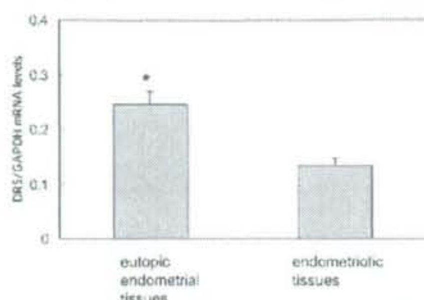


**Figure 5** DR5 mRNA levels in eutopic endometrial tissues and endometriotic tissues of women with endometriosis. Both tissues were collected from eight women with endometriosis. Total RNA isolated from the tissues was reverse transcribed and amplified by real-time PCR. The levels of RNA were calculated by subtracting the signal threshold cycles of the internal standard (GAPDH) from the threshold cycles of DR5. Values are the mean ± SEM. *$P < 0.005$ versus endometriotic tissues.

**Table I Percentage of apoptotic cells in eutopic endometrial cells from women with ($n = 4$) or without ($n = 3$) endometriosis.**

| | Without endometriosis | With endometriosis |
|---|---|---|
| control | $10.9 \pm 0.8^a$ | $11.5 \pm 3.7$ |
| Tunicamycin | $27.1 \pm 4.4^b$ | $18.3 \pm 4.5$ |
| TRAIL | $13.8 \pm 1.3^a$ | $12.9 \pm 3.7$ |
| Tunicamycin + TRAIL | $29.5 \pm 4.4^b$ | $27.0 \pm 4.7$ |

Endometrial stromal cells of women with or without endometriosis were cultured with or without tunicamycin (2 μg/ml) for 16 h, then treated with or without tumor necrosis factor-related apoptosis-induced ligand (TRAIL, 200 ng/ml) for 24 h. Values are mean ± SEM. $P < 0.05$, a versus b.

In women with endometriosis, tunicamycin in combination with TRAIL appeared to enhance the apoptosis of eutopic endometrial cells. However, the levels of apoptosis induced by tunicamycin with TRAIL in the eutopic endometrial cells were low, about a third of that observed in ESC. These findings suggest that ESC are more sensitive to the tunicamycin-enhanced TRAIL-induced apoptosis than eutopic endometrial cells. Whether this characteristic might have any relevance to the pathophysiology of endometriosis would be a matter of future study.

In summary, tunicamycin induces ER stress and increases levels of DR5 mRNA, a death receptor for TRAIL, in ESC. Tunicamycin also substantially increases TRAIL-induced apoptosis in ESC in a caspase-dependent manner, and the effect is mediated by increasing DR5 mRNA.

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# Interleukin (IL)-1$\beta$ Stimulates Migration and Survival of First-Trimester Villous Cytotrophoblast Cells through Endometrial Epithelial Cell-Derived IL-8

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**IL-1, secreted by human embryos and trophoblast cells, is important for successful implantation and pregnancy. We previously reported that IL-1$\beta$ induced IL-8 production in human endometrial stromal cells (ESCs) and that induction was regulated by substances implicated in implantation. In the present study using human primary cells in culture, we measured IL-1$\beta$-induced production of IL-8 from endometrial epithelial cells (EECs) and ESCs and examined effects of the endometrium-derived IL-8 on migration and number of first-trimester villous cytotrophoblast cells (vCTs). Both basal and IL-1$\beta$-induced IL-8 levels of cell supernatants were much higher in EECs than ESCs. Addition of IL-1$\beta$ to EECs increased the chemotactic activity of the supernatants to vCTs, and this effect was suppressed by immunoneutralization with anti-IL-8 antibody. Supernatants of IL-1$\beta$-stimulated EECs yielded significantly higher number of vCTs compared with those of untreated EECs, and the effect was inhibited by IL-8 antibody. These findings suggest that IL-1 promotes implantation by stimulating EECs to produce IL-8, which subsequently induces migration of vCTs and contributes to survival of vCTs. (*Endocrinology* 150: 350–356, 2009)**

Migration and survival of trophoblast cells are essential for establishing a pregnancy. These phenomena are controlled elaborately by various substances that are secreted from the embryo, the endometrium, or both during the implantation process (1–5). In particular, molecular cross talk between the embryo and endometrium is indispensable for their coordinated development, which is required for successful implantation (6, 7).

IL-1 is a typical cytokine that affects the implantation process at the interface between the embryo and endometrium (8–10). In view of the findings that IL-1 increases secretion of prostaglandin E2 and leukemia inhibitory factor (LIF) and expression of the integrin $\beta$3-subunit in human endometrium (11–13), IL-1 may be one of the first signals that the blastocyst exerts on the endometrium. The notion is also supported by evidence of IL-1 expression in human embryo and trophoblast as described below. It has been shown that successful implantation after *in vitro* fertilization is correlated positively with high concentrations of IL-1$\alpha$ and IL-1$\beta$, two active ligands of IL-1, in the embryos' culture media (14, 15). Immunohistochemical studies have demonstrated the localization of IL-1$\beta$ in first-trimester villous cytotrophoblast cells (vCTs) (16, 17). In addition, an *in vivo* study in mice has shown that administration of IL-1 receptor antagonist significantly reduces the number of implanted embryos (18).

With the aim of gaining a better understanding of the interaction between the human endometrium and trophoblast, a recent study identified the gene expression profile of endometrial stromal cells (ESCs) cocultured with first-trimester trophoblast explants (19). The study revealed that one of the most up-regulated genes was chemokine IL-8, which was up-regulated more than 300-fold. In another study, IL-8 from decidual natural killer cells promoted invasion of first-trimester extravillous trophoblast cells (20). These findings imply that endometrial IL-8 is involved in the process of implantation under the stimulus of trophoblast.

Endometrial IL-8 acts as not only a chemoattractant of leukocytes but also an autocrine growth factor (21). IL-8 acts on cells through IL-8 chemokine receptors CXCR-1 and CXCR2, which are expressed in endometrium (22). In contrast to the roles

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Abbreviations: CCK-8, Cell Counting Kit-8; CXCR, chemokine receptor; DNase I, deoxyribonuclease I; EEC, endometrial epithelial cell; EEC-SN, supernatant of EEC; ESC, endometrial stromal cell; ESC-SN, supernatant of ESC; F12, Ham's F12 medium; FBS, fetal bovine serum; HPF, high-power field; IL-8Ab, IL-8 antibody; LIF, leukemia inhibitory factor; mIgG, mouse IgG1; PF, peritoneal fluid; PL, peritoneal leukocyte; vCT, villous cytotrophoblast cell.



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of IL-8 on endometrium, its chemotactic and proliferative effects on first-trimester vCTs still remain obscure. As for expression of the receptors, a study has shown that CXCR2 is expressed in primary first-trimester trophoblast cells and a trophoblast cell line BeWo cells but has not examined the CXCR1 expression (23).

We reported previously that IL-1 induces IL-8 production in human ESCs and the induction is modulated by substances that are implicated in implantation (24, 25). Combined with the above findings, we hypothesized that the development of trophoblast in early pregnancy is affected by endometrial IL-8, which is up-regulated by embryo-derived IL-1. To address this issue, we first examined IL-1$\beta$-induced production of IL-8 from human endometrial epithelial cells (EECs) and ESCs. We then assessed effects of the culture media from EECs stimulated by IL-1$\beta$ on migration and number of human first-trimester vCTs.

