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17 β -Estradiol promotes endometrial cancer proliferation and invasion through IL-6 pathway

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Abstract

Accumulating evidence revealed that the leading risk factor of endometrial cancer is exposure to endogenous and exogenous estrogens, while the exact mechanism underlying estrogen contribution to endometrial cancer progression has not been elucidated clearly. Interleukin (IL)-6 has been verified to be critical for tumor progression in several human cancers. In this study, we provided evidence that 17 β -estradiol (E₂) could significantly promote endometrial cancer cells viability, migration and invasion through activation of IL-6 pathway, which involved in its downstream pathway and target genes (p-Stat3, Bcl-2, Mcl-1, cyclin D1 and MMP2). Meanwhile, utilization of IL-6-neutralizing antibody could partially attenuate the increased cancer growth and invasion abilities in Ishikawa and RL95-2 endometrial cancer cell lines and an orthotopic endometrial cancer model. We established a causative link between estrogen and IL-6 signaling activation in the development of endometrial cancer. The molecular mechanism defined in this study provided the evidence that E₂ promotes endometrial carcinoma progression via activating the IL-6 pathway, indicating that interruption of IL-6 might be an essential therapeutic strategy in estrogen-dependent endometrial cancer.

Key Words

- ▶ endometrial carcinoma
- ▶ estrogen
- ▶ IL-6
- ▶ proliferation
- ▶ invasion

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Introduction

Endometrial cancer is the fifth most common cancer in females (4.8% of cancers in women), who have a accumulative risk of 1% of developing the disease by age 75 years (1). Additionally, endometrial adenocarcinomas is separated into two distinct types: Type I is estrogen-dependent and derived from endometrial hyperplasia owing to overexposure of estrogen, which occurs more frequently in young women and is related to obesity, early age at menarche, nulliparity, late-onset menopause and use of tamoxifen, while type II is more aggressive and estrogen independent (2, 3). Disturbance of cell invasion and aggressive behavior is one of the main malignant characters of cancer cells (4). Therefore, understanding the underlying mechanism of cancer proliferation and invasion bio-behavior will be of great

significance for the clinical treatment of malignant tumor patients.

As one of the most important hormone-dependent cancers, the tumorigenesis and progression of endometrial cancer is often correlated with exceptional estrogen changes and estrogen-induced signaling (5). We previously verified that 17 β -estradiol (E₂) upregulated interleukin (IL)-6 expressions in endometrial cancer, which lead to the local E₂ synthesis in tumor tissues (6). IL-6 belongs to the main member of the IL-6 cytokine family and regulates multitudinous functions related to biological metabolism and immunity (7). Abnormal IL-6 cytokine expression and its aberrant downstream signaling activation are important contributors to worse clinical outcomes of malignant tumors (8).

The pro-tumorigenic behaviors of IL-6 cytokine family members are initiated by cancer cell activities or indirect effects, such as regulation of inflammation, immunosuppression and angiogenesis (9). The roles of IL-6 in the promotion of endometrial cancer invasion and migration abilities have been elucidated (10). However, the detailed mechanism of E₂ and IL-6 activation in regulation of endometrial cancer invasive and metastatic behavior is unknown.

In this paper, we determine to study whether the E₂-induced IL-6 signaling pathway participated in the proliferation, migration and invasive potential of endometrial cancer. Furthermore, the inhibition effect of IL-6-neutralizing antibody (IL-6 Ab) on increased tumor proliferation and invasion was examined not only in endometrial cancer cell lines but also in cancer orthotopic xenografts mice model. For the first time, our data define a novel mechanism of E₂ promotion on endometrial cancer and provide an effective therapeutic strategy to conquer cancer progression.

Materials and methods

Reagents and antibodies

Recombinant human IL-6 was from Peprotech (Rocky Hill, NJ, USA). E₂ was bought from Sigma. IL-6 neutralizing antibody was from R&D systems. Anti-total Stat3, anti-phospho Stat3 (p-Stat3), anti-Bcl-2, anti-myeloid cell leukemia-1 (Mcl-1), anti-CyclinD1, anti-matrix metalloproteinases 2 (MMP2) antibodies and rabbit IgG were from Epitomics (CA, USA).

