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ORIGINAL ARTICLE ***Reproductive biology***

T-cadherin inhibits invasion and migration of endometrial stromal cells in endometriosis

Qinsheng Lu[1,†], Yanqing Huang[2,†], Jiabao Wu[3], Yutao Guan[4], Miaomiao Du[2], Fenghua Wang[5], Zhihong Liu[2], Yali Zhu[2], Guifang Gong[2], Huomei Hou[1], Min Zhang[1], Joy Yue Zhang[1], Fen Ning[1], Lixin Chen[6,*], Liwei Wang[6,*], and Gendie E. Lash[1,*]

[1]Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, 510623, Guangdong, P.R. China [2]Department of Obstetrics and Gynecology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou 510623, P.R. China [3]NHC Key Laboratory of Male Reproduction and Genetics, Family Planning Research Institute of Guangdong Province, Guangzhou, Guangdong 510600, P.R. China [4]Department of Obstetrics and Gynecology, the First People's Hospital of Foshan, Foshan, Guangdong 528000, P.R. China [5]Department of Pathology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong 510623, P.R. China [6]Department of Physiology, Medical College, Jinan University, Guangzhou, Guangdong 510632, P.R. China

*Correspondence address. Gendie E. Lash, Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong 510623, P.R. China. Tel.: +86-(0)20-38367682; E-mail: gendie.lash@hotmail.com (G.E.L.) or Liwei Wang and Lixin Chen, Medical College, Jinan University, Guangzhou 510632, P.R. China. Tel.: +86-(0)20-85228865; E-mail: twangliwei@jnu.edu.cn (L.W.), tchenlixin@jnu.edu.cn (L.C.)

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STUDY QUESTION: What is the expression level of T-cadherin in endometriosis, and does T-cadherin play a role in regulating invasion and migration of endometrial stromal cells?

SUMMARY ANSWER: T-cadherin expression was reduced in ectopic endometriotic lesions compared to eutopic endometrium, and T-cadherin overexpression inhibited the invasion and migration of endometrial stromal cells.

WHAT IS KNOWN ALREADY: Endometriosis is a disease that involves active cell invasion and migration. T-cadherin can inhibit cell invasion, migration and proliferation in various cancer cells, but its role in endometriosis has not been investigated.

STUDY DESIGN, SIZE, DURATION: We explored the expression status of T-cadherin in 40 patients with and 24 without endometriosis. We also isolated endometrial stromal cells to study the invasion, migration and signaling pathway regulation of T-cadherin overexpression.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Patients were recruited at the Guangzhou Women and Children's Medical Center to study the expression levels of T-cadherin. The expression of T-cadherin was detected by immunohistochemistry staining and western blot. H-score was used to evaluate the staining intensity of T-cadherin. The correlation between T-cadherin expression levels (H-score) and endometriosis patients' age, stage, lesion size and adhesion was analyzed. Endometrial stromal cells from patients with and without endometriosis were isolated, and cell invasion and migration were detected by transwell assays after T-cadherin overexpression. The expression of vimentin in T-cadherin-overexpressed cells was detected by western blot. After T-cadherin overexpression, the phosphorylation profile of signaling pathway proteins was detected with the Proteome Profiler Human Phospho-Kinase Array Kit.

MAIN RESULTS AND THE ROLE OF CHANCE: There was no difference in the expression of T-cadherin in the normal endometrium of control patients and the eutopic endometrium of endometriotic patients, but it was significantly decreased in the ectopic endometrium of endometriotic patients, compared with control endometrium and eutopic endometrium of endometriosis patients ($P < 0.0001$, for both). Western blot analysis also showed that the expression of T-cadherin was decreased in ectopic endometriotic lesions, but not the normal control endometrium or the endometriotic eutopic endometrium. The results of transwell assays indicated that T-cadherin overexpression inhibited the invasion and migration of endometrial stromal cells. In addition, T-cadherin overexpression promoted the phosphorylation of HSP27 (S78/S82) and JNK 1/2/3 (T183/Y185, T221/Y223) and decreased the expression of vimentin, MMP2 and MMP9 in eutopic endometriosis stromal cells.

LARGE-SCALE DATA: N/A.

[†]These authors contributed equally to this work.

LIMITATIONS, REASONS FOR CAUTION: The control group were patients with benign gynecological conditions (e.g. uterus myoma, endometrial or cervical polyp), which may have genetic or epigenetic variations associated with T-cadherin expression and signaling pathways. The case numbers of involved endometriosis and control patients were limited. This study only used endometrial stromal cells from patients with or without endometriosis. Ideally, ectopic endometrial stromal cells of the ovarian endometriotic lesions should also be utilized to explore the function of T-cadherin.

WIDER IMPLICATIONS OF THE FINDINGS: Further investigation of the role of T-cadherin in endometriosis may generate new potential therapeutic targets for this complex disorder.

STUDY FUNDING AND COMPETING INTEREST(S): This study was supported by the Natural Science Foundation of Guangdong Province (2016A030313495), National Natural Science Foundation of China (81702567, 81671406, 31871412), the Science and Technology Programs of Guangdong (2017A050501021), Medical Science Technology Research Fund of Guangdong Province (A2018075), the Science and Technology Programs of Guangzhou City (201704030103), Internal Project of Family Planning Research Institute of Guangdong Province (S2018004), Post-doc initiation fund of Guangzhou (3302) and Post-doc science research initiation fund of Guangzhou Women and Children's Medical Center (20160322). There are no conflicts of interest.

Key words: T-cadherin / endometriosis / invasion / migration / signaling transduction / phosphorylation

Introduction

Endometriosis is a benign gynecological disease defined as the presence of endometrial glands and stroma outside the uterus (Vercellini *et al.*, 2014). It can be classified as superficial or peritoneal, ovarian and deep-infiltrating endometriosis (Vercellini *et al.*, 2014). As an estrogen-dependent chronic inflammatory condition, endometriosis affects 5–10% of women in their reproductive years, with a peak between 25 and 35 years of age (Vercellini *et al.*, 2014). There are no valid biomarkers for endometriosis (Coutinho *et al.*, 2019). The noninvasive diagnosis of endometriosis may use imaging methods, such as transvaginal ultrasound and magnetic resonance imaging (Peiris *et al.*, 2018). Current treatment options for endometriosis include surgical removal of the endometriotic lesions and pharmacotherapy, using combined hormonal contraceptives and progestins, gonadotropin-releasing hormone (GnRH) antagonists or aromatase inhibitors (AIs) (Barra *et al.*, 2019). After surgery, hormonal contraceptive therapies are often adopted to relieve endometriosis related pain and decrease the probability of endometriosis recurrence (Grandi *et al.*, 2019), due to the high rate of disease recurrence (40 and 50% in 5 years) (Vercellini *et al.*, 2014). However, hormonal contraceptive therapies may inhibit ovulation and are therefore not suitable for patients desiring to become pregnant.

