In Vitro and In Vivo Effects of Tumor Suppressor Gene PTEN on Endometriosis: An Experimental Study

Juan Lv AEG Qiaoying Zhu C Xuemei Jia C Ningzhu Yu BDF Qian Li

Corresponding Author: Qiaoying Zhu, e-mail: zqy2101@sohu.com

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Background: Endometriosis can cause dysmenorrhea and infertility. Its pathogenesis has not yet been clarified and its treatment continues to pose enormous challenges. The protein tyrosine phosphatase (PTEN) gene is a tumor suppressor gene. The aim of this study was to investigate the role and significance of PTEN protein in the occurrence, development, and treatment of endometriosis through changes in apoptosis rate, cell cycle, and angiogenesis.

Material/Methods: PTEN was overexpressed and silenced in lentiviral vectors and inserted into primary endometrial cells. The changes in cell cycle and apoptosis in the different PTEN expression groups were evaluated using flow cytometry. Vessel growth mimicry was observed using 3-dimensional culture. A human-mouse chimeric endometriosis model was constructed using SCID mice. Hematoxylin and eosin staining and immunohistochemistry were used to detect pathological changes in ectopic endometrial tissues and the expression of VEGF protein in a human-mouse chimeric endometriosis mouse model.

Results: PTEN overexpression significantly increased apoptosis and inhibited the cell cycle compared with the silenced and control groups. Furthermore, cells expressing low PTEN levels were better able to undergo vasculogenic mimicry, and exhibited significantly increased angiogenesis compared to cells overexpressing PTEN. We found that ectopic foci were more easily formed in the endometrial tissue of SCID mice with low PTEN expression, and the VEGF expression in this group was relatively high.

Conclusions: PTEN inhibits the occurrence and development of endometriosis by regulating angiogenesis and the apoptosis and cell cycle of endometrial cells; therefore, we propose that the PTEN gene can be used to treat endometriosis.

MeSH Keywords: Angiogenesis Inducing Agents • Apoptosis • Cell Cycle • Endometriosis • PTEN Phosphohydrolase • Vascular Endothelial Growth Factor A

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Background

Endometriosis (EM) refers to a condition in which endometrial tissues grow outside the uterine mucosa. These ectopic endometrial tissues exhibit growth, invasion, planting, and cytocical bleeding under the influence of sex hormones. EM is a chronic disease, and its pathogenesis has not yet been clarified; therefore, its treatment continues to pose enormous challenges. Treatment of EM with drugs is associated with several adverse effects, including liver damage, weight gain, gastrointestinal reactions, and menopause-like symptoms. Furthermore, low estrogen-related adverse effects limit the long-term use of drugs, as the treatment can affect ovulation and thus reduce fertility potential, and the recurrence rate after stopping medication is high [1]. The symptoms of EM include severe dysmenorrhea and infertility, and it has a higher 5-year recurrence rate after conventional surgery; therefore, it greatly affects the quality of life and reproductive health of women. The morbidity of EM has significantly increased over the past few years, and the pathogenesis, biological characteristics, and alternative treatment approaches of EM have now become an important focus of fundamental gynecology research.

The protein tyrosine phosphatase (PTEN) gene is a tumor suppressor gene (approximately 55 kD in size) located on chromosome 10q23.3. It is composed of 9 exons, including 1209 nucleotides that can encode the 403-amino-acid protein tyrosine phosphatase (PTEN) [2]. This enzyme plays a role in dephosphorylation of threonine, serine, and tyrosine, and PTEN with C-terminal mutations will lose the phosphatase activity. Many scholars have studied the expression of the PTEN gene in endometrial, breast, prostate, and ovarian cancer, confirming a higher frequency of deletions or mutations in PTEN in many types of tumors [3–6]. Furthermore, it was reported that PTEN inactivation can lead to loss of tumor suppressor function, which is associated with the occurrence and progression of tumors.

PTEN has been implicated as one of the housekeeping genes in the endometrium [7]. Therefore, its expression in endometriosis is of special significance. Govatati et al. analyzed the PCR genes in the ectopic endometrium and found a high (84.4%) frequency of loss of heterozygosity in chromosome 10q23.3, which is structurally the site of the PTEN gene. Structural frameshift and insertion mutations occurred at 10: 89692992–89692993, which is an important N-terminal phosphatase region of PTEN. Govatati et al. suggested that the PTEN-P13K/Akt-Bad axis may be involved in the pathogenesis of endometriosis [8].