## Materials and Methods

### Reagents and materials

Type I collagenase, antibiotics, and magnesium sulfate ($MgSO_4$) were purchased from Sigma (St. Louis, MO). DMEM and Ham's F12 medium, DMEM/F12, 0.25% trypsin and 0.25% trypsin/EDTA, were from Life Technologies (Rockville, MD). Charcoal-stripped fetal bovine serum (FBS) was from HyClone (Logan, UT). Ficoll-Paque Plus (1.077 g/ml) was from Amersham Biosciences (Piscataway, NJ). Mouse monoclonal antihuman IL-8 antibody (IL-8Ab), human recombinant IL-1$\beta$ and human recombinant IL-8 were from R&D Systems (Minneapolis, MN). Isotype control mouse IgG1 (mIgG) and mouse monoclonal antibodies to human pan-cytokeratin (AE1/AE3), human vimentin, and human CD45 were from Dako (Glostrup, Denmark). Mouse monoclonal antibody to human cytokeratin 7 was from AbD Serotec (Oxford, UK). Deoxyribonuclease I (DNase I) was from Takara (Tokyo, Japan).

### Tissue sources

Endometrial tissues were obtained from a total of 20 women (43.7 $\pm$ 4.2 yr, mean $\pm$ SD) undergoing hysterectomy for benign gynecological conditions such as uterine fibroids without endometrial pathologies. Although the relatively high reproductive age range of the subjects and the myometrium pathology results may place some limitations on the present study, we used these samples due to the unavailability of endometrial tissue in healthy young women. All subjects had regular menstrual cycles and had not received hormone therapy for at least 6 months before surgery. Placental tissues between 5 and 8 wk of gestation (7.4 $\pm$ 6.7 wk) were obtained from a total of 25 women (29.6 $\pm$ 6.7 yr) undergoing elective terminations of pregnancy for isolation of first-trimester vCTs. Peritoneal leukocytes (PLs) were used as a positive control in the experiment of RT-PCR. To obtain PL, peritoneal fluid (PF) with PL was obtained from a woman with endometriosis undergoing laparoscopy. PF was collected via a laparoscopic cannula introduced into the cul-de-sac before starting any manipulative procedures. The Institutional Review Board of the University of Tokyo approved this study, and written informed consent for use of the tissue samples was obtained from each woman. The tissues were collected under sterile conditions and were processed for primary cell cultures.

### Isolation, purification, and culture of EECs, ESCs, first-trimester vCTs, and PLs

The isolation and culture of human EECs and ESCs was carried out as described previously (2, 26). Endometrial tissues were minced and incubated in DMEM/F12 containing 0.25% type I collagenase and 15 U/ml DNase I for 60 min at 37 C. The resulting dispersed endometrial cells were separated by filtration through a 40-$\mu$m nylon cell strainer (Becton Dickinson, Lincoln Park, NJ). Intact endometrial epithelial glands were retained by the strainer, whereas dispersed ESCs passed through the strainer into the filtrate. ESCs in the filtrate were collected by centrifugation and resuspended in DMEM/F12 containing 5% FBS and antibiotics. ESCs were then plated in a 100-mm culture plate and incubated at 37 C in a humidified 5% $CO_2$-95% air atmosphere. At the first passage, ESCs were plated at a density of $2 \times 10^5$ cells/well into 12-well culture plates for experimental use. Cells enriched with endometrial epithelial glands were collected by backwashing the strainer with DMEM/F12, plated in a 100-mm plate, and incubated at 37 C for 60 min to allow any contaminating stromal cells to attach to the plate wall. The nonattached epithelial glands formed a monolayer of EEC after attachment to culture plates. EECs at a density of $2 \times 10^5$ cells/well in 12-well culture plates were used for the experiments.

The isolation and culture of first-trimester vCTs were performed as described previously (2). Briefly, the first-trimester placental tissues were washed in PBS, and the soft villous material was cut away from connective tissue and vessels. The washed tissue was incubated in sterile PBS containing 1 mM $MgSO_4$, 0.125% trypsin, and 30 U/ml DNase I for 30 min at 37 C with mild stirring. The suspension was then filtered through a 100-$\mu$m nylon cell strainer, and the cells were centrifuged at $200 \times g$ for 5 min to obtain a cell pellet, which was resuspended in DMEM/F12 with 5% FBS. The cell suspension was layered onto Ficoll-Paque Plus and centrifuged at $150 \times g$ for 15 min. The cells recovered from the interface were washed with PBS and resuspended in DMEM/F12. The remaining leukocytes were removed by plating the cells for 30 min, followed by aspiration of the supernatant enriched with cytotrophoblast cells. The cells were washed with PBS, the medium was changed to DMEM/F12 with 10% FBS, and the cells were placed in type IV collagen-coated plates (BD Biosciences, Bedford, MA) and incubated at 37 C in a humidified 5% $CO_2$-95% air atmosphere. The cells were plated at a density of $2 \times 10^4$ cells/well in 96-well plates for cell number assays, and at a density of $4 \times 10^5$ cells/well in 6-well plates for RT-PCR and *in vitro* migration assay. After incubation for 24 h, the cells were ready for experimental use.

PLs were collected as previously described (27). PF containing peritoneal leukocytes was centrifuged at $200 \times g$ for 5 min, and the supernatant removed. The cell pellet was resuspended in PBS, layered onto Ficoll-Paque Plus and centrifuged at $150 \times g$ for 30 min. PLs were recovered from the interface.

We confirmed the purity of EECs, ESCs, vCTs, and PLs by immunocytochemistry as we described previously (2). The purity of EEC preparations was greater than 95%, as judged by positive cellular staining for pan-cytokeratin (a marker for epithelial cells), negative cellular staining for vimentin (a marker for stromal cells), and CD45 (a marker for leukocytes). The purity of ESC preparations was greater than 98%, as judged by positive cellular staining for vimentin and negative cellular staining for pan-cytokeratin and CD45. The purity of vCT preparations was greater than 90%, as judged by positive cellular staining for cytokeratin 7 (a marker for trophoblast cells) and negative staining for vimentin and CD45. The purity of PLs was greater than 90%, as judged by positive cellular staining for CD45.

### Treatment of endometrial cell cultures

When ESCs and EECs approached confluence, the complete media were removed and replaced with fresh media and antibiotics, and the cells were cultured for 24 h. The wells were then replenished with FBS-free media containing different concentrations of IL-1$\beta$ (0, 0.1, 1, and 10 ng/ml) and incubated for an additional 24 h. After IL-1$\beta$ treatment, the cell supernatants of ESCs and EECs (ESC-SN and EEC-SN, respectively) were collected, centrifuged and stored at −80 C for subsequent analysis.

### Measurement of IL-8 in endometrial cell supernatants

Concentrations of IL-8 in endometrial cell culture media were measured using human IL-8 ELISA kit (R&D Systems) following the manufacturer's protocol. Absorbance was read at 450 nm with the DigiScan microplate reader (ASYS Hitech GmbH, Eugendorf, Austria). The total amount of IL-8 in the culture medium was calculated from the liquid

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volume and the concentration of IL-8. After collection of the culture media, the cultured cells were homogenized, and the amount of total protein in the homogenized cells was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Data of IL-8 content in the culture media were normalized against the total protein content of the cell lysates.