There are three proposed pathogenic theories of endometriosis, of which the retrograde menstruation theory is the most convincing, shredded endometrial fragments implant and grow in extrauterine sites e.g. the peritoneum and abdominal organs, especially the ovaries (Vercellini *et al.*, 2014). Cell migration and invasion are important in the formation of endometriotic cysts, but the molecular mechanism is not yet clear. Therefore, we need to further explore the pathogenesis of endometriosis to develop more targeted therapies.

T-cadherin (T-cad), also named H-cadherin, is coded by the *CDH13* gene. T-cadherin is a member of the cadherin family, which also includes E-cadherin, N-cadherin and other important cell surface glycoprotein adhesion molecules. Their functions mainly include regulating calcium-mediated cell adhesion and intercellular junctions, cell polarity and morphogenesis, and participating in cell recognition and signal transduction (Moelans *et al.*, 2011). The T-cadherin gene is on the long arm of human chromosome 16q24, which is a region where allele loss often occurs in many tumors. T-cadherin has no histidine, alanine and valine tripeptide sequence at its amino terminal; its repeat sequence constitutes an extracellular domain like the classical cadherin molecular structure. However, T-cadherin lacks the transmembrane and cytoplasmic regions of the classical cadherin molecules and is attached to the cell membrane by a glycosyl phosphatidylinositol anchor (Ranscht and Dours-Zimmermann, 1991). Recent studies have found that the expression of T-cadherin is decreased in many tumors, such as gastric cancer (Lin *et al.*, 2017), bladder cancer (Chen *et al.*, 2016), lymphoma (Alkebsi *et al.*, 2016) and prostate cancer (Maslova *et al.*, 2015). It is speculated that T-cadherin may be a tumor suppressor and play important roles in tumorigenesis and cancer development. The downregulation of T-cadherin is related to the invasion and metastasis of tumors (Lin *et al.*, 2013; Maslova *et al.*, 2015), while overexpression of T-cadherin can inhibit tumor cell invasion and migration (Lin *et al.*, 2017). Endometriosis is a disease with active cell migration and invasion. Though T-cadherin plays roles in the regulation of cell migration and invasion in different types of cancers, no study has been performed to explore the relationship between T-cadherin and endometriosis.

In the current study, we investigated the expression of T-cadherin in eutopic and ectopic endometria of patients with endometriosis, and the endometrium of normal patients without endometriosis by western blot and immunohistochemistry. We also studied the role(s) of T-cadherin on migration and invasion of isolated endometrial stromal cells from normal and endometriotic patients, and potential downstream signaling pathways.

Materials and Methods

Ethical approval

This study was approved by the Research Ethics Committee of the Guangzhou Women and Children's Medical Center (No. 2015090106), and written informed consent was obtained from each subject prior to tissue sample collection.

Patients and tissue sample collection

In the current study, the guidelines for subject involvement were according to our previous study (Guan *et al.*, 2016). All involved

Table I Clinical characteristics of patients with and without endometriosis.

		Controls	Endometriosis
Cases		24	40
Age, years	Mean (range)	35.7 (27–49)	35.8 (23–54)
	20–29	3	8
	30–39	12	17
	40–49	8	14
	50–59	0	1
Stage			
	I	/	9
	II	/	3
	III	/	9
	IV	/	19
Diameter, cm			
	<5	/	20
	≥5	/	20
Adhesion			
	None	/	6
	Mild	/	12
	Moderate	/	1
	Severe	/	21
Classification			
	Ovarian endometriosis	/	27
	Pelvic endometriosis	/	3
	Mesenteric endometriosis	/	3
	Fallopian tube endometriosis	/	1
	Abdominal wall endometriosis	/	1
	Pelvic endometriosis with adenomyosis	/	3
	Pelvic endometriosis with adenomyoma	/	2

Data are number of instances unless stated otherwise

subjects had not received any hormonal treatment and did not use an intrauterine contraceptive device in the last 3 months prior to examination and sampling. The 40 endometriosis cases included ovarian endometriosis and pelvic endometriosis, with or without adenomyosis or adenomyoma. Eutopic endometrium and ectopic endometrial lesions were collected from endometriosis patients. The 24 control patients without endometriosis had uterine myoma, and endometrial polyp or cervical polyp provided eutopic endometrium. Patient details are shown in Table I and Supplementary Table SI. The presence or absence of endometriosis was confirmed by laparoscopic surgery and post-operative histological examination. Samples were obtained during the proliferative phase of the menstrual cycle, which was determined by preoperative history and histological examination. For all subjects, some of the collected tissue was fixed in 4% w/v paraformaldehyde and paraffin-embedded, for pathological diagnosis and immunohistochemistry (IHC) assessment, and some was snap-frozen in liquid nitrogen and stored at −80°C until use, for tissue protein extraction and western blot. For some patients, further tissue was used for isolation of endometrial stromal cells.