Cell culture

Ishikawa and RL95-2 cells, human endometrial carcinoma cell lines, were purchased from the American Type Culture Collection (ATCC). Cells are maintained in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (FBS) according to the provider's instruction. In IL-6 Ab experiments, cells were incubated for the entire 24-h period in IL-6-neutralizing antibody or respective IgG control before addition of E₂ (10 nM). E₂, Ig G and IL-6 antibody were all added to the culture medium containing 0.1% FBS and cells were incubated for indicated time.

Cell proliferation assay

To examine cell proliferation ability, exponentially growing endometrial cancer cells were seeded into 96-well

culture plates with 2000 cells/well and was divided into the five groups: control, only in the presence of IL-6 Ab (2.5 mg/mL), E₂ (10 nM) stimulation group, E₂ plus IL-6 Ab and E₂ plus Ig G. At the time of the assay, the cells were stained with 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma) and made soluble in 150 μL of DMSO subsequently. Absorbance was examined at 570 nm using a microtiter plate reader.

Wound-healing assays

Ishikawa and RL95-2 cells were seeded into six-well plates in a monolayer with nearly complete confluence. Lesions were made using a 10 μL plastic pipette tip, and then cells were washed with PBS three times in order to remove the debris. Before stimulation with E₂ (10 nM) and incubation with IL-6 Ab (2.5 mg/mL), cells were serum starved for 24 h. The five groups were same as those in the MTT assay. The monolayer was cultured in culture medium containing 0.1% FBS for 24 h, and photographs were taken by a phase-contrast microscopy.

Cell invasion assays

Ishikawa and RL95-2 cells were trypsinized and resuspended. Approximately 2×10^5 cells in containing 0.1% FBS were plated into the upper chamber of 24-well transwell plates (Corning, 8-μm-diameter pore size) coated with Matrigel (Corning). E₂, IL-6 Ab and Ig G were all added in the top chambers. DMEM/F12 containing 10% FBS was placed into the lower chamber. After 24-h incubation, top cells on the upper chamber were gently removed with a cotton swab and the migrated cells (on the bottom of chamber) were fixed in formalin for 30 min and stained with 0.1% crystal violet solution for 20 min. The migrating cell numbers in five high fields were counted, and the means for each chamber were determined. Experiments were repeated three times.

Western blot

Whole-cell protein extracts from endometrial cancer cells were prepared with RIPA Lysis Buffer (Santa Cruz) according to the manufacturers' instructions. Total proteins were fractionated by SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were then probed with primary antibodies primary antibodies (total Stat3, p-Stat3, Bcl-2, Mcl-1, CyclinD1, MMP2 and β-actin) at 4°C overnight. After washing with TBST three times, the membranes were incubated with horseradish

peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (Cell Signaling Technology). The labeled proteins were detected by enhanced chemiluminescence. β -actin was used as an internal control.

Development of an orthotopic endometrial carcinoma model in nude mice

Orthotopic surgical endometrial cancer model was developed using athymic female nude mice (BALB/c, 4–6 weeks old, $n=6$ every group). The animals were kept in specific pathogen-free (SPF) conditions. The animal procedures were approved by the Department of Laboratory Animal Science Fudan University, Shanghai Medical College, following the Guide for the Care and Use of Laboratory Animals.

The orthotopic implantation operations were performed as the procedure previously described (11). Estrogen was supplied to the mice subcutaneously using 17 β -estradiol 90-day release pellets (0.72 mg/pellet). Mice received a weekly dose of IL-6-neutralizing antibody. Drug treatments were given right after implantation of tumor tissue.

After 6 weeks, the animals were killed and the peritoneal cavity was examined macroscopically. Each uterus and tumor lesions were processed for hematoxylin and eosin staining and histological examination. Procedures for immunohistochemical analysis of p-Stat3, Bcl-2, Mcl-1, CyclinD1, MMP2 and Ki-67 were done as described previously (6). Briefly, the paraffin tissue was dehydrated and underwent antigen retrieval by microwave. Then, the tissue was incubated with primary antibody and peroxidase-labeled secondary antibody. Finally, the sections were counterstained with hematoxylin.