### *In vitro* migration assay

*In vitro* migration assay was performed in 24-well plates containing Transwell permeable supports with an 8-μm polycarbonate membrane (Costar, Cambridge, MA) for vCTs as previously described (2). EEC-SNs that had or had not been stimulated by 0.1, 1, and 10 ng/ml IL-1β for 24 h [IL-1β (0.1, 1, 10 ng/ml) EEC-SN and control EEC-SN, respectively] or FBS-free medium with or without IL-8 (100 pg/ml) was added to the lower chambers. In the experiments using the neutralizing antibody, control EEC-SN and EEC-SN that had been stimulated by 1 ng/ml IL-1β for 24 h [IL-1β (1 ng/ml) EEC-SN] were preincubated for 1 h with 1 μg/ml of IL-8Ab or isotype control mIgG and plated in the lower chambers. Cultured vCTs were plated at a density of $2 \times 10^5$ cells/well in the upper chamber of the Transwell membranes, which contained 100 μl FBS-free DMEM/F12, and were incubated for 72 h at 37 C in a 5% $CO_2$ atmosphere. After the incubation, the upper surface of the membranes was gently cleansed with a cotton swab. Then the cells that had migrated through the pores were fixed with acetone/methanol and stained with hematoxylin and eosin. The filter was gently cut from the chamber, and the migrated cells were counted from the underside of the filter. The number of vCTs that migrated across the filters was counted in 10 randomly selected high-power fields (HPFs) per filter under the light microscope.

### Measurement of vCT number

To measure the cell number, we used the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) as we previously described (28).

The cultured medium was removed from vCTs and replaced with fresh medium supplemented with antibiotics 24 h before treatment. Cultured vCTs were treated with control and IL-1β (0.1, 1, 10 ng/ml) EEC-SN or FBS-free medium with or without 100 pg/ml IL-8. In the experiments using neutralizing antibody, control and IL-1β (1 ng/ml) EEC-SN were preincubated for 1 h with 1 μg/ml IL-8Ab or isotype control mIgG before the treatment of vCTs was started. After 72 h incubation, CCK-8 assay was performed.

In the CCK-8 assay, CCK-8 solutions containing the tetrazolium salt WST-8 were added and incubated at 37 C for an additional 2 h. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product in culture medium. The amount of formazan, which is directly proportional to the number of living cells, was evaluated by measuring the OD at 450 nm in the DigiScan microplate reader (ASYS Hitech).

### RT-PCR of *CXCR1* and *CXCR2* mRNA

RT-PCR was performed as reported previously (29). Total RNA was extracted from vCTs and PLs using the RNAeasy minikit (QIAGEN, Hilden, Germany). Reverse transcription was performed using ReverTra Ace-α (Toyobo, Tokyo, Japan). One microgram of total RNA was reverse transcribed in a total volume of 20 μl, and cDNA was amplified using oligonucleotide primers based on the human *CXCR1* and *CXCR2* sequences. PCR was performed using the ReverTra Dash kit (Toyobo) according to the manufacturer's instructions. *CXCR1* primers (sense, 5'-TACTGTTGGACACACCTGGC-3'; antisense, 5'-TAGACATCAGTGACGGAGCG-3') were designed to amplify a 294-bp fragment. *CXCR2* primers (sense, 5'-CACAGTGAAGACATCGGTGG-3'; antisense, 5'-AGGGATTCTGGTTCACATGG-3') were designed to amplify a 246-bp fragment. The expression levels of *CXCR1* and *CXCR2* mRNA were normalized to *GAPDH* mRNA, which was used as an internal control and as a loading control. Human *GAPDH* primers (Toyobo) were designed to amplify a 452-bp fragment. The PCR conditions for *CXCR1* consisted of 35 cycles at 98 C for 10 sec, 60 C for 4 sec, and 74 C for 12 sec. The PCR conditions for *CXCR2* consisted of 35 cycles at 98 C for 10 sec, 60 C for 4 sec, and 74 C for 12 sec. PCR products were purified using the QIAEX II gel extraction kit (QIAGEN), and their sequence identities confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

### Statistical analysis

Data were evaluated using ANOVA with *post hoc* analysis using Fisher's protected least-significance difference test. $P < 0.05$ were accepted as significant.

## Results

### Both basal and IL-1β-induced IL-8 levels of culture media are higher in EECs than ESCs

Both ESCs and EECs have been reported to produce IL-8 in response to IL-1 (24, 25, 30). IL-1 has two bioactive ligands, IL-1α and IL-1β, possessed of similar biological effects (31). Therefore, in the present study, we used IL-1β as a representative of IL-1 ligands, and compared IL-8 production in response to IL-1β between ESCs and EECs. As shown in Fig. 1, both the basal IL-8 levels and the IL-1β-induced IL-8 levels were higher in EEC-SN than in ESC-SN. The basal IL-8 level of EEC-SN was 72-fold higher than that of ESC-SN. The IL-8 level of media in EECs stimulated by 1 ng/ml IL-1β was 5 times higher than that of ESCs. Based on these results, we chose to use the EEC-SN in all subsequent experiments to evaluate the effects of endometrial IL-8 on first trimester vCT.

### Migration of first-trimester vCTs is stimulated by EEC-SN treated with IL-1β via IL-8

To study the chemotactic effects of EEC-derived IL-8 on first-trimester vCTs, *in vitro* migration assay was performed. As illustrated in Fig. 2A, more vCTs migrated to IL-1β (0.1, 1, 10 ng/ml) EEC-SN than control EEC-SN. The effect appeared to be maximal at 1 ng/ml. Recombinant IL-8 also promoted the chemotaxis of

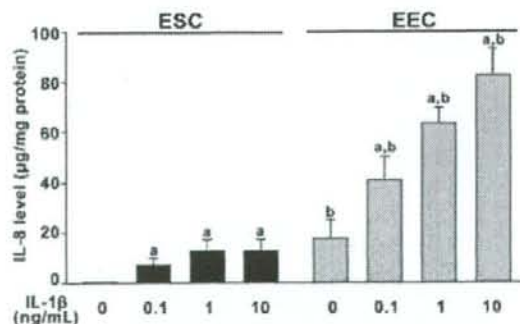


FIG. 1. IL-1β-stimulated IL-8 secretion from ESCs and EECs. Human ESCs (n = 10) and EECs (n = 10) were cultured in FBS-free media with different doses of IL-1β for 24 h. The supernatants were collected and assayed for IL-8 by ELISA. Values are normalized to the total protein content of the cell lysate. The values (total IL-8 content of the supernatants/total protein content of the cell extract, micrograms per milligram protein) are presented as the mean ± SEM of the combined data of separate experiments using different ESC and EEC preparations. a, $P < 0.05$ when compared with the same cell type without IL-1β stimulation; b, $P < 0.05$ when compared with ESCs stimulated by the same dose of IL-1β.