Direct or indirect interactions among PTEN, VEGF, and VEGFR play an important role in occurrence and development of many human tumors, angiogenesis, tumor invasion, and metastasis [9]. Moreover, research has shown that PTEN can play an important role in angiogenesis and regulate the expression of vascular endothelial growth factor (VEGF) through the PI3K/AKT signaling pathway, suppressing the generation of new blood vessels [10,11]. Thus, we speculated that the PTEN gene can inhibit the generation of new blood vessels by inhibiting the PI3K/Akt signaling pathway and inhibiting VEGF generation. Thus far, very few studies have determined whether re-initiating the expression of the PTEN gene can prevent and treat endometriosis; therefore, further studies are required to explore the involvement of the PTEN gene in endometriosis.

In this study, we investigated the role of PTEN in endometriosis in primary cultured human endometrial cells and a human-mouse chimeric EM animal model. Furthermore, we studied the relationship between the PTEN expression level and the development of EM, as well as the preventive and therapeutic effects of re-initiating PTEN expression on EM by studying the cell cycle changes in primary endometrial cells after PTEN transfection, apoptosis, angiogenesis, and VEGF expression of ectopic endometrial tissues in the animal model. We hope to identify approaches to prevent and treat EM by re-expression of the PTEN gene in the near future.

Material and Methods

Reagents and tissue sources

Female severe combined immunodeficiency (SCID) mice were purchased from Shanghai B&K Universal Group Limited (license number: SCXK (Shanghai) 2013-0016). Endometrial samples were obtained from patients with hysteromyoma who had undergone hysterectomies at the Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University in August 2013. Endometrial tissue samples were collected aseptically during the operation, and treated within 1 h after collection by rinsing repeatedly in cold phosphate-buffered saline (PBS) and storing in sterile Dulbecco’s modified Eagle’s medium (DMEM) until analysis.

Ethics approval

The specimens were obtained after approval of the Ethics Committee of the Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, and informed consent was obtained from all the included patients.

Culture and identification of primary endometrial cells

Fresh endometrial specimens were placed in a sterile culture dish and washed 3 times with phosphate-buffered saline (PBS) to remove surface impurities and blood. The tissue samples were cut into 1-mm³ blocks, and 2–3 mL of 0.1% I

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Collagenase was added to the samples, which were then incubated with shaking in a 37°C thermostatic water bath for 60–100 min to digest tissues. The reaction was stopped when the tissue blocks disappeared and single cells were observed under the microscope. The cell suspension was filtered through a 300-mesh cell strainer, and the filtrate was centrifuged at 800 rpm for 5 min. The epithelial cell culture broth was shifted to a culture flask for continuous culture when the mesenchymal cells grew with adherence. Two kinds of cells were incubated at 37°C with 5% CO₂, and the medium was replaced after 24 h to remove dead non-adherent cells and blood cells. Mouse anti-human cytokeratin antibodies and mouse anti-human vimentin antibodies were selected as the specific markers, fluorescein immunothiocyanate (FITC) was used to label secondary antibodies, and the isolated cells were identified by immunofluorescence staining.

**Overexpression of PTEN and construction of silent lentiviral vector**

The PTEN gene was subcloned into the pLV-IRES-PURO plasmid after synthesis; the siRNA and siNC of the 3 PTEN genes were synthesized, and 5’-AAGTAAGGACCAGAGACAA-3’ was determined as a valid sequence from the 3 sequences. This sequence was designed as short-hairpin RNA (shRNA) and constructed into the pLV2-shPTEN and pLV2-shNC vectors. They were then transfected into 293T cell-packed lentivirus after plasmid extraction to infect primary endometrial cells. PTEN expression was measured using Western blot analysis.

**Detection of cell cycle by flow cytometry**

The samples were divided into 5 groups and each group was subjected to different conditions. Endometrial cells in the blank group were cultured in control medium. Cells in the vector and over-PTEN groups were transfected with pLV-NC and pLV-PTEN, respectively, with the pLV-NC acting as a negative control. Cells in the siNC and si-PTEN groups were transfected with lentivirus carrying the scrambled and si-PTEN sequences, respectively, to knockdown the expression of PTEN.

