate the changes of protein expression. Protein expression of N-cadherin, Twist1, and Vimentin increased, whereas E-cadherin expression decreased with TRADD knockdown in EuSCs. Conversely, the protein expression of N-cadherin, Twist1 and Vimentin reduced with TRADD upregulation, and decreased with TRADD downregulation in EcSCs. **P<0.05.

TRADD overexpression significantly reduced IL-6 level in patients with endometrial homogenization-treated THP-1-derived macrophages (P<0.01 and P<0.001 for both). Conversely, IL-10 production was reduced by TRADD knockdown in normal endometrial homogenization-treated THP-1-derived macrophages, and TRADD upregulation increased IL-6 in endometrial homogenization-treated THP-1-derived macrophages (P<0.01 and P<0.001 for both) (*Figure 5D*).

TRADD overexpression suppressed ectopic endometrial cysts formation in rat model

To evaluate if TRADD was involved in the progression of endometriosis *in vivo*, a rat model of endometriosis was established. As shown in *Figure 6A*, there was no significant difference of body weight between Sham, Endometriosis + EV or Endometriosis + TRADD group post-surgery. TRADD overexpression was realized by injection of TRADD expression lentivirus. IHC staining revealed that TRADD overexpression dramatically decreased ectopic endometrium formation compared with empty lentivirus control (EV) group (*Figure 6B*). The ectopic endometrium cyst volume of Endometriosis + TRADD group was also reduced compared with Endometriosis + EV group (*Figure 6C*). The above results indicated that TRADD overexpression suppressed ectopic endometrial cysts formation *in vivo*.

TRADD overexpression activated NF-κB and MAPK signaling in EcSCs and rat models

To explore the underling molecular mechanism of TRADD in endometriosis, activation of downstream pathways was