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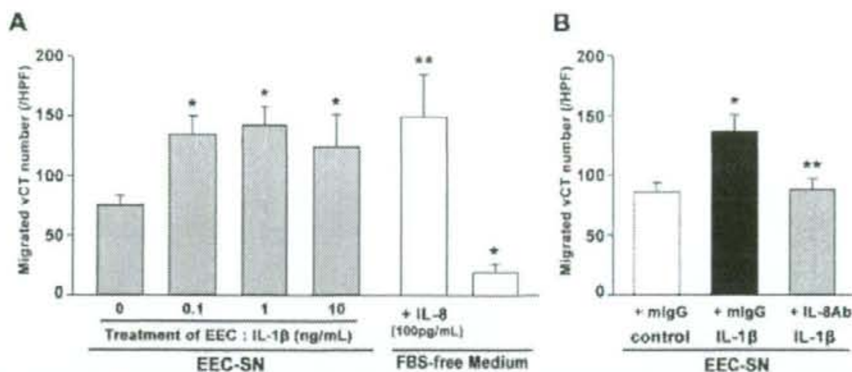


FIG. 2. Stimulatory effect of EEC-SN treated with IL-1β on migration of first-trimester vCTs through IL-8. *In vitro* migration assay was performed to determine whether migration of human first-trimester vCTs was affected by endometrial IL-8 expression. Cultured vCTs were plated at a density of $2 \times 10^5$ cells in the upper chambers of Transwell membranes for 72 h. After incubation, vCTs that had migrated through the pores were fixed and counted. The number of vCTs that had migrated across the filters was counted in 10 randomly selected HPFs per filter under the light microscope. Values represent the cell number per HPF. A, EEC-SN that had or had not been treated with IL-1β for 24 h (IL-1β EEC-SN and control EEC-SN, respectively) or FBS-free media with or without IL-8 were plated in the lower chambers. Values are the mean ± SEM of the combined data from six independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN; **, $P < 0.05$ when compared with FBS-free media without IL-8. B, Control EEC-SN and EEC-SN that had been treated with 1 ng/ml of IL-1β for 24 h [IL-1β (1 ng/ml) EEC-SN] were preincubated for 1 h with 1 μg/ml of anti-IL-8Ab or isotype control mIgG and plated in the lower chambers. Values are the mean ± SEM of the combined data from 10 independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN + mIgG; **, $P < 0.05$ when compared with IL-1β (1 ng/ml) EEC-SN + mIgG.

vCTs. In contrast, immunoneutralization with IL-8Ab abolished the chemotactic activity of IL-1β (1ng/ml) EEC-SN (Fig. 2B).

### EEC-SN treated with IL-1β yielded higher number of first-trimester vCTs via IL-8

To study the effects exerted by EEC-derived IL-8 on number of first-trimester vCTs, we used CCK-8 assay. As illustrated in Fig. 3A, when compared with control EEC-SN, IL-1β (0.1, 1, 10 ng/ml) EEC-SN up-regulated the vCT number. Recombinant IL-8 also increased in the vCT number. The effects of IL-1β (1ng/ml) EEC-SN were eliminated by immunoneutralization with IL-8Ab (Fig. 3B).

### *CXCR1* and *CXCR2* mRNA are expressed in first-trimester vCTs

To examine the expression of IL-8 receptors in first-trimester vCTs, RT-PCR was performed using primers specific to *CXCR1* and *CXCR2* (receptors for IL-8). A sample of PLs was used as a positive control for IL-8 receptor-expressing cells (32–35). As demonstrated in Fig. 4, mRNAs of IL-8 re-

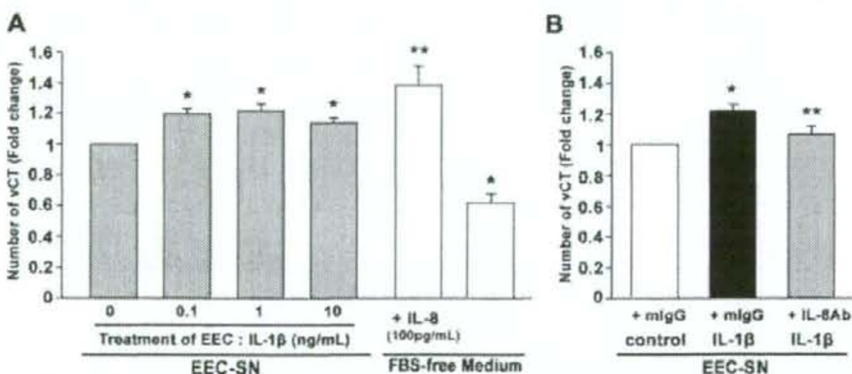


FIG. 3. Stimulatory effect of EEC-SN treated with IL-1β on increase in number of first-trimester vCTs through IL-8. The CCK-8 assay was performed to determine whether number of human first-trimester vCTs was affected by endometrial IL-8 expression. Before the assay, vCTs were treated for 72 h. A, vCTs were treated with control EEC-SN and IL-1β (0.1, 1, and 10 ng/ml) EEC-SN or FBS-free media with or without 100 pg/ml of IL-8. The values represent the ratio of the cell number relative to control EEC-SN. Values are the mean ± SEM of the combined data from seven independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN; **, $P < 0.05$ when compared with FBS-free media without IL-8. B, Control EEC-SN and IL-1β (1 ng/ml) EEC-SN were preincubated for 1 h with 1 μg/ml of IL-8Ab or mIgG. The values represent the ratio of the cell number relative to control EEC-SN with mIgG. Values are the mean ± SEM of the combined data from nine independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN + mIgG; **, $P < 0.05$ when compared with IL-1β (1 ng/ml) EEC-SN + mIgG.

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**FIG. 4. Expression of IL-8 receptors *CXCR1* and *CXCR2* mRNA in human first-trimester vCTs.** *Total RNA isolated from human first-trimester vCTs was reverse transcribed and amplified by PCR using primers for CXCR1 and CXCR2. Amplification of the internal control GAPDH was used to ensure RNA quality and as a loading control. The results are from four vCT samples (vCT 1–4) of different individuals. Gestational age of the vCT samples were: vCT1, 6 wk; vCT2, 6 wk, vCT3, 7 wk, vCT4, 8 wk. DNA marker, φX174/HinfI; positive control, peritoneal leukocytes; negative control, water without cDNA.*

ceptors *CXCR1* and *CXCR2* mRNA were expressed in first-trimester vCTs.

## Discussion

In the present study, we demonstrated that IL-1β induced the production of IL-8 from endometrial cells and that endometrial cell-derived IL-8 promoted migration of first-trimester vCTs and yielded higher number of vCTs.

IL-1 is produced by human embryo and first-trimester vCTs (8, 9) and is thought to play a regulatory role in human embryo implantation. It has been reported previously that IL-1 up-regulates integrin-β3, a marker of uterine receptivity in endometrial epithelial cells (12). Furthermore, IL-1β can induce the expression of prostaglandins (36) and LIF (37), factors that are important for the implantation process (6), in human endometrial and decidual cells. Our findings that IL-1β stimulated migration of first-trimester vCTs and increased the cell number via endometrium-derived IL-8 suggest a novel function for IL-1β and extend the notion that IL-1β is a pivotal and multifunctional factor in human embryo implantation.