Cell isolation and culture

Endometrial samples from endometriosis and non-endometriosis patients were collected under sterile conditions and kept in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, USA) medium supplemented with 1% v/v penicillin–streptomycin (100 units/mL and 100 μg/mL, respectively) (Cat#15140122, Gibco, USA) and 10% v/v fetal bovine serum (FBS, Cat#SFBS, Bovogen, VIC, Australia) on ice. The tissue was processed within 6 h of collection. Endometrial stromal cells were isolated according to Guan *et al*. (2016) with a few modifications. The tissues were rinsed with PBS and minced into small pieces. The tissue was digested with collagenase I (1 mg/mL, Cat #C2674, Sigma-Aldrich, St. Louis, MO, USA) and DNase I for 30 min (1 mg/mL, Gibco) at room temperature with constant agitation. After filtering through a sterile 100-mesh (150 μm) gauze, the remaining larger pieces were subjected to a second round of digestion. The filtrate from the 100-mesh (150 μm) gauze was then filtered through a 40-μm gauze, epithelial cells were left in the sieve and the filtrate contained stromal cells and a few epithelial cells. The sieve was washed with PBS several times and then turned upside down to collect the epithelial cells. The glands and filtrate were centrifuged at 200*g* for 5 min, the supernatant discarded and the cells were resuspended and cultured in DMEM/F-12 complete medium. After 20 min of culturing and attachment, the cells remaining in the culture medium (most are stromal cells) were transferred to a new flask to reduce glandular cell contamination. When cells reached about 90% confluence, cells were trypsinized (0.5× Trypsin/EDTA, 5 min, 37°C) and floating glandular cells discarded, and the remaining cells were predominantly stromal cells. Cell purity was confirmed by flow cytometry stained with EpCAM (mouse monoclonal, conjugated with Phycoerythrin (PE), Cat#ab112068, abcam, Cambridge, MA, USA) and CD13 (mouse monoclonal, conjugated with allophycocyanin (APC), Cat#557454, BD Biosciences, San Jose, CA, USA) antibodies. Cellular phenotype was also confirmed by immunocytochemistry staining with anti-vimentin (Cat#10366-1-AP) and anti-cytokeratin 18 (Cat#66187-1-Ig) antibodies (both from ProteinTech, Wuhan, Hubei, China). The purity of isolated stromal cells was more than 95%.

Western blotting

Cells were lysed in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% w/v N-P40, 0.1% w/v SDS, 0.5% w/v sodium deoxycholate and protease inhibitor) and incubated for 30 min at 4°C. After centrifugation at 18 800*g* for 30 min at 4°C, lysate supernatants were immediately shock-frozen and stored at −80°C. Frozen tissues were ground into powder with a mortar and pestle and liquid nitrogen, lysed in RIPA buffer and processed as above for cells. Lysates (40 μg protein per sample) were separated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with WB blocking solution (Cat#P0023B, Beyotime, Shanghai, China) for 1 h at room temperature. The following primary antibodies were used anti-T-cadherin (1 in 1000 dilution; Abgent, San Diego, CA), anti-MMP2 (Cat#AM1844b, mouse, 1:500,

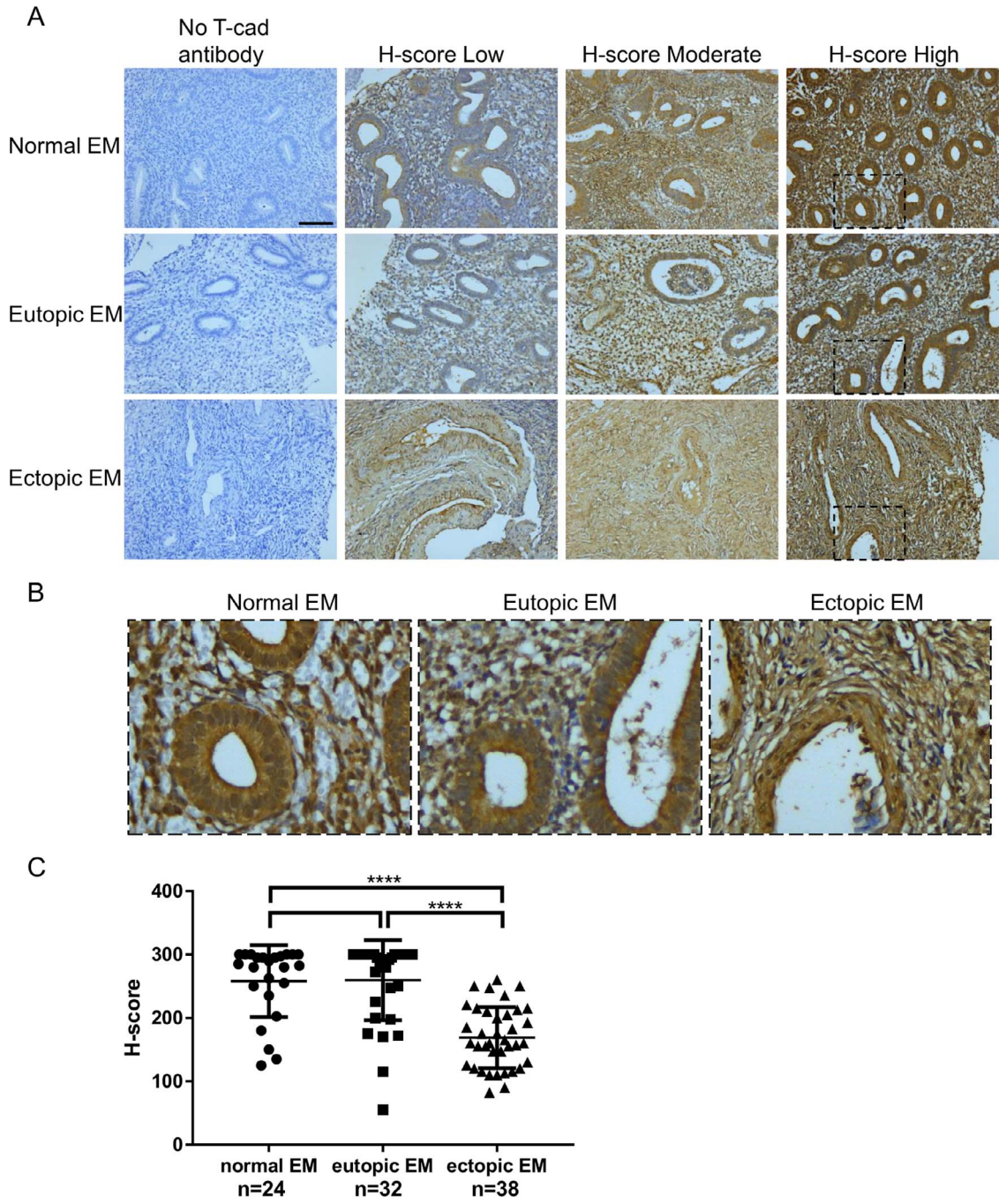

Figure 1 The expression of T-cadherin was decreased in endometriotic lesions. The expression of T-cadherin was detected by immunohistochemistry (IHC) in normal control endometrium and endometriotic endometrial lesions. (**A**) Representative IHC images, from left to right; negative control staining without T-cadherin antibody, low expression, moderate expression and high expression of T-cadherin, respectively. The original magnification was 200×, and the scale bar represented 100 μm. The dotted boxes in the right panel were the regions zoomed in **B**. (**B**) The regions within dotted boxes enlarged 4-fold. (**C**) Dot plot of the *H*-score. Bars show mean ± SD. Differences were analyzed by Student's *t* test. $^{****}P < 0.0001$. T-cad: T-cadherin, normal EM: normal control endometrium, eutopic EM: eutopic endometriotic endometrium, ectopic EM: ectopic endometriotic lesion.

