

# Roles of cell migration and invasion mediated by Twist in endometriosis

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## Abstract

**Aim:** To investigate the roles of cell migration and invasion mediated by Twist in endometriosis.

**Methods:** The protein levels and locations of Twist, N-cadherin and E-cadherin were measured by Western blot and immunohistochemistry in ectopic endometrium and eutopic endometrium of ovarian endometriosis as well as normal endometrium of nonendometriosis patients. The messenger RNA (mRNA) expressions of Twist, N-cadherin and E-cadherin in these tissues were measured by quantitative reverse transcription polymerase chain reaction. Stable overexpression of Twist in eutopic endometrial stromal cells was transfected with a plasmid-mediated delivery system. The protein and mRNA expressions of N-cadherin and E-cadherin were detected by western blot and reverse transcription polymerase chain reaction. The changes of migration and invasion of endometrial stromal cells were explored by transwell.

**Results:** Levels of protein and mRNA of Twist and N-cadherin showed the highest expression in ectopic endometrium of ovarian endometriosis, while lowest in normal endometrium of nonendometriosis patients. On the contrary, the expression of E-cadherin showed highest in normal endometrium of nonendometriosis patients. The overexpression of Twist after transfection significantly upregulated the protein and mRNA expression of N-cadherin, while downregulated the protein and mRNA expression of E-cadherin. There is significant difference between groups. For transwell, the overexpression of Twist in eutopic endometrial stromal cell significantly promoted cell migration and invasion.

**Conclusion:** Twist might be related with the increase of migration and invasion in endometrial stromal cells, mediated by epithelial-to-mesenchymal transition.

**Key words:** cadherin, cell migration and invasion, endometriosis, epithelial-to-mesenchymal transition, Twist.

## Introduction

Endometriosis is a disorder in which abnormal growth of tissue, histologically resembling the endometrium, present in locations other than the uterine lining. It is a common disease. There is 10–15% of women in reproductive age suffering from it, especially who aged between 25 and 40 years old, with an increasing trend in recent years.<sup>1</sup> The symptoms of endometriosis, such as chronic pelvic pain, dysmenorrhea and infertility, significantly impacted

women's health and life quality.<sup>2</sup> Endometriosis was first described by Von Rokitansky in 1860, but its pathogenesis remains to be unclarified.<sup>3,4</sup> The hypothesis suggested by Sampson, 'retrograde menstruation and implantation', has been widely accepted. However, retrograde menstruation was common among reproductive-age women with an occurrence rate of 76–90%, while the incidence of endometriosis is only 10–15%. This suggests that retrograde menstruation might be only a precipitating factor, while the biological behaviors of ectopic

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endometrium is key in pathogenesis of endometriosis, such as the migration and invasion of the endometriosis, adhesion and infiltration to pelvic organs such as ovary or peritoneum.

Epithelial-mesenchymal transition (EMT) plays an essential role in the embryogenesis and tumor metastasis.<sup>5–7</sup> Various studies indicate that the EMT plays a crucial role in the metastasis of tumors. Similar to tumor metastasis, various published studies indicate that the EMT also plays a significant part in the initial formation of endometriosis.<sup>8–10</sup> N-cadherin is a member of the superfamily of integral membrane glycoproteins that regulates cell adhesion and cell motility. N-cadherin plays an important role in EMT, which epithelial cells gain migratory and invasive capacity.<sup>11,12</sup> In experimental models, E-cadherin depletion leads to mesenchymal morphology and increased cell migration and invasion.<sup>13,14</sup> Nevertheless, the pathogenesis of the EMT in the endometriosis remains to be proved.

Twist1, which usually called Twist, a basic helix–loop–helix domain-containing transcription factor and a highly conserved protein which can suppress apoptosis, whose functions include inducing EMT and enhancing migration and invasion of tumor cells, inhibiting cell apoptosis, promoting tumor angiogenesis, as well as causing chromosome instability.<sup>15–17</sup> Importantly, Twist has been characterized as a critical transcription factor that regulates the expression of N-cadherin and E-cadherin in cancer cells.<sup>18,19</sup> An overexpression of Twist was shown in ectopic endometrium.<sup>20,21</sup> However, how gene Twist plays a role in the pathogenesis of endometriosis needs to be delineated.

In the present study, we investigated the effect of Twist on the migration and invasion of eutopic endometrial cells and explored the possible pathways that might be involved in the pathogenesis of endometriosis.

## Materials and Methods

### General information

All tissues were derived from surgical resection of patients admitted to our hospital from January 2013 to July 2016. Among them, 30 cases of ectopic endometriosis and eutopic endometrium of ovarian endometriosis were included, aged between 25 and 43 years old with a median age of 35 years old. Thirty cases of normal endometrium from infertility patients aged from 26 to 40 years old (with a median age of 32 years old) and were collected and included as

control group. All patients who had had no hormonal treatment for 3 months before biopsy were subjected to laparoscopy or laparotomy, and pathologic diagnosis of biopsy specimens was made retrospectively, which were confirmed by postoperative pathology. All endometriosis were III–IV staged by revised American fertility society.