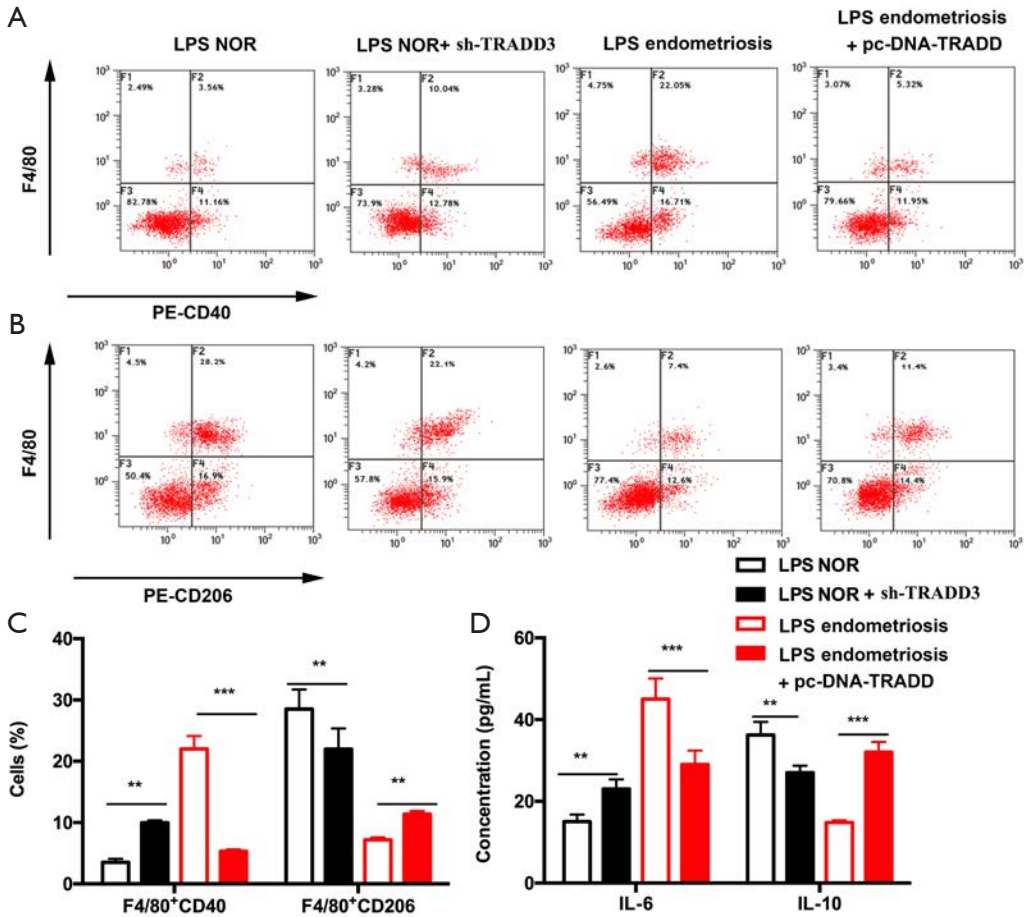

Figure 5 Tumor necrosis factor receptor type 1-associated death domain (TRADD) upregulated M1/M2 polarization into macrophages. Cells were treated with lipopolysaccharide (LPS) for 24 h and divided into the LPS normal group (LPS NOR), LPS normal (LPS NOR) + sh-TRADD3 group, LPS endometriosis group, and LPS endometriosis + pc-DNA-TRADD group. (A,B,C) Flow cytometric assay was performed to detect M1/M2 polarization in experimental cells. F4/80$^+$CD40 positive cells of normal endometrial homogenization-treated THP-1-derived macrophages increased with TRADD knockdown, and F4/80$^+$CD40 positive cells from patients with endometrial homogenization-treated THP-1-derived macrophages were reduced with TRADD upregulation of. Conversely, F4/80$^+$CD206 positive cells of normal endometrial homogenization-treated THP-1-derived macrophages were reduced by TRADD inhibition, and F4/80$^+$CD206 positive cells from patients with endometrial homogenization-treated THP-1-derived macrophages partially increased with TRADD overexpression. (D) Enzyme-linked immunosorbent assay was used to measure the production of inflammatory cytokines. IL-6 was increased with TRADD silencing in normal endometrial homogenization-treated THP-1-derived macrophages, and reduced with TRADD upregulation from patients with endometrial homogenization-treated THP-1-derived macrophages. Moreover, IL-10 production reduced by TRADD knockdown in normal endometrial homogenization-treated THP-1-derived macrophages, and increased with TRADD overexpression from patients with endometrial homogenization-treated THP-1-derived macrophages. **P<0.05, ***P<0.001.

evaluated by western blot. We were surprised to find that TRADD silencing reduced NF-κB and MAPK activation in EuSCs, and overexpression of TRADD increased NF-κB and MAPK activation in EcSCs (*Figure 7A,B*). TRADD silencing increased the expression of IκBα and reduced the phosphorylation of p-p65 and p-MAPK, while TRADD overexpression showed contrary effects (*Figure 7A,B*). In the rat model of endometriosis, TRADD overexpression significantly suppressed the expression of IκBα and upregulated the phosphorylation of p-p65 and p-MAPK, indicating that TRADD overexpression activated NF-κB and MAPK signaling *in vivo* (*Figure 7C,D*). Taken togther,