Statistical analysis

All statistical analyses were analyzed employing Statistical Package for the Social Sciences version 17.0 (SPSS). Student's *t*-test was performed for analyzing the differences between two groups. A value of $P < 0.05$ was considered statistically significant.

Results

E₂ could increase the growth ability of endometrial cancer cells through IL-6 pathway

In survival analysis, administration of E₂ was found to significantly increase cells growth ability of Ishikawa and RL95-2 cells. The significant difference was seen from day 5, whereas simultaneous treatment with IL-6-neutralizing antibody could reduce the increased proliferation ability more than 60%. No obvious changes of cell proliferation were observed when addition of antibody alone. When Ishikawa and RL95-2 cells were simulated with E₂ plus Ig G, the increased cell proliferation abilities induced by E₂ were not significantly reduced (Fig. 1A and B).

E₂ enhanced the endometrial cancer cells migration and invasion ability

Wound-healing assay was performed to investigate the migratory ability of endometrial cancer cells. The wounded gap closure was observed accelerating in the E₂ incubation group compared to the control (1.68-fold and 2.37-fold for Ishikawa and RL95-2 cell respectively), which could not be observed in the IL-6 Ab-treated alone group. The accelerated wound healing stimulated by E₂ was partially attenuated by 58 and 85% for Ishikawa

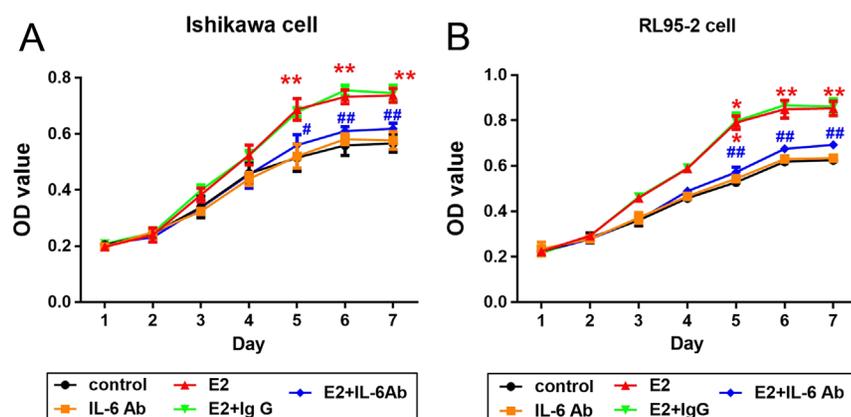


Figure 1
E₂ promotes endometrial cell lines proliferation and increases IL-6 protein production. Growth curves of Ishikawa (A) and RL95-2 cells (B) treated with E₂, in the presence or absence of IL-6-neutralizing antibody. Cell growth was measured by MTT assay. IL-6 Ab: IL-6-neutralizing antibody. Ig G was used as a negative control. ** $P < 0.01$ versus control group; ## $P < 0.01$ versus E₂-treated group.

and RL95-2 cells respectively, when in the presence of IL-6 Ab (Fig. 2A).

In the transwell invasion experiment, E₂ enhanced the numbers of cancer cells in the upper chamber that migrated to the lower chamber part compared to the control group (3.96-fold and 3.50-fold for Ishikawa and RL95-2 cells

respectively). The elevation cell numbers were reduced by adding IL-6 Ab (Fig. 2B). No obvious changes of cell migration and invasion were observed in E₂ plus Ig G group compared with E₂-treated group. From these observations, we found out that IL-6 participated in E₂-triggered the endometrial cancer cells migration and invasion.