The study had received written approval from the Ethics and Committee of Women's Hospital of Zhejiang University for Clinical Research. All of the patients participated in the study signed the informed consent form.

### Immunohistochemistry

For the immunohistochemical study, paraffin slides (5  $\mu$ m thick) were deparaffinized with xylene and serial ethanol dilutions. Endogenous peroxidase activity was blocked for 30 min with a buffer solution containing peroxide (0.5% H<sub>2</sub>O<sub>2</sub> in phosphate citrate) followed by antigen retrieval (boiling in citrate buffer [PH6.0], 20 min). Slides were incubated overnight with the anti-Twist (1:500; Abcam), E-cadherin (1:200; Abcam) and N-cadherin (1:200; Abcam) antibody followed by the secondary antibody. All slides were mounted, and all sections were developed with diaminobenzidine followed by hematoxylin counterstaining. Before the slides were mounted, all sections were dehydrated in alcohol and xylene. Percentage and intensity of positively stained nuclei and cytoplasm were estimated by an experienced pathologist. A semiquantitative subjective scoring system to evaluate the localization, quantity and intensity of immunoreactivity was employed using light microscopy (200 $\times$  magnification). In each sample, the staining for glandular epithelial cells and stromal cells was scored separately. The intensity of the staining was scored using a four-point scoring scale (0, negative staining; 1, weak staining; 2, moderate staining, 3, strong staining). The percentage of positively stained cells was again scored by a four-point scoring scale (0, negative staining; 1, 1–35% positive cells; 2, 36–70% positive cells; 3, >70% positive cells). The two scores were combined by multiplication to derive a final immunohistochemistry (IHC) score (0–9).

### Protein extraction and western blot analysis

Proteins were extracted by using radio-immunoprecipitation assay lysis buffer containing protease and phosphatase inhibitor cocktail. Proteins were then separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and subjected

to gel electrophoresis. Resolved proteins were transferred to a polyvinylidene fluoride membrane. Membranes were then blocked in 5% nonfat dry milk in Tris-buffered saline with Tween for 1 h and then incubated with antibodies to glyceraldehyde phosphate dehydrogenase (GAPDH), anti-Twist (1:500; Abcam), anti-N-Cadherin (1:500; Abcam), and anti-E-Cadherin (1:500; Abcam) 4°C overnight. The membrane was washed thrice with Tris-buffered saline with Tween and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The blotting bands were visualized after the chemiluminescence reaction by using a digital imaging system (Image Quant LAS 4000 mini; GE Healthcare). GAPDH was used as a loading control.

### The extraction of total RNA and the reverse transcriptional reaction in endometriosis tissues and endometrial tissues

Cells were seeded at  $10^6$  cells per 10 cm dish and were allowed to grow to 80% confluency in complete media. Cells were removed with 0.025% ethylenediaminetetraacetic acid and centrifuged for 5 min at 1100 rpm. Cell pellets were resuspended in 1 mL of Trizol (Invitrogen Corporation), and RNA was extracted according to the manufacturer's protocol. Total RNA in unstained paraffin-embedded samples was extracted with the RNeasy FFPE Kit (Qiagen). The extracted total RNA was quantitatively analyzed by ultraviolet spectrophotometer, and reversed transcribed using the PrimerScript reverse transcription kit. The production of cDNA was stored at  $-20^{\circ}\text{C}$ .

For all polymerase chain reaction (PCR) reactions, GAPDH was used as an endogenous control, and cycle threshold values were normalized to levels of GAPDH expression. Sequences used to analyze RNA expression include the following: Twist, forward: 5'-AGACCGAGAAGGCGTAGC-3' and reverse: 5'-TGAGCAAGATTCAGACCC-3'; E-cadherin, forward: 5'-GGCGGGTAGTGTAAGTGTC-3' and reverse: 5'-CTCAAATGGATGTTGGGTCA-3'; N-cadherin, forward: 5'-AAAGAGTGAGCGACAAGGAA-3' and reverse: 5'-AAAGTAGGCAGAACAAGGTG-3'; GAPDH, forward: 5'-ATGGAAATCCCATCACCA TCTT-3' and reverse: 5'-CGCCCCACTTGATTTTGG-3'. Fluorescence quantitative PCR kits were purchased from TAKARA. Real-time PCR instrument (7900 Type; Applied Biosystems Inc.) was used to amplify. And the PCR reaction condition was: denaturation for 5 s at  $95^{\circ}\text{C}$ , then turned to 30 s at  $60^{\circ}\text{C}$ , with

40 circulations. The relative quantitative analysis results were analyzed with  $2^{-\Delta\Delta\text{Ct}}$  method.