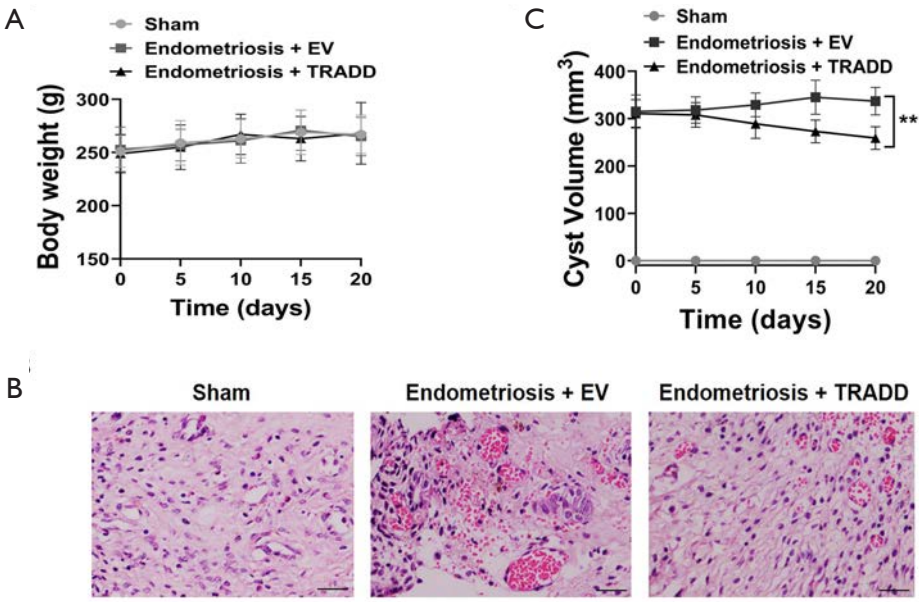

Figure 6 Tumor necrosis factor receptor type 1-associated death domain (TRADD) overexpression suppressed ectopic endometrial cysts formation in rat model. (A) The body weight of rats in each group. (B) Hematoxylin staining of the endometrial tissues from each group. Scale bar =50 μm. (C) Ectopic endometrial cyst volume of each group. **P<0.05.

TRADD regulated NF-κB and MAPK activation in EcSCs and rat models.

Discussion

Endometriosis is a chronic inflammatory disease that is associated with the production of cytokines and differential expression of immune inflammatory genes in the symptomatic peritoneal and ectopic endometrium (21,22). Endometriosis is not a cancer; however, is has several cancer-like characteristics, such as proliferation, migration, invasion, and anti-apoptosis (23). In the present study, we investigated the potential role of TRADD in the invasion, migration, EMT, M1/M2 polarization of THP-1-derived macrophages and a rat model of endimetriosis.

Migration and invasion play an essential role in the development of endometriotic cysts. Endometrial tissues are found to invade host tissues (24). According to Harada *et al.*, VEGF promotes angiogenesis in endometrial implants (25). MMP expression in the ectopic endometrium from patients with endometriosis is different from that in normal patients. The protein expression of MMP-9 of ectopic endometrium from patients with endometriosis is notably higher than that of normal patients (26). It has been reported that MMP-9 is involved in invasion, and MMP-9 and VEGF are downstream molecules of the Wnt/β-catenin pathway (27-29). Therefore, the protein expression of MMP-14 and VEGF was measured. In the present study, the expression of MMP-14 and VEGF was promoted by TRADD silencing in EuSCs, while the expression of MMP-14 and VEGF was prohibited by TRADD overexpression in EcSCs, implying that TRADD can modulate migration and invasion *in vitro*.

Although endometriosis is not a malignant disease, its biologic behaviors, such as invasiveness, are similar to those of malignant tumors (30). Increasing research has indicated that invasiveness is related to the downregulation of E-cadherin, that is, epithelial cell phenotype damage is the result of the EMT cellular process (31). EMT is a complicated cellular process that transforms immobile epithelial cells into movable mesenchymal cells during embryonic growth, cancer, and physical responses to injury (32). Accumulating evidence has indicated that EMT may be associated with the migration and invasion in endometrial epithelial cells, and the process plays an essential part in the formation of endometriosis (33). EMT is characterized by reduced E-cadherin expression and elevated expression of mesenchymal markers, such as fibronectin and Vimentin (34). Additionally, existing research has revealed the key role of EMT in the