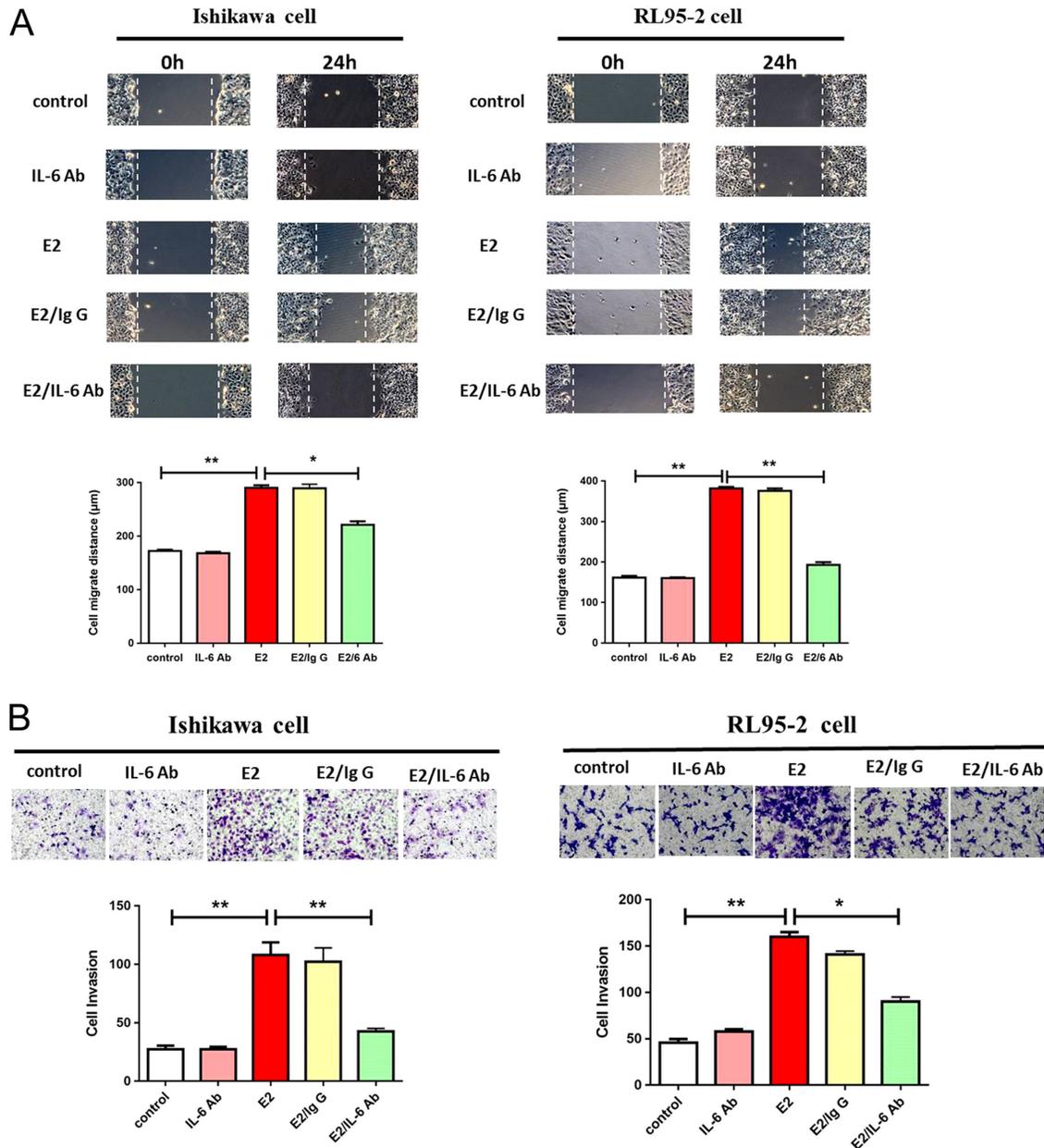


Figure 2

E₂ increased the migration and invasion of Ishikawa and RL95-2 cells. (A) Ishikawa and RL95-2 cells treated with E₂ demonstrated increased wound recovery at 24 h after wounding, which was attenuated by IL-6-neutralizing antibody. (B) Transwell assay results showed that E₂ promoted cell invasive capacity of Ishikawa and RL95-2 cells, which was inhibited by IL-6-neutralizing antibody. IL-6 Ab: IL-6 neutralizing antibody. Ig G was used as a negative control. **P* < 0.05, ***P* < 0.01. Experiments were repeated three times.