### Cell transfection

The eutopic stromal cells of endometriosis were primarily cultured and identified in complete dulbecco's modified eagle medium (DMEM) medium until the convergence degree reached about 70%. Stable overexpression of Twist with plasmid-mediated delivery system (cmv-mcs-3flag-sv40-Neom-Twist) was transfected efficiently to them. The protein expression of N-cadherin and E-cadherin was detected by western blot. The mRNA expression of N-cadherin and E-cadherin was tested by reverse transcription PCR.

### Cell migration and invasion

The changes of migration and invasion of endometrial stromal cells were explored by transwell. A 24-well Transwell plate (Corning) was used to measure migratory and invasive ability *in vitro*. A total of  $5 \times 10^4$  cells in 0.2-mL serum culture medium were plated on the bottom of each well. For the migration assay,  $5 \times 10^4$  cells were plated in the top chamber with a noncoated membrane for 24 h. For the invasion assay,  $5 \times 10^4$  cells were seeded in the top chamber coated with Matrigel (BD Biosciences) for 48 h. In both assays, cells were cultured in DMEM containing 10% fetal bovine serum with the indicated treatment. After incubation, migrated cells were fixed and stained with the crystal violet (for invasion) and counted in six random fields. The experiments were performed in triplicate wells, and each experiment was performed at least two or three times as indicated.

After Twist transfection for 48 h, cellular total RNA and protein were extracted to examine.

### Statistical analysis

SPSS 16.0 statistical software was used to analyze all the data. The measurement data were expressed as mean  $\pm$  standard deviation, which tested by normality test. The comparison between two groups was tested by one-way ANOVA. The multicomparison between groups was tested by least significant difference test.  $P < 0.05$  was considered as significant difference. Spearman analysis was tested by correlation analysis.

**Table 1** Comparison of Twist expression among different groups by immunohistochemistry

| Groups | <i>n</i> | Twist |    |    |    | Positive (%) | <i>P</i> -value |
|--------|----------|-------|----|----|----|--------------|-----------------|
|        |          | +++   | ++ | +  | -  |              |                 |
| EMs    | 30       | 5     | 11 | 13 | 1  | 29 (96.7)    | <0.05           |
| EuM    | 30       | 0     | 3  | 8  | 19 | 11 (36.7)    |                 |
| EN     | 30       | 0     | 1  | 3  | 26 | 4 (13.3)     |                 |

EMs, ovarian endometriosis; EN, normal endometrium from infertility patients; EuM, eutopic endometrium of ovarian endometriosis.