Abgent), anti-MMP9 (Cat#PA5-16851, rabbit, 1:1000, Thermo Fisher Scientific, USA), anti-vimentin (Cat#3932S, rabbit, 1:2000, Cell Signal Technology, USA), anti-HSP27 (Cat#66767-1-Ig, mouse, 1:1000, ProteinTech), anti-pHSP27 (Cat#AF2197, rabbit, 1:1000, Beyotime), anti-JNK (Cat#9252S, rabbit, 1:1000, Cell Signaling Technology, USA), anti-pJNK (Thr183/Tyr185) (Cat#4668S, rabbit, 1:1000, Cell Signaling

Table II Correlation analyses between H-score of T-cadherin in ectopic endometriosis lesions and clinical parameters.

	Spearman's rho	*P* value
Age, years	0.065	0.697
Stage	0.076	0.652
Diameter, cm	0.077	0.647
Adhesion	0.038	0.821

Technology) and anti-GAPDH (1 in 10 000 dilution; Abgent), incubated overnight at 4°C. After three washes with TBST (TBS with 0.1% v/v Tween 20), membranes were incubated with HRP-coupled secondary anti-mouse (Cat#HAF007, R&D Systems, Minneapolis, MN, USA) or anti-rabbit (Cat#HAF008, R&D Systems) IgG in TBST for 1 h at room temperature, as appropriate. Immunoreactive proteins were visualized by Immobilon Western Chemiluminescent HRP Substrate (Cat#WBKLS0500, Merck Millipore, Billerica, MA, USA), and images were captured by Bio-Rad Molecular Imager ChemiDoc XRS+ Imaging System and relative densitometry analysis performed.

IHC

IHC was performed on 4-µm sections from formalin-fixed paraffin-embedded endometriosis and control endometrium tissue samples. Following de-paraffinization and antigen retrieval using 10 mM sodium citrate pH 6.0 by a pressure cooker for 1 min, slides were blocked for endogenous peroxidase by 3% (v/v) H_2O_2 in methanol for 15 min at room temperature, slides were then incubated with non-specific antigen in antibody diluent (Cat#GT100910, GeneTech, Shanghai, China) for 1 h at room temperature and slides were then incubated with anti-T-cadherin antibody (1:200, Abgent, Cat#PRS3583, rabbit, Sigma-Aldrich, MO, USA) overnight at 4°C. A universal HRP-labeled anti mouse/rabbit secondary antibody (Dako REAL EnVision HRP Rabbit/mouse, Cat#K5007, Dako, Denmark) was incubated 1 h at room temperature. The slides were visualized by 3,3′-diaminobenzidine (DAB; Cat#K5007, Dako, Denmark) staining followed by counter-staining with hematoxylin. Negative controls were performed without primary antibody incubation. Images were captured by a Leica DM4 B microscope. *H*-score was performed to compare the expression of T-cadherin in IHC staining based on the percentage of cells stained at each intensity level, ranging from 0 for negative staining to 3 for the most intense staining using the following formula: *H*-score = % cells with intensity level 1 + (% cells with intensity level 2 ∗ 2) + (% cells with intensity level 3 ∗ 3). Data are shown as mean ± SD.

Transfection experiments

Primary endometrial stromal cells of control and endometriosis patients were transfected with full-length T-cadherin expression vector pCMV3-CDH13 (Cat#HG11249-UT G11MA08M001, Sino Biological, Beijing, China) and the empty control vector pCMV3-untagged-NCV (Cat# CV011 G10JL15M007, Sino Biological, Beijing, China), using Qiagen transfection reagent (Cat#301427, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The sequence accuracy of pCMV-T-cad was confirmed by the manufacturer. The transfection was performed in the presence of serum, but not penicillin–streptomycin. Six hours post-transfection, the transfection medium was changed to complete culture medium.

Measurement of migration and invasion

Twenty-four-well 8-µm transwell chambers (BD BioCoat Matrigel invasion chamber, Becton Dickinson, NJ, USA) were used for migration and invasion assays. Twenty-four hours post-transfection, cells were digested to a single-cell suspension, washed in PBS and resuspended in DMEM/F12 without FBS. For the migration assay, 2×10^5 cells in 200 µL DMEM/F12 without FBS were seeded in the upper chamber of transwells. The lower chamber was filled with 500 µL DMEM/F12 containing 10% v/v FBS. Cells were cultured in standard conditions, and after 24 h, cells on the upper surface of the filter were removed by wiping with a wet cotton swab and washed several times in PBS. The migrated cells on the lower surface of the upper chamber were fixed in methanol for 10 min, dried at room temperature, stained with 0.1% w/v crystal violet (Cat#G1062, Solarbio, Beijing, China) for 30 min and washed with tap water. The migrated cells were visualized and photographed under a Leica DMi8 microscope. Experiments were

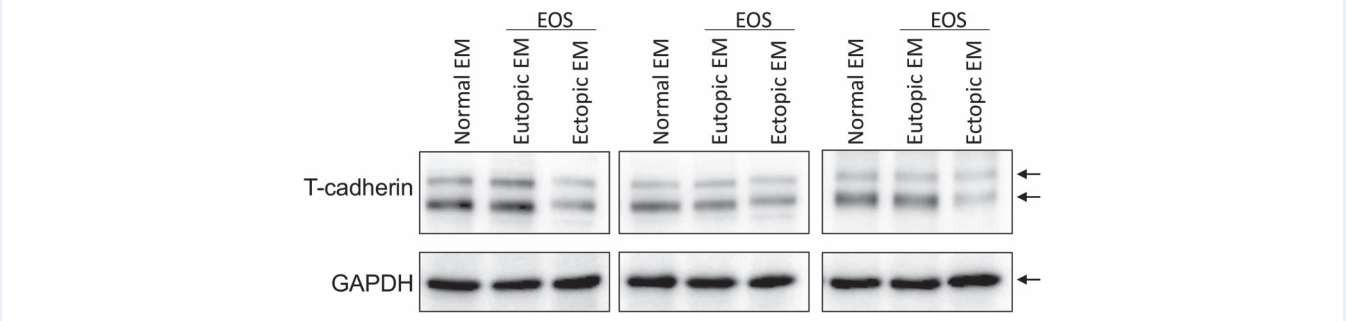

Figure 2 T-cadherin protein expression levels in endometrial tissues with and without endometriosis. Western blot was used to detect the T-cadherin protein expression levels in endometrial tissues with or without endometriosis. Representative results of three normal control cases without endometriosis and three cases with endometriosis were shown. Arrows indicate the specific bands on the blot for each antibody. GAPDH was the internal loading control. EOS patient with endometriosis, normal endometrium was from control women.