The CXC chemokine IL-8 participates in the migration of leukocytes such as neutrophils and T lymphocytes (20, 21, 38–41). As well as being able to encourage the accumulation of leukocytes, uterine IL-8 is thought to have unique roles in endometrial angiogenesis, apoptosis, proliferation, and differentiation (42). These events are crucial to preparing the endometrium for implantation. Combined with the promotive effects on migration of first-trimester vCTs and maintenance of the number of live cells in the present study, IL-1β-induced secretion of IL-8 may orchestrate the implantation process and therefore affect both the endometrium and trophoblast. As a note, because IL-8 is secreted by first-trimester extravillous trophoblast cell line HTR8 (43) and its secretion is enhanced by IL-1 in third-trimester trophoblast cells (44), IL-1 secreted by first-trimester vCTs may induce IL-8 secretion from vCTs themselves as well as endometrial cells. IL-1, therefore, might be involved in implantation via trophoblast-derived IL-8. However, in the present study, we did not observe any difference between cell-free IL-1β EEC-SN and FBS-free medium without any additions and between control EEC-SN with IL-8Ab and control EEC-SN with isotype mIgG on the migration and cell number of vCTs (data not shown). Further studies are warranted to elucidate the detail mechanism.

The current study demonstrated that both basal and IL-1β-induced IL-8 levels were much higher in EEC-SN than ESC-SN. The implication for this difference is not clearly understood at the moment. However, it is interesting to note that our recent study has demonstrated that CXCL11, which stimulates migration of first-trimester vCTs, is also inducible in epithelial cells but not stromal cells (2). It can therefore be speculated that like CXCL11, epithelium-derived IL-8 is involved in a relatively early-phase of implantation, assuming that the endometrial epithelium is an important component in initiating the molecular interactions between embryo and endometrium (2, 45).

Migration of first-trimester trophoblast cells needs to be a tightly regulated process for successful implantation and for circumventing undesirable complications of pregnancy such as poor fetal growth or preeclampsia. The chemokines CX3CL1, CCL14, CCL4, CXCL9, CXCL10, and CXCL11 promote the migration of trophoblast cells into the endometrium (1, 2). Furthermore, decidual natural killer cell-derived IL-8 promotes the invasion of first-trimester extravillous trophoblast cells into the decidua (20). Our findings are interesting in that trophoblast may control its own activity through cross talk with EECs. Collectively, the molecular mechanism that fine-tunes trophoblast migration is believed to work in a spatiotemporally specific manner.

A previous study demonstrated that the supernatants of first-trimester primary decidual cells inhibit the proliferation of trophoblast cell line BeWo cells, whereas those of BeWo cells and first-trimester primary trophoblast cells promote the proliferation of decidual cells (46). These findings indicate that a regulatory loop to control the growth of fetal and maternal cells exists at the fetomaternal interface. Thus, cross talk between trophoblast and endometrium seems to be important for maintenance of trophoblast cell numbers. The studies on the inhibitory role of IL-1β in the proliferation of trophoblast cell lines BeWo cells and JAR cells (47, 48) imply a possibility that IL-1β is one of the regulating factors in the growth of placenta. In light of our findings that IL-8 derived from IL-1β-stimulated EECs could yield a higher number of vCTs, it can be speculated that endometrial IL-8 may act as one of the survival factors for first-trimester vCTs and facilitate placental growth in early pregnancy. Thus, not only IL-1β but also subsequent IL-8 may participate in the regulatory loop of placental growth at the fetomaternal interface.

In summary, we have shown that IL-1β is able to induce secretion of IL-8 from EECs and that EEC-derived IL-8 is able to stimulate the migration of human first-trimester vCTs and yield a higher number of vCTs. These findings suggest that human vCTs may regulate their own status via IL-8 secreted by IL-1β-stimulated EECs to accomplish successful implantation.

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## Acknowledgments

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*Metabolic, Endocrine and Genitourinary Pathobiology*

# Interleukin-4 Stimulates Proliferation of Endometriotic Stromal Cells

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**Several lines of evidence indicate that the Th2 immune response is associated with endometriosis. Although an increased concentration of interleukin (IL)-4, a typical Th2 cytokine, has been reported in endometriotic tissues, the implication of this for endometriosis has not been determined. To investigate a possible role of IL-4 in the development of endometriosis, we examined the presence of IL-4-producing cells in endometriotic tissues and the effect of IL-4 on proliferation of endometriotic stromal cells. Endometriotic stromal cells were isolated from endometriotic tissues obtained from women undergoing surgery for endometrioma. Immunohistochemistry of endometriotic tissues revealed that IL-4-positive cells were abundant in the stroma. The effect of IL-4 on proliferation of endometriotic stromal cells was studied using cell counting and BrdU incorporation assays. IL-4 (0.1 to 10 ng/ml) significantly increased cell number and BrdU incorporation in a dose-dependent manner, and the proliferative effect of IL-4 was inhibited by anti-IL-4 receptor antibody. IL-4-induced activation of mitogen-activated protein kinases in endometriotic stromal cells was examined by Western blotting. IL-4 induced phosphorylation of p38 mitogen-activated protein kinase, stress-activated protein kinase/c-Jun kinase, and p42/44 mitogen-activated protein kinase and inhibitors of these kinases suppressed IL-4-induced proliferation of endometriotic stromal cells. These findings suggest that proliferation of endometriotic stromal cells induced by locally produced IL-4 is involved in the development of endometriosis.** ***(Am J Pathol 2008, 173:463–469; DOI: 10.2353/ajpath.2008.071044)***

Endometriosis is an enigmatic disease that deteriorates the health of women of reproductive age.[1,2] A widely believed etiology is that endometrial debris in retrograde menstruation implants, survives and grows in the peritoneal cavity.[3] However, it remains unknown why endometrial implants develop to substantial endometriotic lesions. Numerous lines of evidence suggest that aberrant immune responses and inflammatory reactions are involved in the pathogenesis of endometriosis.[4–6]

Women with endometriosis have characteristics of autoimmune disease, such as increased polyclonal B-cell activity, abnormalities in T- and B-cell function, and familial inheritance.[5–7] High prevalence of autoimmune disease in endometriotic women supports an autoimmune aspect of endometriosis.[8] Allergies and asthma are also reported at high rates in endometriotic women. In addition, a recent genome-wide transcriptional profiling study revealed that endometriosis exhibits a gene expression signature reminiscent of other autoimmune disorders.[9]

It is well known interleukin (IL)-4 is a distinguished molecule in autoimmunity and allergy.[10,11] In view of the autoimmune and allergic background of endometriotic women, IL-4 is speculated to play a role in the pathogenesis of endometriosis. The notion is underpinned by the evidence that the levels of IL-4 mRNA and protein in peripheral blood monocytes and peritoneal fluid cells are elevated in women with endometriosis.[12,13] However, localization of IL-4 and effects of IL-4 in endometriotic cells have been unknown.

IL-4 exerts its effect on immune cells.[11] In addition, actions of IL-4 on several nonimmune cells have been reported.[10] Interestingly, IL-4 stimulates or inhibits cell proliferation in different cells and settings.[14–19] The biological function of IL-4 is mediated by a specific IL-4 receptor that is linked to several different intracellular

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signal cascades.[11] To address the possible implication of IL-4 in endometriosis, we studied localization of IL-4 in endometriotic tissues and effects of IL-4 on the proliferation of endometriotic stromal cells (ESCs).