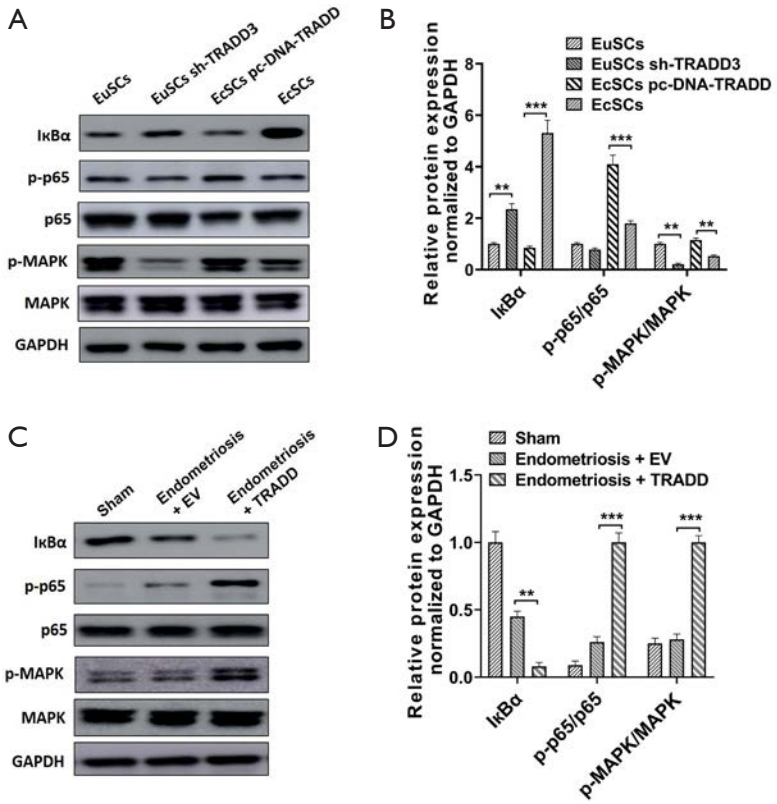

Figure 7 Tumor necrosis factor receptor type 1-associated death domain (TRADD) overexpression activated NF-κB and MAPK signaling in EcSCs and rat models. (A,B) EuSCs were transduced with sh-TRADD3, and EcSCs were transduced with pc-DNA-TRADD, then collected cell lysates for western blot (A). Relative protein expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was shown (B). (C,D) Cell lysates from ectopic endometrial cyst or endometriosis tissues were used for western blot (C). Relative protein expression normalized to GAPDH was shown (D). **P<0.05, ***P<0.001.

occurrence and development of endometriosis (35). In the present study, we analyzed the expression of E-cadherin, N-cadherin, Twist1, and Vimentin. The data showed that that TRADD upregulation reduced migration, invasion, and EMT in EcSCs, whereas TRADD silencing had the opposite effect in EuSCs. These findings indicate that TRADD functions as an anti-EMT effector in endometriosis.

Previously published studies have implied that the disruption of the homeostasis of M1and M2 phenotypes may be involved in the pathogenesis of endometriosis (21,22). According to Bacci *et al.*, endogenous macrophages are involved in tissue remodeling during the formation of endometriosis, and M1/M2 polarization is needed for the development of ectopic lesions (36). The M1/M2 polarization of macrophages is implied in the susceptibility and resistance to endometriosis; however, this is still controversial (1). A previous study found that, compared with the control group, the number of macrophages in the abdominal cavity of patients with endometriosis was remarkably promoted, but the proportion of CD163+ M2 cells was consistent (37). Bacci *et al.* found that peritoneal macrophages from patients with endometriosis and normal patients also express CD86 receptors (36). In the present study, we found that TRADD silencing increased the percentage of CD40 macrophages after eutopic homogenates under LPS exposure, while TRADD overexpression decreased the percentage of CD40 macrophages after ectopic endometrial homogenates under LPS exposure. Meanwhile, TRADD demonstrated opposite effects on the percentage of CD206 macrophages after the same treatments. Therefore, M1/M2 polarization was facilitated by TRADD in THP-1-derived macrophages, implying that TRADD silencing can prohibit the

development of endometriosis to a certain degree. This was also supported by the production of IL-6 and IL-10.

In summary, we explored the effect of TRADD on migration, invasion, and EMT in endometriosis. The results implied that TRADD overexpression exerted inhibitory functions on migration, invasion and EMT in EuSCs and EcSCs. TRADD overexpression prohibited migration, invasion in EcSCs. In addition, TRADD overexpression promotes M1/M2 polarization in THP1 derived macrophages. In a rat endometriosis model, TRADD suppresses ectopic endometrial cysts formation. These effects may due to the activation of NF-κB and MAPK signaling by TRADD in EcSCs and rat models.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Both human and animal experiments were approved by the Medical Ethics Committee of Beijing Obstetrics and Gynecology Hospital (No. SYXK (Jing) 2018-0003). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Signed informed consent form was obtained from each patient prior to study participation. All animal handling and experimental procedures were in accordance with guideline of the Animal Experimental Ethnics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University for animal care and use.

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