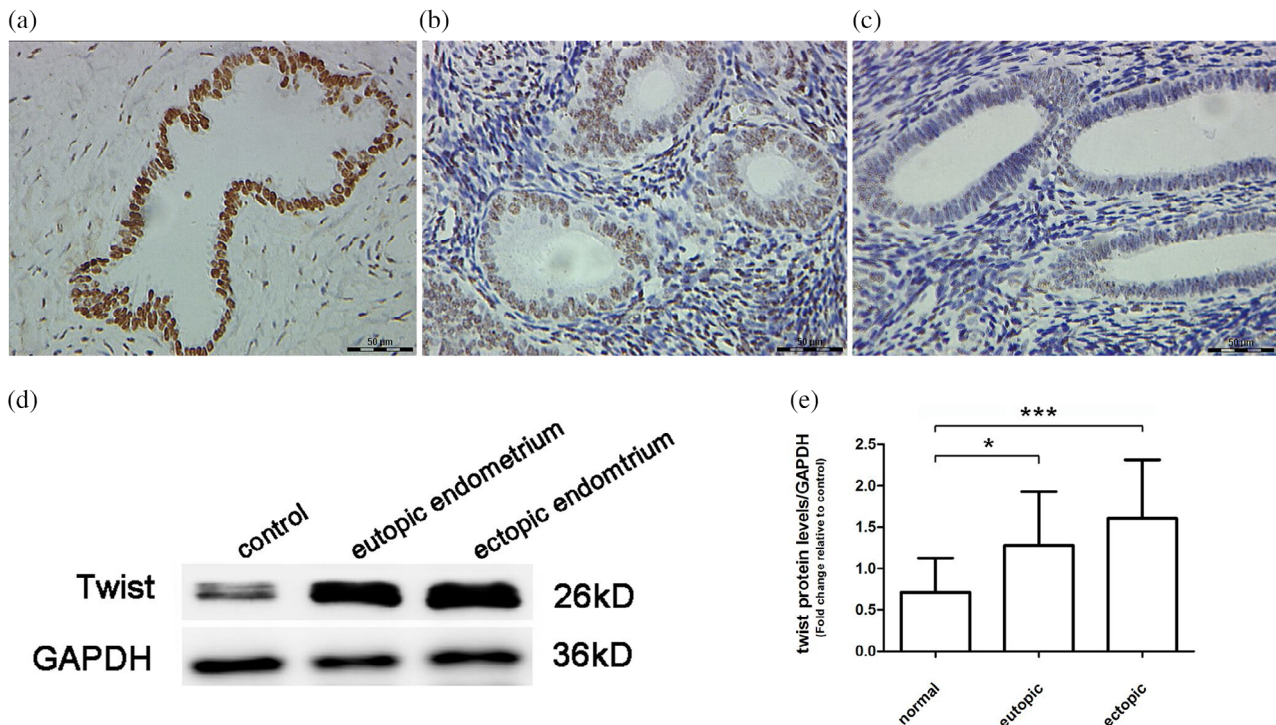
## Results

### Protein expression and relationship of Twist, E-cadherin and N-cadherin among ectopic endometrium, eutopic endometrium of ovarian endometriosis and normal endometrium

Results from IHC showed that Twist, N-cadherin and E-cadherin were expressed in both stromal cells and glandular epithelium. Mean percentage of stained endometriosis nuclei of Twist was 96.7%. As expected, Twist (Table 1 and Fig. 1) and N-cadherin (Table 2 and Fig. 2) showed the highest expression in ectopic endometrium of ovarian endometriosis, while lowest in normal endometrium of nonendometriosis patients. On

the contrary, the expression of E-cadherin (Table 2 and Fig. 2) showed highest in normal endometrium of non-endometriosis patients. No association between Twist expression and clinicopathologic data was found. The differences between different groups were significant ( $P < 0.05$ ). Spearman analysis showed positive correlation between N-cadherin and Twist (Pearson correlation coefficient = 0.519), while negative correlation between E-cadherin and Twist (Pearson correlation coefficient = -0.425).

The expression of Twist (Fig. 1) and N-cadherin protein (Fig. 2) measured by Western blot decreased stepwise in ectopic endometrium of ovarian endometriosis, eutopic endometrium of ovarian endometriosis and



**Figure 1** Expressions and localizations of Twist. The expression level of Twist in EMs (ovarian endometriosis) and EuM (eutopic endometrium of ovarian endometriosis) was significantly higher than that in EN (normal endometrium from infertility patients). (a–c) Immunohistochemistry in EMs, EuM and EN, respectively. (d and e) Western blotting results.



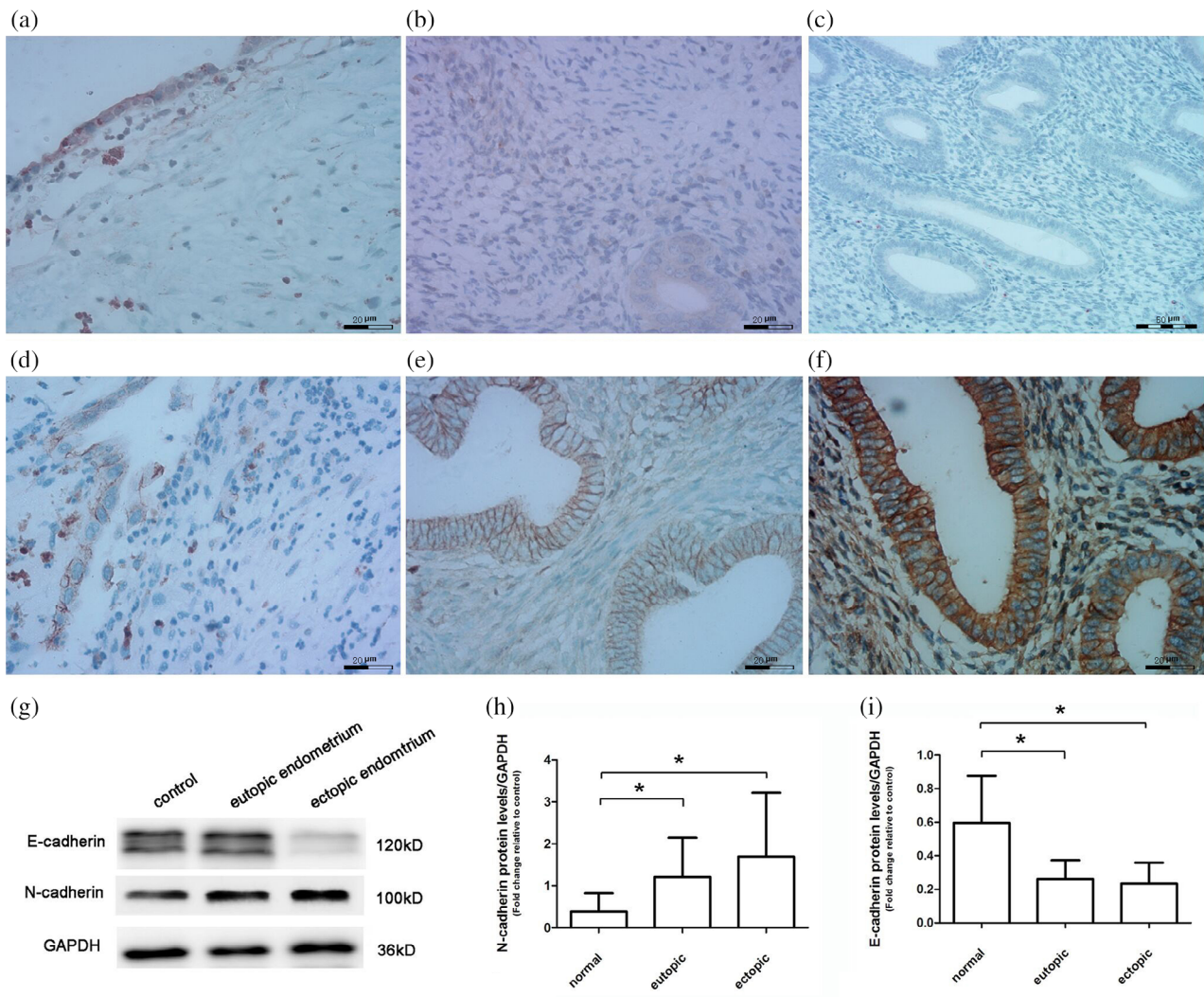
**Table 2** Comparison of N-cadherin and E-cadherin expressions among different groups by immunohistochemistry