In order to collect the cells in the different groups, the cultures were centrifuged, the supernatant was discarded, the pellet was washed twice with cold PBS, and the cells were fixed by slowly adding 1 mL of pre-cooled 70% ethanol at 4°C for 30 min after homogeneous mixing. The cells were centrifuged again, washed with pre-cooled PBS to remove ethanol, centrifuged, resuspended in 0.2 mL PI dye liquor after discarding the supernatant, mixed with RNaseA to achieve a final concentration of 50 μg/mL, and allowed to stand at 4°C for 30 min. The precipitate was then discarded after centrifugation, the supernatant was removed, and the cells were resuspended in 0.2 mL of PBS before flow cytometry analysis.

**Detection of cell cycle and apoptosis by flow cytometry**

The cells in each of the 5 groups were collected by centrifugation and washing with PBS as described previously. The cells were suspended in 200 μL of 1× buffer and 5 μL of Annexin V, incubated in the dark for 15 min, and collected by centrifugation. Then, the cells were washed with 1× buffer and resuspended in 200 μL of 1× buffer, and tested on the flow cytometer after adding 5 μL of PI.

**Analysis of vasculogenic mimicry of cell growth**

Matrigel solution (0.5 mL in medium at a 1: 1 ratio) was seeded in the wells of a 24-well plate and incubated at 37°C for 30–90 min to promote solidification. The primary log-phase endometrial cells in all the 5 groups were washed 3 times with serum-free PBS and resuspended at the concentration of 5×10⁶ cells/mL in complete medium containing DMEM after digestion. The cells were seeded in a 24-well plate (50 μL/well) and incubated at 37°C for 72 h, and angiogenesis was observed and photographed under a phase-contrast microscope.

**Construction of human-mouse chimeric endometriosis model**

Eighteen female SCID mice were used in this study. The mice were reared in cages at 20–22°C and a relative humidity of 40–60%, and given access to food and water ad libitum. They were randomly divided into 3 groups of 6 mice each. The endometrial samples were obtained from women in the operating room of Nanjing Maternity and Child Health Care Hospital, and immediately rinsed several times with sterile PBS in a petri dish, cut with ophthalmic scissors to obtain approximately 1-mm³ tissue blocks, and placed into 5-mL centrifuge tubes. The supernatant was discarded after centrifuging at 500 rpm for 5 min, and the underlying endometrial tissue blocks were placed into the wells of a 24-well plate with DMEM/F12 containing 10% FBS, 100 U/mL penicillin, and 10 nmol/L 17β-oestradiol. Complete medium was added after conventional infection with different lentiviruses for 6 h, and the medium was renewed after 24 h. The endometrial tissue was collected after 48 h of culture, washed 3 times with PBS containing 2% FBS, and placed in 300 μL of PBS containing 2% FBS. All the above-mentioned procedures were carried out under aseptic conditions. The endometrial tissue blocks of the over-PTEN, si-PTEN, and blank groups were collected in 1.5-mL Eppendorf tubes with DMEM/F12 medium. Five 1-mm³ tissue blocks were separately injected into the abdominal skin of 6 SCID mice to establish SCID models of mice from the PTEN overexpression, PTEN knockdown, and control groups. The mice received intramuscular injections of 2 mg/kg estradiol benzoate by 5 days after implanting, and the mice were sacrificed by cervical dislocation after 20 days.
Immunohistochemical analysis of lesion tissue sections of human-mouse chimeric endometriosis model

The lesions were obtained by dissection of the mice. The specimens were embedded in paraffin and sectioned to obtain 5-μm serial sections. Hematoxylin and eosin (HE) staining was carried out for part of the biopsy tissues, and the changes in the glands, mesenchyme, and epithelium were observed under a light microscope. Two-step immunohistochemistry was used to detect the changes in the expression levels of PTEN and VEGF for part of biopsy tissues; the paraffin sections were dewaxed in xylene and hydrated in an ethanol gradient. Microwave antigen retrieval was carried out, and the sections were washed with PBS, after which serum was added. The primary antibodies were added after blocking the nonspecific binding sites, and PBS was used instead of the primary antibodies in the negative control. The mixture was then incubated in the refrigerator at 4°C overnight. The secondary antibodies were added after washing with PBS, and incubated at 37°C for half an hour. A color developing agent was added after adding SABC, and the samples were stained by hematoxylin, dehydrated, and mounted.