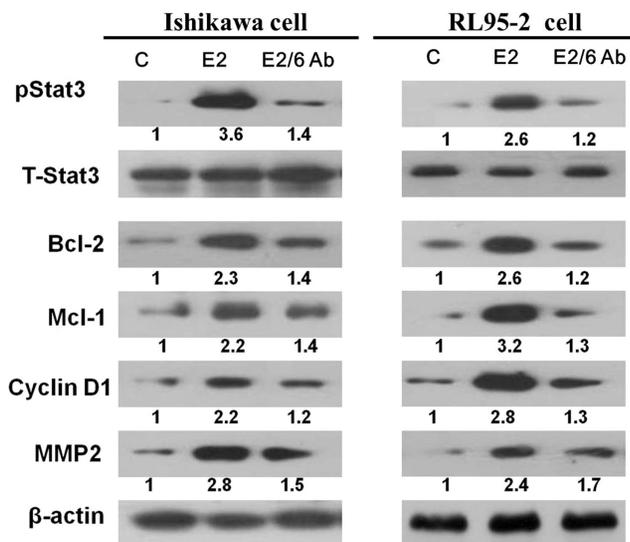


Figure 3
Western blot analysis to measure the phosphorylated and total Stat3 levels and its target genes expression in Ishikawa and RL95-2 cells after treatment with E₂ with or without IL-6-neutralizing antibody.

E₂ activated the IL-6 downstream signaling pathway and its target genes

Next, we explored the mechanism underlying E₂-triggered IL-6 downstream pathway. An increased expression of Stat3 phosphorylation was found when Ishikawa and RL95-2 cells were incubated with E₂, while simultaneous addition with IL-6 Ab could attenuate the enhancement. Meanwhile, changes of total Stat3 expression was not observed, showing that the upregulation of Stat3 phosphorylation was not attributed to the increased protein expression. Furthermore, we investigated the expression of IL-6/Stat3 target genes including Bcl-2, Mcl-1, CyclinD1 and MMP2. The results showed that stimulation with E₂ elevated Bcl-2, Mcl-1, CyclinD1, MMP2 protein expression in endometrial cancer cells, while IL-6 Ab partially attenuated the enhanced expression of these proteins Fig. 3.

Effects of E₂ and IL-6 neutralizing antibody in an orthotopic endometrial carcinoma model

Histological examination verified that the orthotopic tumor was observed growing from the uterine and was surrounded with normal endometrial stromal and epithelial cells. The mean tumor volume in E₂-treated mice group elevated obviously compared with the control group, and IL-6 Ab treatment could reduce the elevated tumor volumes (Fig. 4).

Using immunohistochemistry, we examined an enhancement in nuclear staining of p-Stat3, Bcl-2, CyclinD1, Ki-67 and cytoplasm staining of Mcl-1, MMP2 in E₂-treated animals, which was decreased by IL-6 Ab (Fig. 5). The percentages of positive staining of every parameter were also calculated.

Discussion

The main risk factor for the tumorigenesis and development of endometrial cancer is associated with a prolonged estrogen exposure and aberrant estrogen excess (12). Then, comprehension of the regulatory mechanisms of estrogen that control these actions is pivotal to increase endometrial cancer patient survival. The relationship of estrogen and IL-6 has been elucidated in several studies (13, 14).

Our previous findings elucidate the mechanism that E₂ could stimulate IL-6 expression in Ishikawa and RL95-2 cells, which upregulated aromatase expression in stromal cells (6). Other groups also confirmed that IL-6 was highly upregulated in human cancers and correlated with a negative prognosis in these patients (15). In this report, we determined to investigate whether the IL-6 pathway was involved in the regulation of E₂-induced proliferation, migration and invasive potential of endometrial cancer cells. We found that E₂ could significantly promote endometrial

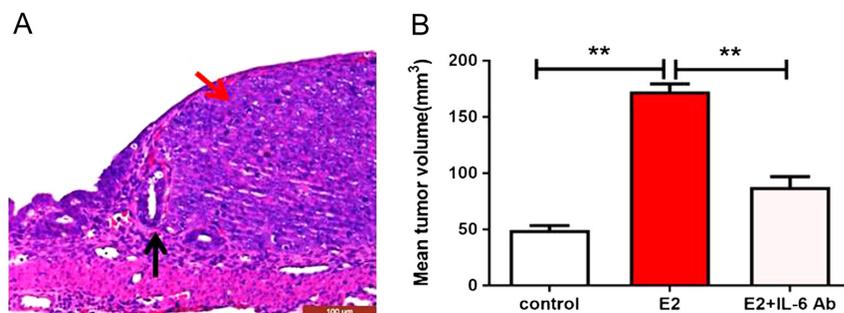


Figure 4
Development and characterization of an orthotopic endometrial cancer model. (A) Microscopic view of an orthotopic tumor. Black arrow, normal endometrium; red arrow, orthotopic tumor. Original magnification ×200. Scale bar, 100 μm. (B) Mean orthotopic tumor volumes of control, E₂-treated and IL-6-neutralizing antibody administration group. ***p* < 0.01.