## Materials and Methods

### Reagents and Materials

Type I collagenase and antibiotics (mixture of penicillin, streptomycin, amphotericin B) were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12) and 0.25% trypsin-ethylenediaminetetraacetic acid were from Life Technologies (Rockville, MD). Mitogen-activated protein kinase (MAPK) inhibitors SB202190, SP600125, and PD98059 [inhibitors for p38 MAPK, stress-activated protein kinase/c-Jun kinase (SAPK/JNK), and p42/44 MAPK, respectively], a PKA inhibitor H89, and a nuclear factor (NF)-κB inhibitor SN50 were from Calbiochem (La Jolla, CA). Rabbit antibodies of total p38 MAPK, phosphorylated (phospho-) p38 MAPK, total SAPK/JNK, phospho-SAPK/JNK, total p42/44 MAPK, and phospho-p42/44 MAPK were from New England BioLabs (Beverly, MA). Mouse anti-human IL-4 antibody (MAB304), mouse anti-human IL-4 receptor antibody (MAB230), and recombinant human IL-4 were from R&D Systems (Minneapolis, MN). Isotype mouse IgGs (IgG1 and IgG2a) were from Dako Cytomation (Glostrup, Denmark). Charcoal/dextran-treated fetal bovine serum was from Hyclone (Logan, UT). Deoxyribonuclease I was from Takara (Tokyo, Japan).

### Collection of Tissues

Endometriotic tissues were obtained from patients ($n$ = 32) with ovarian endometriomas undergoing laparoscopy or laparotomy after obtaining written informed consent under a study protocol approved by the institutional review board of the University of Tokyo. The mean age of the patients was 35.2 years (SD, 5.7). These patients had not received hormones or GnRH agonist for at least 3 months before surgery. The stages of endometriosis were III ($n$ = 14) and IV ($n$ = 18), and the mean rASRM score was 56.6 (SD, 34.9). Endometriotic tissues were obtained from the cyst wall of ovarian endometrioma. Samples were collected under sterile conditions and transported to the laboratory on ice in DMEM/F12.

### Immunohistochemistry

Endometriotic tissue samples were washed in phosphate-buffered saline (PBS), embedded in OCT compound (Sakura, Tokyo, Japan), and snap-frozen in liquid nitrogen. Cryosections were cut at an 8-μm thickness and mounted on poly-L-lysine-treated slides. Sections were fixed in acetone for 30 minutes on ice and washed in PBS for 5 minutes twice. Sections were treated with 3% $H_2O_2$ for 15 minutes to eliminate endogenous peroxidase. After blocking with nonspecific staining blocking reagent, the sections were incubated with 100 μg/ml of anti-human IL-4 antibody or 100 μg/ml of mouse IgG1 isotype control for 60 minutes at room temperature and incubated with peroxidase-conjugated goat anti-mouse secondary antibody (labeled polymer-horseradish peroxidase anti-mouse, Dako Cytomation) for 30 minutes. Staining was detected with the vector novaRED substrate kit (Funakoshi, Tokyo). All sections were counterstained with hematoxylin and evaluated under a light microscope. As a positive control, we stained amniochorionic membranes.[20]

### Isolation, Purification, and Culture of ESCs

The procedure was performed as described previously.[21–24] Briefly, endometriotic tissue was minced into small pieces, incubated in DMEM/F12 with type I collagenase (2.5 mg/ml) and deoxyribonuclease I (15 U/ml) for 1 to 2 hours at 37°C, and filtered through nylon cell strainers with apertures of 100 μm, and then 70 μm. Stromal cells remaining in the filtrate were centrifuged at 200 × $g$ for 5 minutes, washed with PBS, resuspended in DMEM/F12, and plated onto 100-mm dishes and allowed to adhere at 37°C for 30 minutes, after which nonadhering epithelial cells and blood cells were removed with PBS rinses. ESCs were cultured in DMEM/F12 containing 5% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. When the cells became confluent in 2 or 3 days, they were dissociated with 0.25% trypsin-ethylenediaminetetraacetic acid, harvested by centrifugation at 200 × $g$ for 5 minutes, replated in six-well plates at $2 \times 10^5$ cells/well for reverse transcription and polymerase chain reaction (RT-PCR) and Western blotting, or 96-well plates at $0.5 \times 10^4$ cells/well for cell proliferation assay, and incubated at 37°C in a humidified 5% $CO_2$/95% air environment for 24 hours. The complete media were then removed and replaced with fresh serum-free media containing antibiotics, and the cells were cultured for an additional 24 hours. Purification of the stromal cell population was determined by immunocytochemical staining before confluency for the following antibodies: vimentin (stromal cells), cytokeratin (epithelial cells), CD45 and CD68 (monocytes and other leukocytes), and von Willebrand factor (endothelial cells). The purity of the stromal cell was more than 98%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin, CD45, CD68, and von Willebrand factor.

### Treatment of ESCs

To evaluate dose effects of IL-4 on cell proliferation of ESCs, the wells were replenished with serum-free media with different concentrations of IL-4. To evaluate the effect of anti-IL-4 receptor neutralizing antibody on IL-4-induced proliferation of ESCs, the cells were preincubated with the antibody or isotype IgG2a for 30 minutes before IL-4 treatment (1 ng/ml). To evaluate effects of inhibitors of MAPK, PKA, and NF-κB on IL-4-induced

proliferation of ESCs, the cells were preincubated with SB202190 (10 μmol/L), SP600125 (10 μmol/L), PD98059 (25 μmol/L), mixture of all of the MAPK inhibitors, H89 (5 μmol/L), or SN50 (50 μmol/L) for 1 hour before IL-4 treatment. To evaluate proliferative effect of tumor necrosis factor (TNF)-α and IL-4 on ESCs, the cells were treated with 1 ng/ml of IL-4 and/or 0.1 ng/ml of TNF-α. The conditions of the treatment were determined with reference to our previous studies.[21,25–27]

### Cell Proliferation Assay

To measure the proliferative activity of ESCs, we measured the cell number of ESCs and BrdU incorporation. The number of ESCs was measured using cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, ESCs were treated with IL-4 for 72 hours, and 10-ml CCK-8 solutions with tetrazolium salt WST-8 were added and incubated at 37°C for an additional 2 hours. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product in culture medium. The amount of formazan, which is directly proportional to the number of living cells, was evaluated by measurement of the optical density at 450 nm in the DigiScan microplate reader (ASYS Hitech GmbH, Eugendorf, Austria). The BrdU proliferation assay was performed as reported previously[23,24,28] using the Biotrak cell proliferation enzyme-linked immunosorbent assay system (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions. Briefly, ESCs were treated with serum-free medium with different concentrations of IL-4 (0.1 to 10 ng/ml) for 48 hours, and 100-μl BrdU solutions were added and incubated at 37°C for an additional 2 hours. After removing the culture medium, the cells were fixed and the DNA denatured by the addition of 200 μl/well fixative. The peroxidase-labeled anti-BrdU bound to the BrdU incorporated in the newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm in the DigiScan microplate reader.