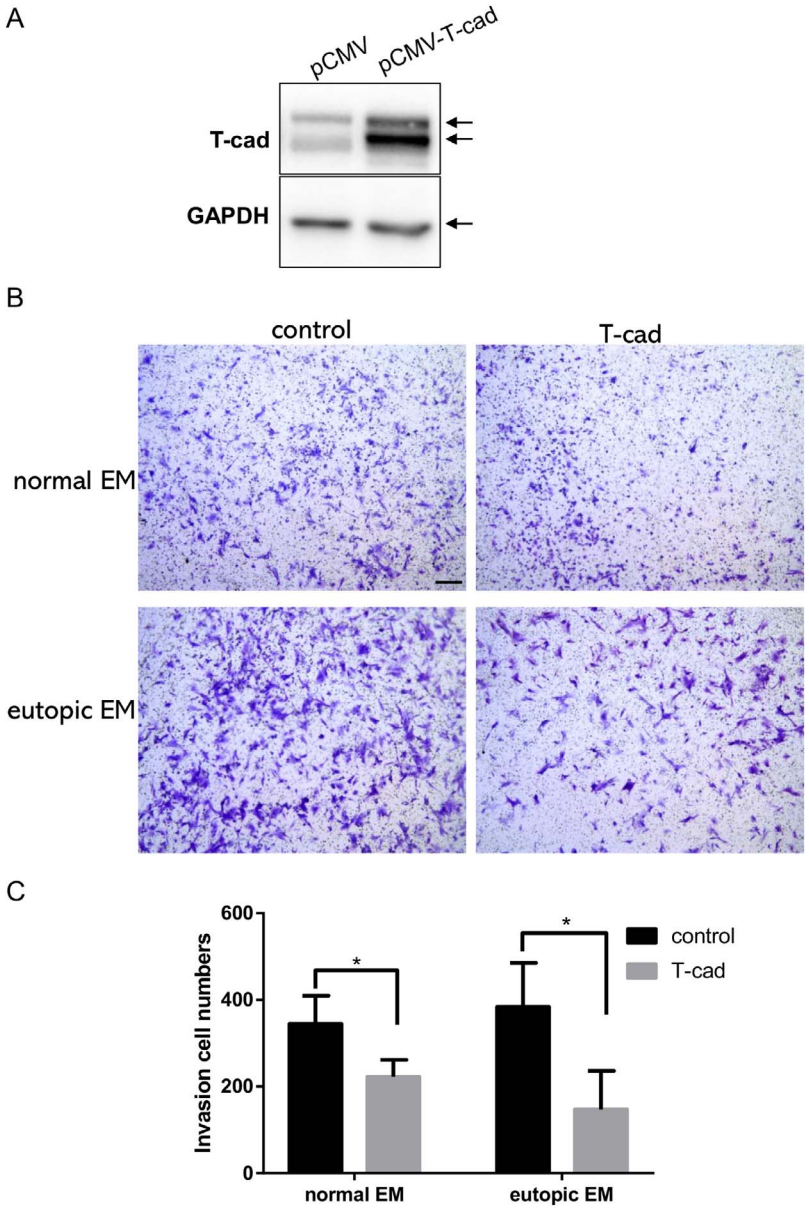

Figure 3 **T-cadherin overexpression inhibited cell invasion in endometrial cells.** (**A**) Normal endometrial stromal cells were transfected by the empty control vector pCMV and the T-cadherin expression vector pCMV-T-cad, and protein samples were collected at 24 h post transfection and western blot was used to confirm T-cadherin overexpression. GAPDH serves as the internal control gene. (**B**) Representative invasion results of T-cad overexpression in normal control and endometriosis eutopic EM stromal cells. The original magnification was 50×. The scale bar represented 200 μm. (**C**) Invasion cell numbers were counted, and data shown as mean ± SD ($n = 3$), analyzed by Student's t test. $^*P < 0.05$.

performed in duplicate for each condition, and the cells in five random fields on each well were selected for counting. For the invasion assay, 5×10^5 cells were seeded in the upper chamber of each transwell pre-coated with 10 μL Matrigel (R&D Systems, USA). After 24 h invasion, cells were fixed, stained and assessed as for the migration assays above. Cell numbers are presented as mean ± SD.

Phosphorylation antibody array assay

Endometriosis eutopic stromal cells were transfected with the empty control vector pCMV and the T-cadherin expression vector pCMV-T-cad, and protein samples collected at 24 h post-transfection. The phosphorylation profile of signaling pathway proteins was detected with the Proteome Profiler Human Phospho-Kinase Array Kit (Cat#ARY003B, R&D Systems, USA), with the 200 μg of T-cadherin overexpression and control total protein samples, respectively. This assay was performed according to the manufacturer's instructions, from protein sample harvest to final blot visualization. Images were captured by Bio-Rad Molecular Imager ChemiDoc XRS+ Imaging System. Blot densities were analyzed by quantitative densitometry. Each dot intensity was normalized to the positive control dots' intensities, which were set as 1.