PTEN expression was considered positive when brown granules were observed in the cytoplasm or nucleus. VEGF stained claybank was localized in vascular endothelial cells. A score of 0 indicated no positive cells; 1 indicated 1–25% positive cells; 2 indicated 26–49% positive cells; while a score of 3 indicated >50% positive cells. The color intensity was graded as follows: 0, no color; 1, pale yellow; 2, claybank; and 3, brown. Based on their scores, each section was scored as – (0), + (2–3), ++ (4–5), and +++ (>5) by calculating the negative and positive rates in each group and calculating the P value of the difference between the groups based on the positive rate.

Statistical analysis

All values are expressed as mean ±s. Significant differences among the groups were determined using one-way analysis of variance (ANOVA). A value of P<0.05 was considered statistically significant.

Results

Culture and identification of primary endometrial cells

The isolated cells completed basic adherence after 24 h, and the epithelial cells grew into a dense monolayer with swirling arrangement 3 days after inoculation. The cells were polygonal or tadpole-shaped and exhibited clear cell boundaries. Interstitial cells were fusiform cells with fibroblast morphology (Figure 1). The isolated cells were covered with 25-cm² cell culture flasks for about 5–7 days, then digested by trypsin for passage. The endometrial epithelial cells expressed cytokeratin, and the interstitial cells expressed vimentin. Mouse anti-human cytokeratin antibodies and mouse anti-human vimentin antibodies were used for immunofluorescence staining (Figure 2).

Identification of PTEN overexpression and the interference silencing efficiency by Western blot analysis

After infecting the endometrial cells with PTEN overexpressing lentivirus, the PTEN expression was upregulated 2-fold; after infecting the cells with lentiviral vector mediating PTEN interference, the silencing efficiency was about 70% (Figure 3).

Apoptosis and changes in the cell cycle

Results of the flow cytometry revealed the percentages of apoptosis in all the groups. The percentages of apoptosis in the blank, vector, and over-PTEN groups were 11.7±0.03%, 7.90±0.07%, and 15.8±0.14%, respectively. In the siNC and si-PTEN groups, the percentages of apoptosis were 9.47±0.11% and 5.33±0.08%, respectively (Figure 4). As shown in Figure 4 and Table 1, the overexpression of PTEN significantly increased the rate of apoptosis of primary endometrial cells, whereas silencing its expression significantly reduced the apoptosis rate compared with the control group (* P Value <0.05).

Figure 5 and Table 2 present the results of the cell cycle detection using flow cytometry analysis. After transfection of the lentiviral vector overexpressing PTEN, the proportion of G0/G1 phase cells significantly increased compared with the control group. The proportion of G2/M phase gradually decreased, and the results of the analysis showed cell cycle arrest at the G0/
G1 phase in cells in the overexpression group. On the contrary, the proportion of G0/G1 phase cells significantly decreased in the PTEN knockdown group compared with the control group.

Analysis on vasculogenic mimicry

Figure 6 presents the microscopic analyses of the 5 groups. The micro-tubular structures shown in the Figure are a simulation of angiogenesis. It can be seen that angiogenesis was inhibited after PTEN overexpression in endometrial cells, angiogenesis significantly increased when PTEN expression was low.

HE staining of endometrial tissue

At low magnification, the transplants were seen to have grown into 1 or more cavity-like structures, and there were secretions in the cyst cavity. The epithelium in the inner face of the cavity, lamina propria, and gland-like structures and stromal cells of the lamina propria were similar to the corresponding cell layers in the eutopic uterus and endometrium of normal control mice (Figure 7).

Immunohistochemical results of PTEN and VEGF in the human-mouse chimeric endometriosis model

PTEN-positive staining revealed distribution of the protein in the cytoplasm or nucleus and surrounding the implants, evidenced by the brown stain. Immunohistochemical staining revealed differences in PTEN expression in the mouse endometrial lesions between the control group (Figure 8A), si-PTEN group (Figure 8B), and over-PTEN group (Figure 8C). The rate of PTEN-positive cells was significantly higher in the over-PTEN group than in the control and si-PTEN groups (Figure 9).