### RT-PCR

Total RNA was extracted from ESCs randomly selected out of the study population, using the RNAeasy mini kit (Qiagen, Hilden, Germany). RT was performed using Rever Tra Ace-a (Toyobo, Tokyo, Japan). One μg of total RNA was reverse-transcribed in a 20-μl total volume and cDNA was amplified using oligonucleotide primers. IL-4 receptor primers (sense, 5'-CAAGCTCTTGCCCTGTTTTC-3'; antisense, 5'-TGCACAGAAGCTCCCTTTTT-3') were chosen to amplify a 238-bp fragment. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Toyobo) were used to ensure RNA amounts. The PCR condition of IL-4 receptor was 30 cycles at 98°C for 10 seconds, 60°C for 4 seconds, 74°C for 15 seconds. Each PCR product was purified with a Qiaex II gel extraction kit (Qiagen) and their identities were confirmed using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA).

### Western Blotting

Western blotting was performed as reported previously.[21,29] Cultured cells were homogenized in a lysis buffer containing 50 mmol/L Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 50 mmol/L dithiothreitol, and 0.1% bromophenol blue, and diluted to 1 mg of total protein/ml. Concentrations of total protein in the homogenized cells were measured by a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Samples were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were blotted onto a nitrocellulose membrane and incubated with rabbit antibodies to total p38 MAPK (1:1000), to phospho-p38 MAPK (1:1000), to total SAPK/JNK (1:1000), to phospho-SAPK/JNK (1:1000), to total p42/44 MAPK (1:1000), or to phospho-p42/44 MAPK (1:1000), as primary antibodies, and anti-rabbit horseradish peroxidase antibody (1:1000, Amersham Biosciences) as a secondary antibody. Immune complexes were visualized by the ECL Western blotting system (Amersham Biosciences).

### Statistical Analysis

Data were evaluated using analysis of variance with post hoc analysis (Fisher's protected least significance). A $P$ value less than 0.05 was accepted as significant.

## Results

### Immunoreactive Cells for IL-4 Were Present in the Stroma of Endometriotic Tissue

Figure 1 shows the cells stained for IL-4 in the stroma of endometriotic tissue. The number of IL-4-positive cells was 14% of total cells. No staining was seen when mouse IgG1 was used as a primary antibody.

### Expression of IL-4 Receptor mRNA and Proliferative Effect of IL-4 on ESCs

We first examined gene expression of the IL-4 receptor in ESCs. As shown in Figure 2, RT-PCR analysis demonstrated that IL-4 receptor mRNA was expressed in ESCs. Next, we studied the proliferative effect of IL-4 on ESCs. As depicted in Figure 3A, IL-4 (0.1 to 10 ng/ml) increased the cell number of ESCs in a dose-dependent manner. The increase at a dose of 10 ng/ml was 1.4-fold of the control. IL-4 (0.1 to 10 ng/ml) also increased BrdU incorporation in ESCs in a dose-dependent manner (Figure 3B). To determine whether the proliferative effect is mediated by IL-4 receptor on ESCs, we used anti-IL-4 receptor neutralizing antibody in addition to IL-4. In consequence, anti-IL-4 receptor neutralizing antibody significantly inhibited IL-4-in-

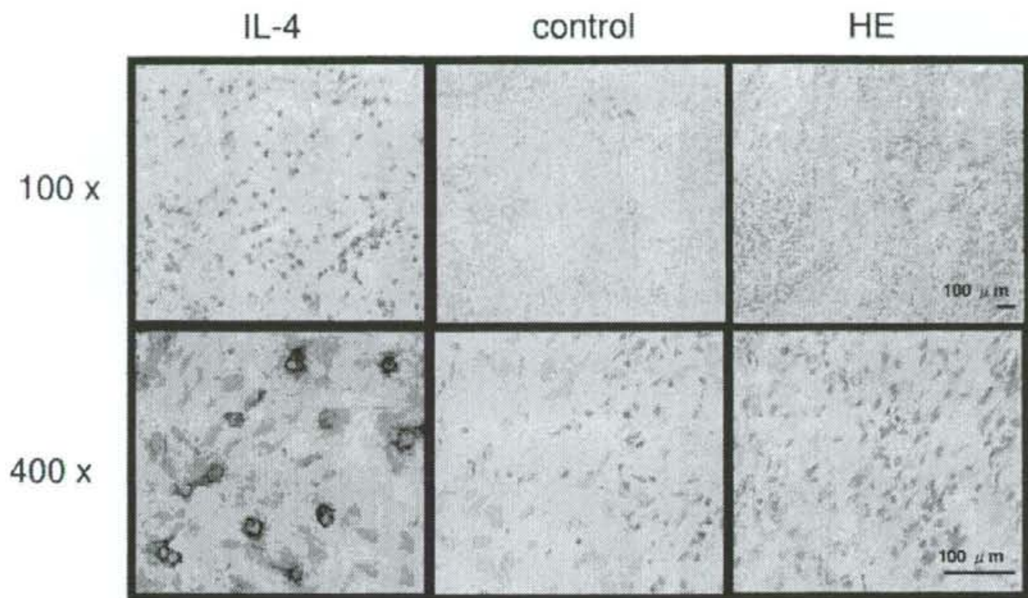



**Figure 1.** Immunohistochemistry of IL-4 in the human endometriotic tissue. Sections were immunostained with anti-human IL-4 antibody (IL-4), mouse IgG1 (control), and H&E. The result is representative of four separate experiments using samples from four different patients. Original magnifications: ×100 (**top**), ×400 (**bottom**).

duced proliferation of ESCs approximately to the control level (Figure 3C).

### *IL-4-Induced Phosphorylation of p38 MAPK, SAPK/JNK, and p42/44 MAPK in ESCs, and Effect of MAP Kinase Inhibitors on IL-4-Induced Proliferation of ESCs*

With the aim to study intracellular signaling involved in IL-4-induced proliferation of ESCs, we first examined phosphorylation of p38 MAPK, SAPK/JNK, and p42/44 MAPK. As shown in Figure 4, IL-4 stimulated the phosphorylation of p38 MAPK, SAPK/JNK, and p42/44 MAPK. We then tested inhibitors of these kinases for the inhibitory effect on IL-4-induced proliferation of ESCs. As shown in Figure 5, inhibitors of p38 MAPK (SB202190), SAPK/JNK (SP600125), and p42/44 MAPK (PD98059) significantly suppressed the IL-4-induced proliferation of ESCs. A mixture of these inhibitors suppressed the IL-4-induced proliferation more markedly. In contrast, inhibitors of PKA and NF-κB did not affect the proliferative effect of IL-4.

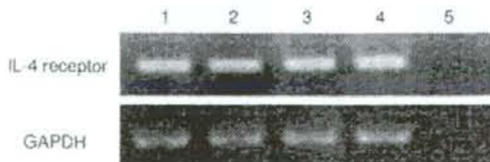



**Figure 2.** Gene expression of IL-4 receptor in ESCs. The samples were randomly selected out of the study population. Total RNA isolated from ESCs of four women with endometriosis was reverse-transcribed and amplified by PCR using primers for IL-4 receptor. Amplification of GAPDH was used to ensure RNA amounts. **Lane 5** is negative control with water.