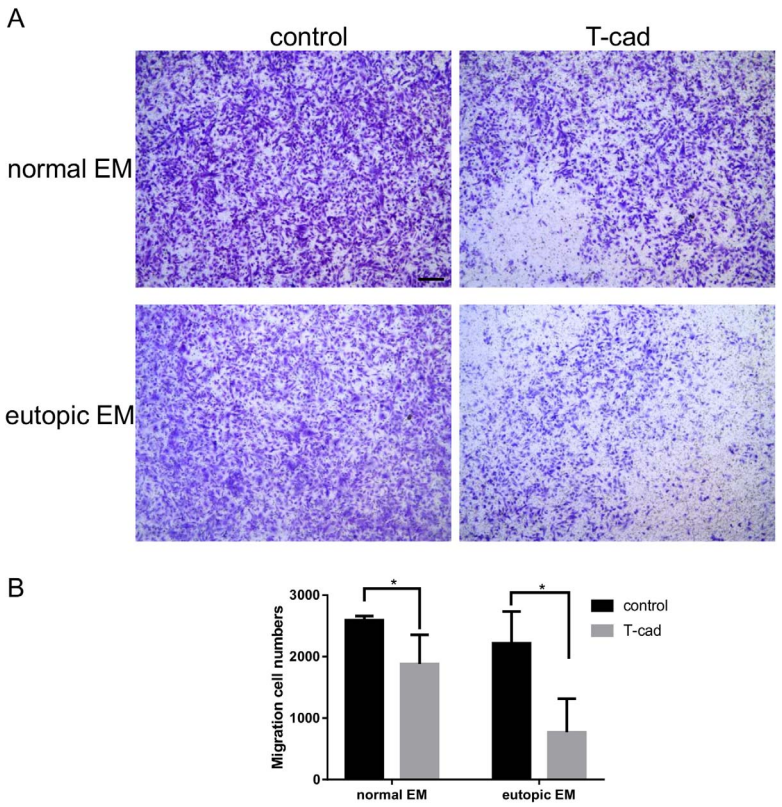

Figure 4 T-cadherin overexpression inhibited cell migration in endometrial cells. (**A**) Representative migration results of T-cadherin overexpression in normal control and endometriosis eutopic EM stromal cells. The original magnification was 50×. The scale bar represented 200 μm. (**B**) Migration cell numbers were counted, and data shown as mean ± SD ($n = 3$), analyzed by Student's t test. *$P < 0.05$.

Data were validated by western blot with more than three cases of stromal cells independent of those used in the array assay.

Statistical analysis

Data are shown as mean ± SD. Data were analyzed by SPSS 23.0 software (SPSS Inc., Chicago, USA). Student's t test was used to analyze the T-cadherin expression levels (H-score), invasion/migration cell numbers and the western blot semi-quantitative data. Spearman correlation was employed to analyze the relationship between clinical characteristics and T-cadherin expression levels (H-score) in endometriosis. $P < 0.05$ was considered to be statistically significant.

Results

T-cadherin expression was decreased in endometriotic lesions

T-cadherin was expressed in the control patients' normal endometrium, the eutopic and ectopic endometrial tissues of endometriosis patients (Fig. 1). The expression of T-cadherin in the normal endometrium of control patients and the eutopic endometrium of endometriotic patients did not differ, but it was decreased in the ectopic endometrium of endometriotic patients, compared with normal endometrial and eutopic endometriotic lesions ($P < 0.0001$, for both). T-cadherin was expressed in both endometrial stromal and glandular cells, with both nuclear and cytoplasmic localization (Fig. 1B). Most cases had a high H-score in normal and eutopic endometriotic lesions, while just a few cases had a high H-score T-cadherin expression in ectopic endometriotic lesions (Fig. 1C). In addition, the correlation between the expression status of T-cadherin and its clinicopathological parameters in endometriosis patients was also investigated. Correlation analyses of H-score and patients' age, stage, lesion size and adhesion in endometriosis were performed. There was no significant correlation found between endometriotic patients' age, stage, lesion size or adhesion and T-cadherin expression (Table II).

To confirm the IHC results, western blot was used to determine T-cadherin protein expression in the endometrium from patients with and without endometriosis. T-cadherin expression was downregulated in ectopic endometriotic tissues, compared with the normal control endometrial and eutopic endometriotic tissues, whose T-cadherin expression had no significant difference (Fig. 2).

T-cadherin overexpression inhibited endometrial stromal cell invasion

Western blotting confirmed the success of transfection and T-cadherin overexpression (Fig. 3A). T-cadherin overexpression significantly inhibited the invasion of both normal control endometrial stromal cells and eutopic endometriotic stromal cells, compared with the

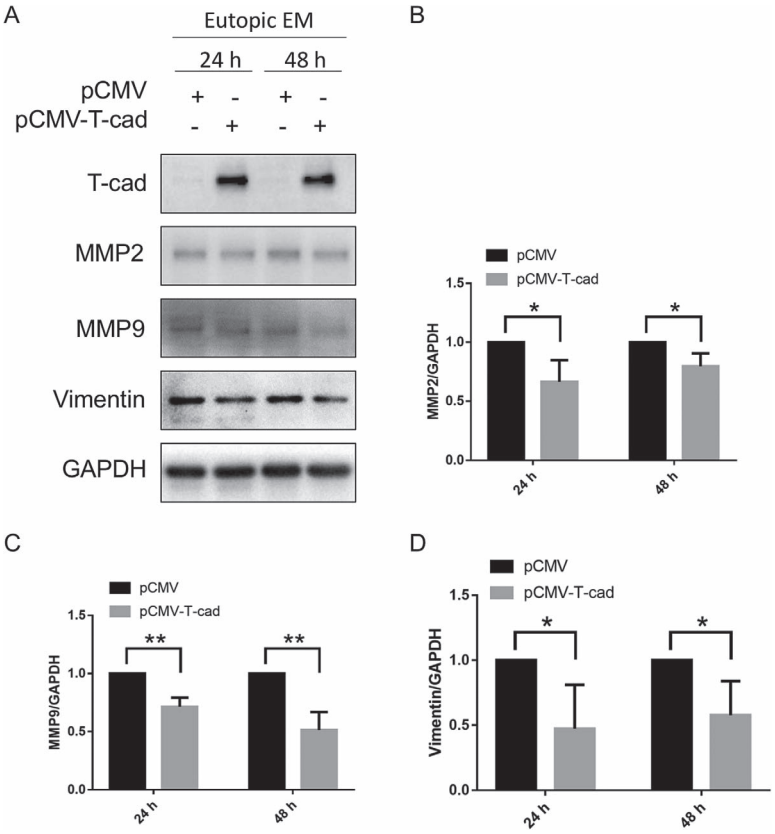

Figure 5 T-cadherin overexpression decreased MMP2, MMP9 and vimentin protein expression. Eutopic endometriotic stromal cells were transfected the empty control vector pCMV and the T-cadherin expression vector pCMV-T-cad, and protein samples collected at 24 and 48 h post-transfection. Western blot was used to detect the expression of T-cadherin, MMP2, MMP9 and vimentin with their specific antibodies. (**A**) Representative images are shown ($n = 3$). (**B**–**D**) Relative densitometry analysis of western blot results in (A), by Student's t test. $^{*}P < 0.05$, $^{**}P < 0.01$.

empty control vector pCMV-transfected cells ($P < 0.05$ for both; Fig. 3B and C).