| Groups | n  | N-cadherin |    |    |    | Positive (%) | P-value | E-cadherin |    |    |    | Positive (%) | P-value |
|--------|----|------------|----|----|----|--------------|---------|------------|----|----|----|--------------|---------|
|        |    | +++        | ++ | +  | -  |              |         | +++        | ++ | +  | -  |              |         |
| EMs    | 30 | 0          | 5  | 13 | 12 | 18 (60)      | <0.05   | 0          | 1  | 5  | 24 | 6 (20)       | <0.05   |
| EuM    | 30 | 0          | 0  | 9  | 21 | 9 (30)       |         | 1          | 5  | 9  | 15 | 15 (50)      |         |
| EN     | 30 | 0          | 0  | 3  | 27 | 3 (10)       |         | 9          | 8  | 12 | 1  | 29 (96.7)    |         |

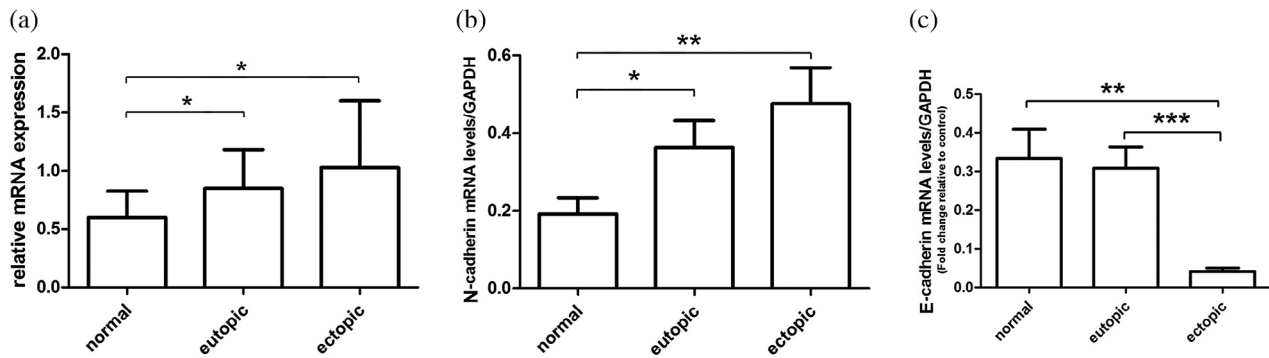
EMs, ovarian endometriosis; EN, normal endometrium from infertility patients; EuM, eutopic endometrium of ovarian endometriosis.

normal endometrium of nonendometriosis patients ( $P < 0.05$ ). The expression of E-cadherin protein (Fig. 2) measured by Western blot increased stepwise in ectopic

endometrium of ovarian endometriosis, eutopic endometrium of ovarian endometriosis and normal endometrium of nonendometriosis patients ( $P < 0.05$ ).