cancer proliferation and invasion, which is consistent with the research results of other groups (16, 17). Furthermore, our data provide evidence that the increased proliferation and the invasion capacity of the endometrial cancer cells elevated by E₂ was partially inhibited by IL-6 Ab, which demonstrated that E₂ might promote cancer progression through IL-6 signaling pathway. Inhibitors of IL-6 pathway, including IL-6, IL-6 receptor and janus kinase (JAK) have all been approved by Food and Drug Administration (FDA) in various malignancies, and other novel inhibitors of the IL-6/JAK/Stat3 signaling pathway are currently in the process of clinical and/or preclinical development (18, 19, 20). In this paper, IL-6-neutralizing antibody was also utilized to block IL-6 pathway and verified to effectively impede endometrial cancer proliferative and invasive potential enhanced by E₂.

Furthermore, we demonstrated that inhibition of IL-6 not only significantly suppressed E₂-induced endometrial cancer cells proliferation and invasion capacity, but also reduced increased expression of p-Stat3. During malignant transformation, in response to IL-6 stimulation, Stat3 is constitutively activated by phosphorylation, and then becomes homo- or hetero-dimers that translocate from cytoplasm to the nucleus, acting as transcriptional activators specific for a series of downstream genes (18). In our study, we found that E₂ could activate IL-6/Stat3 phosphorylation, and IL-6 Ab attenuates the enhancement. This implies that E₂ increased the IL-6 expression, which sequentially activated Stat3 phosphorylation and IL-6-neutralizing antibody blocked Stat3 activation.

There are several lines of evidence supporting the pivotal role of Stat3 activation in initiating the downstream cascade of events in human malignant