T-cadherin overexpression inhibited endometrial stromal cell migration

To investigate the role of T-cadherin in cell migration, a T-cadherin expression vector was transfected into endometrial stromal cells from women with and without endometriosis. T-cadherin overexpression suppressed the migration of endometrial stromal cells of both normal control and endometriotic patients ($P < 0.05$ for both; Fig. 4).

T-cadherin regulated MMP2, MMP9 and vimentin expression in endometrial stromal cells

The expression levels of MMP2, MMP9 and vimentin were decreased in T-cadherin overexpression cells (Fig. 5).

T-cadherin regulated the phosphorylation of signal transduction molecules

Phosphorylation of HSP27 (S78/S82) and JNK 1/2/3 (T183/Y185, T221/Y223) was induced by T-cadherin overexpression ($P < 0.05$) (Fig. 6). Array results were validated by western blot (Fig. 6).

Discussion

Endometriosis is a common gynecologic condition in reproductive age women, with a complex etiology. Generally, hormones, immunity, environment and genetic/epigenetic gene alterations are the causes of endometriosis. Endometriosis is not a cancerous disease, but endometriotic lesions have many cancer-like traits, such as cell invasion, migration, proliferation and anti-apoptosis. In this study, we evaluated the expression status of T-cadherin in endometriosis and explored the effects of T-cadherin on migration and invasion of endometrial stromal cells.

T-cadherin expression is reduced in various types of cancers, including bladder cancer (Chen et al., 2016), lymphoma (Alkebsi et al., 2016), prostate cancer (Maslova et al., 2015) and oral squamous cell carcinoma (Wang et al., 2018a). Currently, it is not clear what factors affect the expression of T-cadherin in endometriosis. Methylation of the T-cadherin promoter, long non-coding RNA (lncRNA) H19, miR-675 and miR-377 can regulate the expression level of T-cad in other diseases (Ellmann et al., 2012; Shi et al., 2014; Sheng et al., 2016; Liu et al., 2018). In the current study, we found that there was no difference in expression levels of T-cadherin between eutopic endometrium from endometriosis patients versus controls, while T-cadherin was significantly downregulated in ectopic endometrium of

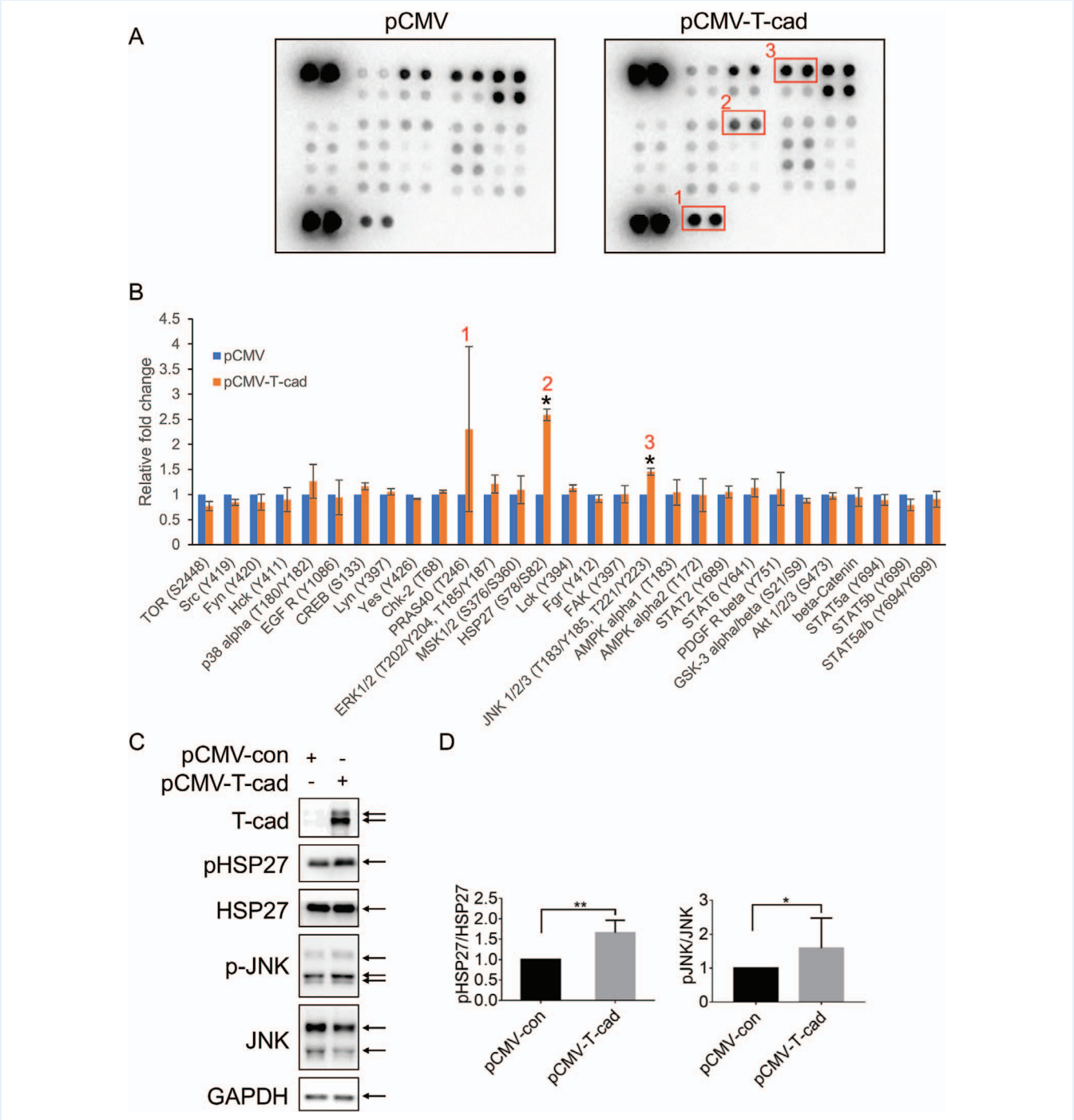

Figure 6 T-cadherin regulated the phosphorylation of signaling transduction proteins. Endometriosis eutopic endometrial stromal cells were transfected by the empty control vector pCMV and the T-cadherin expression vector pCMV-T-cad, and protein samples were collected at 24 h post-transfection and analyzed by protein kinase phosphorylation array assay. (**A**) Representative array results on the blots. Red frames indicate the phosphorylated proteins with fold change above 1.5. (**B**) Quantitative densitometry analysis of the blots. Blot intensities were analyzed normalizing to the positive control dots' intensities which were set as 1. Student's *t* test. $^{*}P < 0.05$. (**C**) The effects of T-cad on the phosphorylation of HSP27 and JNK1/2/3 were validated by western blot. (**D**) Relative densitometry analysis of western blot results in (**C**), by Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$.

endometriotic patients, compared with both. T-cadherin is a tumor suppressor in most cancers, so the downregulation of T-cadherin in endometriotic ectopic lesions may play a role in the pathogenesis of endometriosis.