**Figure 2** Expressions and localizations of N-cadherin and E-cadherin. (a-c) N-cadherin immunohistochemistry in EMs (ovarian endometriosis), EuM (eutopic endometrium of ovarian endometriosis) and EN (normal endometrium from infertility patients), respectively. (d-f) E-cadherin immunohistochemistry in EMs, EuM and EN, respectively. (g-i) Western blot of N-cadherin and E-cadherin. N-cadherin showed the highest expression in EMs, while lowest in EN. On the contrary, E-cadherin showed the highest expression in EN, while lowest in EMs.



**Figure 3** mRNA levels of Twist, N-cadherin and E-cadherin by reverse transcription polymerase chain reaction. It showed Twist and N-cadherin were expressed in EMs > EuM > EN, while E-cadherin was expressed in EN > EuM > EMs. (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). EMs, ovarian endometriosis; EN, normal endometrium from infertility patients; EuM, eutopic endometrium of ovarian endometriosis.

### mRNA expression and relationship of Twist, E-cadherin and N-cadherin among ectopic endometrium, eutopic endometrium of ovarian endometriosis and normal endometrium

Results from reverse transcription PCR showed the mRNA levels of Twist and N-cadherin in ectopic endometrium and eutopic endometrium of ovarian endometriosis were significantly higher when compared with normal endometrium of nonendometriosis patients ( $P < 0.05$ ). However, E-cadherin was expressed highest in normal endometrium of nonendometriosis patients. There were significant differences among groups ( $P < 0.05$ ) (Fig. 3).

### Twist overexpression alters the migration and invasion of eutopic endometrial stromal cells

The overexpression of Twist after transfection significantly upregulated the protein and mRNA expression of N-cadherin, while downregulated the protein and mRNA expression of E-cadherin. There is significant difference between groups ( $P < 0.05$ ) (Fig. 4).

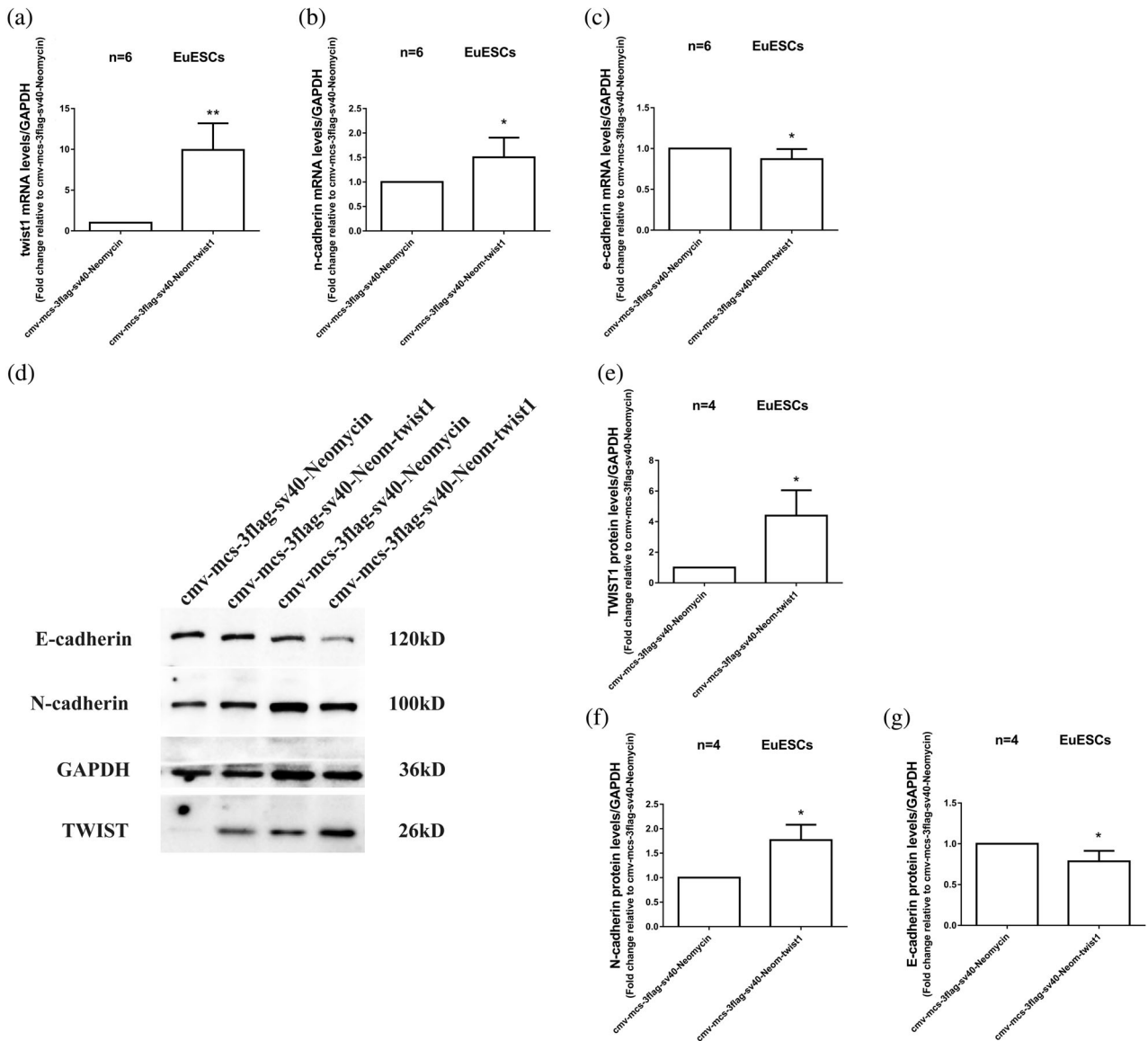
The further experiment showed that after eutopic endometrial stromal cells transfected with Twist, the number of cells in the lower layer of transwell chamber was significantly increased, suggesting that cell migration and invasion were promoted. The difference is significant ( $P < 0.05$ ) (Figs 5 and 6).