VEGF-positive staining was found to be localized in vascular endothelial cells, and some of the capillaries formed a...
Figure 3. Confirmation of PTEN overexpression and knockdown by Western blot. The expression of PTEN was significantly decreased in endometrial cells by si-PTEN (** P Value <0.001 vs. si-control), PTEN was significantly increased by PTEN infection (** P Value <0.001 vs. control).

Figure 4. Effect of PTEN overexpression or knockdown on the apoptosis of human endometrial cells.

Table 1. Statistical data of the apoptosis rate in five groups.

| Group          | N  | Apoptosis rate (%) |
|----------------|----|--------------------|
| Blank group    | 3  | 11.7±0.03          |
| Vector group   | 3  | 7.90±0.07          |
| siNC group     | 3  | 9.47±0.11          |
| si-PTEN group  | 3  | 5.33±0.08*         |
| Over-PTEN group| 3  | 15.8±0.14*         |

Table 2. Cell cycle status in the five groups.

| Group          | N  | G0/G1 (%)   | G2/M (%) |
|----------------|----|-------------|----------|
| Blank group    | 3  | 60.46±0.24  | 5.52     |
| Vector group   | 3  | 60.08±0.32  | 5.82     |
| siNC group     | 3  | 60.88±0.17  | 13.64    |
| si-PTEN group  | 3  | 56.64±0.32* | 3.73     |
| Over-PTEN group| 3  | 66.41±0.19* | 3.85     |
The rate of VEGF-positive cells was higher in the si-PTEN group than in the control and over-PTEN groups (Figure 11), showing that silencing the expression of PTEN may promote the formation of endometrial lesions. In other words, as the rate of VEGF-positive cells was significantly lower in the over-PTEN groups than in the si-PTEN group, PTEN overexpression may inhibit the formation of endometrial lesions.

**Discussion**

The pathogenesis of endometriosis is unknown. There are more than 10 different theories regarding the cause of the disease, but the majority scholars accept the theory of menstrual blood reflux ectopic implantation proposed by Sampson in 1927. According to this theory, EM primarily occurs due to intrauterine menstrual blood reflux into the pelvic cavity through fallopian tubes during the menstrual period, as a result of which endometrial fragments in the menstrual blood implant onto
the ovarian surface, uterus-rectum fossa, other pelvic organs, and peritoneal surfaces, causing hyperplasia, secretion, bleeding, and other ovarian hormone-induced changes. This theory aims to provide a theoretical basis for clinical intervention.

Previous research has suggested that the spontaneous apoptosis of endometrial cells is a key factor in maintaining the normal structure and function of endometrial tissue, and abnormal apoptosis can lead to implantation and survival of endometrial cells outside the uterine cavity. The anti-apoptotic ability of ectopic endometrial cells is enhanced in EM, inhibiting apoptosis. Therefore, the balance between proliferation and death of endometrial cells is destroyed, and the rate of cell death is less than the proliferation rate, and the survival time of the endometrial cells is prolonged, facilitating the proliferation and implantation of these cells outside the uterine cavity [12,13]. PTEN can inhibit cell mitosis by negatively regulating the PI3K pathway to promote apoptosis [14]. The PTEN/Akt signaling pathway can inhibit cell proliferation and induce apoptosis [15,16]. The downregulation of PTEN enhanced the anti-apoptotic ability of ectopic endometrial cells, causing changes in the cell cycle regulatory mechanisms, leading to the ectopic survival and implantation of refluent endometrium. Another study [17] showed that PTEN can inhibit integrin-mediated cell spreading, consequently reducing the adhesive capacity of endometrial cells. Reducing the PTEN expression resulted in increased invasion and metastasis ability of endometrial cells, which was beneficial to the growth of ectopic endometrial cells. We successfully conducted a primary cell culture of human endometrial cells and identified endometrial glandular epithelial cells and stromal cells in the culture, and demonstrated that silencing the PTEN gene expression could significantly reduce the rate of apoptosis of human primary endometrial cells and reduce the proportion of G0/G1 phase

Figure 7. Hematoxylin and eosin staining of lesions transplanted into the peritoneal cavities of severe combined immunodeficiency (SCID) mice (magnification, 200×). (A) Control group, (B) si-PTEN group, (C) Over-PTEN group.