We did not observe any correlation between the expression level of T-cadherin and endometriotic patients' age, stage, lesion size or adhesion although lower T-cadherin expression levels were significantly correlated with advanced clinical stage, higher pathological grade and

poor differentiation in cancers (Tang et al., 2012; Wang et al., 2018a). The difference may be due to our limited endometriosis cases and detection method and that endometriosis is different from cancer. In the current study, most of the endometriosis patients exhibited ovarian endometriosis, but some deep infiltrating endometriosis. Both had similar decreased T-cadherin expression levels, indicating that low expression of T-cadherin may have important roles in endometriosis disease formation or progression. However, the downregulation of T-cadherin seems an acquired property of endometriotic cells when they reach the peritoneal cavity, since T-cadherin had normal expression levels in the eutopic endometrium of endometriotic patients. The microenvironment in the peritoneal cavity in endometriosis is different from the normal condition. It would be interesting to determine the relationship between expression of progesterone receptors and T-cadherin, because both are downregulated in the ectopic lesions in endometriosis (McKinnon et al., 2018). In addition, progesterone resistance was observed when using hormonal contraceptive treatments in some endometriosis patients (Barra et al., 2019). It is not clear whether progesterone resistance in endometriosis is an acquired or innate property (McKinnon et al., 2018). In addition, endometriosis is a chronic inflammatory disease and increased inflammatory cytokines, like interleukin 1β and tumor necrosis factor-α (TNFα), can attenuate the expression of PR (Grandi et al., 2016). However, we do not currently have the data to determine a relationship between T-cadherin down expression, progesterone resistance and inflammatory cytokines, and to illustrate their effects in endometriosis. More studies are needed to explore the clinical roles of T-cadherin down expression in endometriosis.

T-cadherin has been described as a cancer suppressor gene, inhibiting cell migration and invasion in cervical cancer (Zhao et al., 2018), glioma (Shi et al., 2014), bladder transitional cell carcinoma (Lin et al., 2013) and squamous cell carcinoma (Pfaff et al., 2010). T-cadherin overexpression significantly inhibited gastric cancer cell migration and invasion by inducing increased E-cadherin expression and decreased vimentin expression (Lin et al., 2017). MMP2 and MMP9 are vital extracellular metalloproteinases with increased activity in endometriosis and mediate migration and invasion of endometriotic cells (Chen et al., 2010; Lin et al., 2013; Ahn et al., 2015). In the current study, we found that T-cadherin overexpression may inhibit migration and invasion of endometrial stromal cells through the downregulation of vimentin, MMP2 and MMP9. Therefore, T-cadherin may be an endometriotic suppressor gene, which inhibits endometrial stromal cell migration and invasion in ovarian endometriosis.

Based on the structure of T-cadherin, there is increasing evidence that T-cadherin may act as a signaling transduction molecule, anchored on the membrane through an atypical glycosylphosphatidylinositol (GPI). Studies have shown that T-cadherin has various characteristics in different conditions, regulating diverse biological activities through the phosphorylation of Akt and ERK 1/2 (Kipmen-Korgun et al., 2005; Joshi et al., 2007; Lee et al., 2008; Philippova et al., 2008; Adachi et al., 2010; Kyriakakis et al., 2017). However, in our current study, we found that the phosphorylation of HSP27 (S78/S82) and JNK 1/2/3 (T183/Y185, T221/Y223) was dramatically induced by T-cadherin overexpression in endometrial stromal cells. HSP27 had minimal expression in normal cervical glands and is significantly more widespread in tuboendometrial metaplasia/endometriosis (El-Ghobashy et al., 2005). There are no studies on the roles of HSP27 in endometriosis, but some studies showed that HSP27 may regulate tumor cell and myofibroblast migration (Chu et al., 2017; Han et al., 2018; Tanaka et al., 2018; Zhang et al., 2019). JNK has been widely investigated in endometriosis and is involved in cell migration and invasion. Nevertheless, JNK phosphorylation signaling has been shown to have opposing effects in endometriosis, inducing or inhibiting cell migration and invasion (Mei et al., 2013; Miller et al., 2013; Wang et al., 2018b). The current study indicated that T-cadherin inhibited cell migration and invasion by activation of HSP27 and JNK signaling. Therefore, our data also supports T-cadherin as a signaling pathway regulator with roles in signal transduction in endometriosis. However, further investigation is required to fully determine the T-cadherin signaling pathways involved in the invasion and migration of endometrial cells in endometriosis.

In conclusion, we identified that T-cadherin was downregulated in ectopic endometriotic lesions. Overexpression of T-cadherin inhibited cellular invasion and migration in normal control and eutopic endometriosis endometrial stromal cells. T-cadherin regulated the expression of MMP2, MMP9 and vimentin in these cells. Moreover, T-cadherin can be a signaling transduction pathway molecule, regulating the phosphorylation of HSP27 and JNK, which play roles in regulating cell invasion and migration. However, detailed effects and mechanisms of T-cadherin in endometriosis remain to be further studied. Therefore, these data provide new insights into the pathogenesis of endometriosis, and T-cadherin may be a new potential therapeutic target in endometriosis.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

Q.L., Y.H., L.C., L.W. and G.E.L. designed this study, analyzed and interpreted the data and wrote the article. Q.L., Y.H., J.W., M.D., F.W., Z.L., Y.Z. and G.G. collected patients' information and clinical samples. Q.L., Y.G., H.H. and J.Y.Z. isolated the endometrial cells. Q.L., H.H., M.Z. and F.N. performed the experiments. All of the authors read and approved the final version of the article.

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Conflict of interest

No conflict of interest is declared.

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