## Discussion

Although endometriosis is a benign disease, it presents with malignant characteristics, such as invasion to

surrounding tissues, metastasis to distant locations and recurrence following treatment. Sampson's retrograde menstruation hypothesis does not fully explain why most women suffer from retrograde menstruation but only 10 percent of them finally develop endometriosis. Researchers have found that other factors such as altered microenvironment may contribute to the development of endometriosis.<sup>22,23</sup> In the current study, we compared the expression of Twist, E-cadherin and N-cadherin in ectopic endometrium and eutopic endometrium of ovarian endometriosis and normal endometrium of nonendometriosis patients, as well as the changes of migration and invasion of endometrial stromal cells after transfection of Twist genes. We found that aberrant expression of Twist and EMT-related markers existed in ectopic endometrium and eutopic endometrium of ovarian endometriosis. Overexpression of Twist in eutopic endometrial stromal cells promoted the migration, invasion and EMT phenotype.

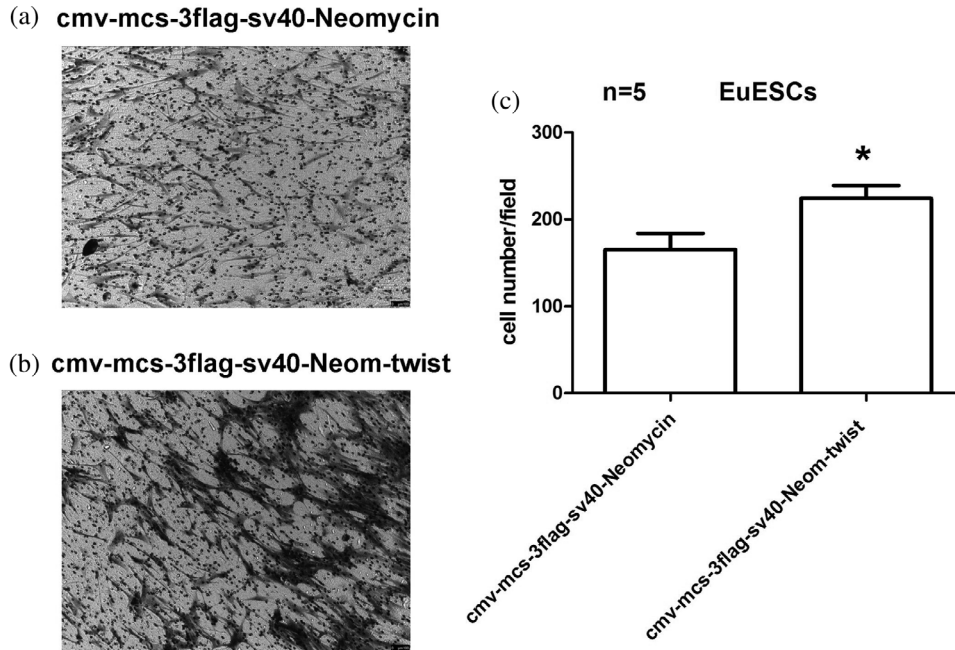
This study had found that protein and mRNA expressions of Twist were significantly higher than those in normal endometrium. And the findings were consistent with the high expression of Twist in endometrial carcinoma and cancer metastasis.<sup>24,25</sup> In order to further observe the influence of Twist on the migration and invasion of endometrial stromal cells, we attenuated the expression of Twist by transfection with Twist, and the findings showed that the migration and invasion ability of endometrial stromal cells were increased correspondingly after the overexpression of Twist, indicating that Twist played a potential role in the migration and invasion of endometrial stromal cells.