Figure 8. PTEN expression in the endometrial lesions of severe combined immunodeficiency (SCID) mice (200×, Bar=100 μm). (A) Control group; (B) si-PTEN group; (C) Over-PTEN group.

Figure 9. Rate of PTEN expression in the endometrial lesions of severe combined immunodeficiency (SCID) mice.
ANGIOGENESIS is a complex process that includes many steps, such as vascular endothelial cell proliferation, migration, and extracellular matrix degradation. A variety of cytokines, growth factors, and sex hormones are involved in the regulation of angiogenesis, and the PI3K/Akt signaling pathway is also activated in this process. Kim et al. found that the enhanced Akt activity had a positive effect on the establishment of endothelial cells, which could be explored as a potential strategy for preventing and treating endometriosis.

The occurrence and development of endometrial lesions must be maintained by building new vessels [18], so angiogenesis is the central link in the pathogenesis of endometriosis. Angiogenesis is a complex process that includes many steps, such as vascular endothelial cell proliferation, migration, and extracellular matrix degradation. A variety of cytokines, growth factors, and sex hormones are involved in the regulation of angiogenesis, and the PI3K/Akt signaling pathway is also activated in this process. Kim et al. found that the enhanced Akt activity had a positive effect on the establishment of ectopic endometrial tissue [19]. Vascular endothelial growth factor (VEGF) is widely recognized as the most important pro-angiogenic factor. PI3K can be activated when VEGF combines with VEGFR, promoting PI2 to generate PI3 by phosphorylation. PI3 acts on the downstream target proteins via the PI3K/Akt signaling pathway to promote the synthesis of VEGF, thereby promoting the formation of blood vessels [20–22]. Ye et al. found that in human liver cancer, the dose-dependent performance of the PTEN inhibitor could recover the expression of VEGF and the ability of human umbilical vein endothelial cells to form capillary structures induced by hepatoma cells [23], which also illustrated that PTEN can affect angiogenesis by acting on VEGF.

Some researchers have found that the serum VEGF concentration in patients with phase I and II endometriosis is significantly higher than the levels in patients with phase III and IV endometriosis and controls, indicating that the increased serum VEGF level may be associated with early endometriosis [24]. Other researchers have speculated that VEGF may promote the occurrence and development of EM by stimulating lymphangiogenesis, intimal lymphatic metastasis, and newly generated capillaries in ectopic lesions [25].

In the present study, angiogenesis was simulated using microtubular structures, and we found that in human endometrial cells, silencing PTEN significantly increased angiogenesis, while its overexpression inhibited angiogenesis. Morphologically, this finding directly indicates the inhibitory effect of PTEN on angiogenesis. The difference of PTEN immunohistochemical staining of mouse endometriosis lesions in the PTEN-overexpression group, PTEN knockdown group, and control group confirmed the successful establishment of an animal model. In the animal model, the rate of VEGF-positive rate was higher in the si-PTEN group than in the control and over-PTEN groups, suggesting that silencing PTEN may promote the formation of endometriotic lesions. However, the proportion of VEGF-positive cells was significantly lower in the PTEN overexpression group than in the other groups, suggesting that PTEN not only promotes the apoptosis of ectopic endothelial cells but may also inhibit the formation of endometriotic lesions by acting on VEGF. Therefore, we consider that the changes in PTEN expression status will affect the occurrence and development of endometriosis, and we propose that the PTEN gene may be used for the treatment of endometriosis. In the latter part of the experiment, we further elaborated the relationship between PTEN and VEGF in the development process of endometriosis.

Figure 10. VEGF expression in the endometrial lesions in severe combined immunodeficiency (SCID) mice (200×, Bar=100 μm). (A) Control group; (B) si-PTEN group; (C) Over-PTEN group.

Figure 11. Rate of VEGF expression in the endometrial lesions of severe combined immunodeficiency (SCID) mice (*** P value <0.05).
by quantitative analysis, and study whether there is another mechanism by which PTEN affects the occurrence and development of endometriosis.

Conclusions

We found PTEN affects endometriosis by regulating angiogenesis, apoptosis, and cell cycle of endometrial cells. Our results demonstrate that PTEN inhibits the occurrence and development of endometriosis, and we propose that the PTEN gene can be used to treat endometriosis.

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Conflicts of interest

The authors have no financial conflicts of interest.