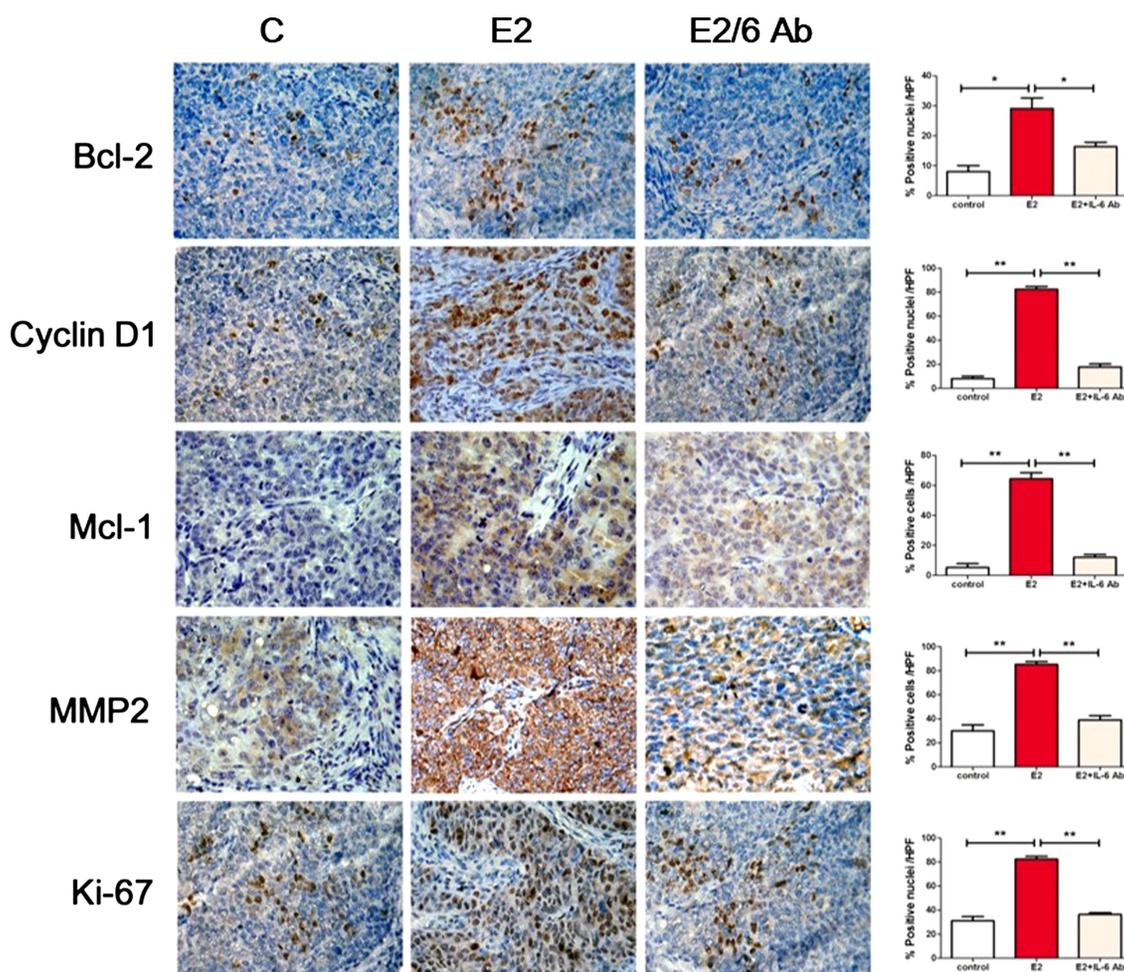


Figure 5 Immunohistochemical studies of Bcl-2, Mcl-1, CyclinD1, MMP2 and Ki-67 expression in tumor samples taken at the time of killing. The percentages of positive staining of were calculated and graphed (*right*). **P* < 0.05, ***P* < 0.01.

tumors, thereby contributing to tumor progression (21, 22). Then, we evaluated the expression of Bcl-2, Mcl-1, CyclinD1 and MMP2, which are all Stat3 target genes (23). Bcl-2 and Mcl-1 are both anti-apoptotic oncoproteins and play an important role in regulating cell survival and proliferation via protein–protein interactions (24). Cyclin D1 has attracted extensive attention due to the prevalence of its aberrant expression in human cancers, which responds to a variety of growth factors and makes cell over proliferation out of control. Nuclear cyclin D1 accumulation leads to abnormal cell cycle progression and drive inappropriate cell division (25). MMP2 is a type IV collagenase and has been considered to be one of the key enzymes in the invasion and metastasis cascade of malignant tumors (26). The upregulation of Bcl-2, Mcl-1 and CyclinD1 might explain the enhanced proliferation capacity induced by E₂ in endometrial cancer cells and elevated invasive potential might be due to increased MMP2 expression. IL-6 Ab interferes with IL-6 binding to IL-6 receptor and interrupts Stat3 downstream genes: Bcl-2, Mcl-1, CyclinD1 and MMP2. These results indicate that the impertinent phosphorylation of Stat3 may be responsible for E₂-triggered endometrial cancer progression by increasing the expressions of target genes. But the detailed mechanism remains to be determined experimentally.

Except for cell lines experiments, we also employed a well-characterized orthotopic xenograft model to illuminate the therapeutic potential of IL-6 Ab. There has been research certifying that orthotopical animal model could better imitate tumor microenvironment and more precisely recapitulate the histological character of human tumors (11, 27). We observed that the tumor volumes are predominately larger in E₂-treated group and administration of IL-6 Ab could reduce the increased tumor volumes. Meanwhile, E₂ could upregulate the expression of Bcl-2, Mcl-1, CyclinD1, MMP2 and Ki-67, and IL-6 Ab attenuated the enhancement. These findings were in accordance with the results *in vitro* and further proved the involvement of IL-6 and its signaling pathway in the E₂-induced endometrial cancer development.

In summary, our results elucidate that the E₂-activated IL-6 pathway may be vital for proliferative and invasive behavior of endometrial cancer, which included the Stat3 signaling pathway and its downstream target genes. These findings imply the significant role of estrogen in regulating malignant tumor progression. A better understanding of E₂ signaling and gene dysregulation in endometrial cancer will lead to the identification of a novel molecular

targets and predictive biomarkers for endometrial cancer intervention.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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