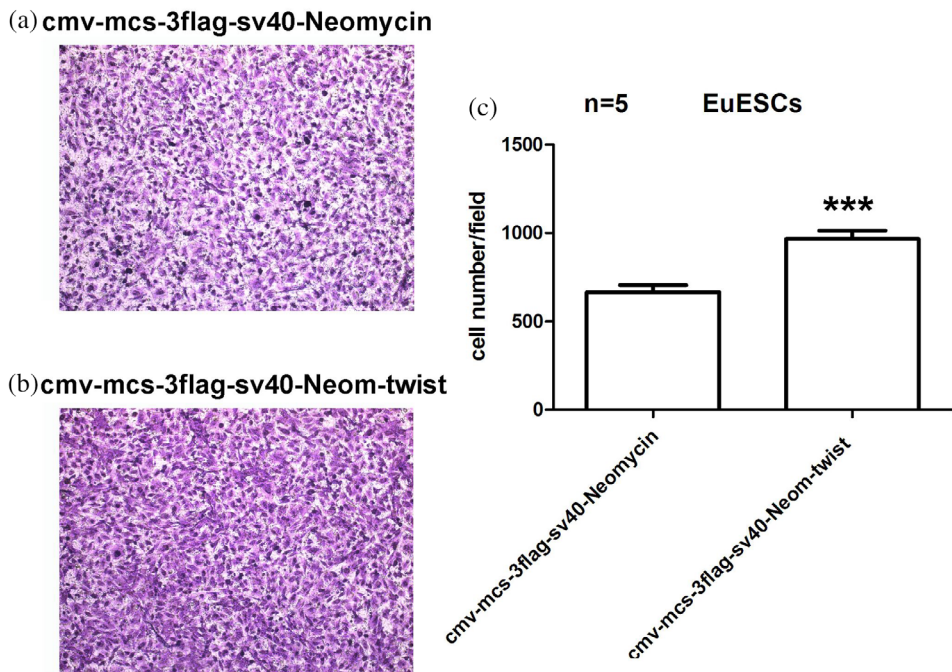
**Figure 4** The changes of protein levels and messenger RNA levels after transfection with cmv-mcs-3flag-sv40-Neom-Twist. (a) Reverse transcription polymerase chain reaction. (b) Western blot. They both showed that N-cadherin expression increased, while E-cadherin decreased after transfection ( $P < 0.05$ ).

One of the important steps in the migration and invasion was the ability of EMT. EMT, playing essential roles in embryogenesis and tumor metastasis, is proved to be prerequisites for the original establishment of endometriotic lesions.<sup>26</sup> Generally, overexpression of N-cadherin and low-expression of E-cadherin are the hallmarks of EMT and metastasis, and Twist plays a key role behind this.<sup>27,28</sup> Twist plays a key function in E-cadherin adjustment. Twist, as a basic helix-loop-helix transcription factor, can recognize and combine with the

E-box sequence on the promoter of the target gene, consequently affecting the E-box promoter expression at the transcriptional level, inhibiting the expression of E-cadherin, and inducing the formation of EMT.<sup>21</sup> On the contrary, Twist enhances the expression levels N-cadherin. In order to further explore the mechanism of Twist affecting the migration and invasion of endometrial stromal cells by regulating E-cadherin and N-cadherin, we detected the expression of N-cadherin and E-cadherin after transfection with Twist. And the results



**Figure 5** Transwell migration analysis. It showed that the migration ability of EuM cells after transfection with Twist (cmv-mcs-3flag-sv40-Neom –Twist) was significantly higher than that of control cells ( $\times 100$ ).



**Figure 6** Transwell invasion analysis. It showed that the invasion ability of EuM, eutopic endometrium of ovarian endometriosis cells after transfection with Twist (cmv-mcs-3flag-sv40-Neom – Twist) was significantly higher than that of control cells ( $\times 100$ ).

showed that overexpression of Twist could significantly stimulate the expression of N-cadherin and inhibit the expression of E-cadherin, indicating that Twist could increase the migratory and invasive capability of eutopic

endometrial stromal cells of ovarian endometriosis by mediating E-cadherin and N-cadherin

It also suggests that Twist gene may be associated with the incidence of endometriosis. The pathogenesis

of endometriosis may be related with the increase of migration and invasion in endometrial stromal cells.

This study was the first to demonstrate that Twist overexpression in endometrial stromal cells altered cell migration and invasion. Twist acted through regulation of EMT. Given its critical role in the pathogenesis of endometriosis, Twist may be a promising therapeutic target for endometriosis treatment. Drugs repressing the expression or inhibiting the function of Twist may suppress the progress and recurrence of endometriosis. However, the therapeutic effect of targeting Twist on endometriosis remains to be investigated in the future.

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## Disclosure

All authors declare no conflict of interests and funding in the manuscript, including financial, consultant, institutional and other relationships that might lead to bias.

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