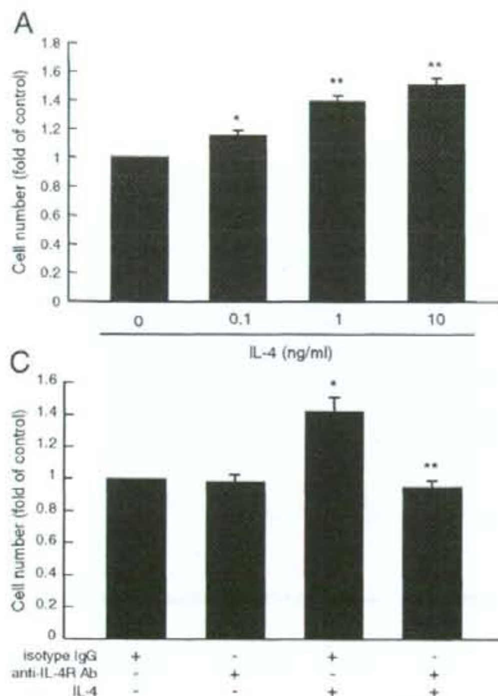
### *Effect of IL-4 on TNF-α-Induced Proliferation of ESCs*

It has been previously reported that TNF-α has proliferative effect on ESCs.[30] We examined whether IL-4 has an additional effect on TNF-α-induced proliferation of ESCs. As shown in Figure 6, addition of IL-4 to TNF-α showed a synergistic effect on ESC proliferation.

## *Discussion*

In the present study, we demonstrated the presence of IL-4-immunoreactive cells in the endometriotic tissue. Expression of IL-4 receptor was detected by RT-PCR in ESCs. We then showed that IL-4 stimulated the proliferation of ESCs, which was inhibited by the addition of anti-IL-4 receptor antibody. IL-4 stimulated the phosphorylation of p38MAPK, SAPK/JNK, and p42/44 MAPK in ESCs, and inhibitors of these MAPKs suppressed the IL-4-induced proliferation of ESCs. IL-4 also exerted a synergistic effect on TNF-α-induced proliferation of ESCs. These findings suggest important roles of IL-4 in the pathophysiology of endometriosis.

It has been shown that multiple immune cells, eg, macrophages, T lymphocyte, NK cells, mast cells, eosin-



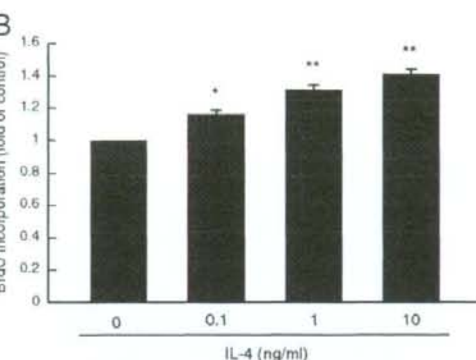




**Figure 3. A:** Proliferative effect of IL-4 on ESCs. ESCs were treated with IL-4 at different concentrations for 72 hours. The proliferation of ESCs was evaluated by cell counting kit-8 (CCK-8). Values are the mean ± SEM of combined data from five independent experiments using different ESC preparations from different women. *$P < 0.01$; **$P < 0.0001$ (each versus control). **B:** Effect of IL-4 on BrdU incorporation in ESCs. ESCs were treated with IL-4 at different concentrations for 48 hours, and then BrdU incorporation into DNA was measured. Values are the mean ± SEM of combined data from four independent experiments using different ESC preparations from different women. *$P < 0.01$; **$P < 0.0001$ (each versus control). **C:** Effect of anti-IL-4 receptor neutralizing antibody on IL-4-induced proliferation of ESCs. After the preincubation with either anti-IL-4 receptor neutralizing antibody or isotype IgG2a for 30 minutes, the cells were treated with or without IL-4 for 72 hours. Proliferation of ESCs was evaluated by CCK-8. Values are the mean ± SEM of the combined data from five independent experiments using different ESC preparations. *$P < 0.0001$ (versus isotype IgG); **$P < 0.0001$ (versus IL-4 plus isotype IgG).

ophils, reside in endometriotic tissues.[14,31,32] The immune cells in endometriotic tissues are suggested to be involved in the development of endometriosis by inducing various events such as inflammation, proliferation, invasion, angiogenesis, and fibrosis.[4] In these events, cytokines from the immune cells play important roles, acting directly on ESCs or modulating other immune cell functions. In the present study, we detected many IL-4-immunoreactive cells in the endometriotic tissue. This finding is consistent with the report showing the increased expression of IL-4 mRNA and protein in lymphocytes in endometriotic tissues.[12] In addition, IL-4 stimulated the proliferation of ESCs, suggesting that IL-4 plays as a local mediator to grow endometriotic lesion. These findings

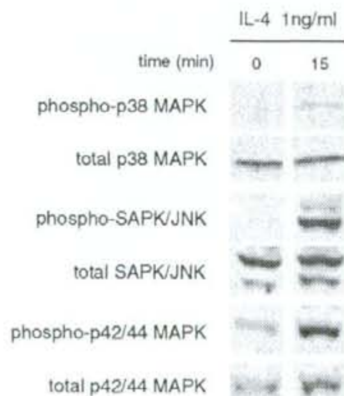



**Figure 4.** IL-4 induced MAPK activation. ESCs were incubated with 1 ng/ml of IL-4 for 15 minutes. Cell extracts were prepared and assayed for phospho-/total p38 MAPK, phospho-/total SAPK/JNK and phospho-/total p42/44 MAPK by Western blotting. The result is representative of three separate experiments.

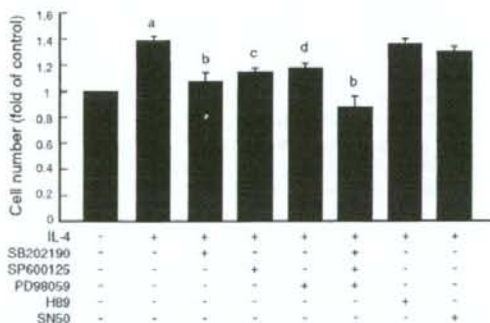



**Figure 5.** After the preincubation with SB202190 (p38 MAPK inhibitor, 10 μmol/L), SP600125 (SAPK/JNK inhibitor, 10 μmol/L), PD98059 (p42/44 MAPK inhibitor, 25 μmol/L), mixture of all of the MAPK inhibitors, H89 (PKA inhibitor, 5 μmol/L), or SN50 (NF-κB inhibitor, 50 μmol/L) for 1 hour, the cells were treated with or without IL-4 for 72 hours. The proliferation of ESCs was evaluated by CCK-8. Values are the mean ± SEM of the combined data from eight independent experiments using different ESC preparations from different women. **a**, $P < 0.0001$ (versus control); **b**, $P < 0.0001$ (versus IL-4); **c**, $P < 0.001$ (versus IL-4); **d**, $P < 0.005$ (versus IL-4).

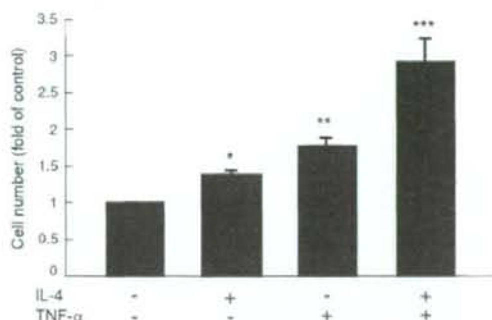



**Figure 6.** Synergistic effect of IL-4 and TNF-α on ESC proliferation. The cells were treated with 1 ng/ml of IL-4 and/or 0.1 ng/ml of TNF-α for 72 hours. The proliferation of ESCs was evaluated by CCK-8. Values are the mean ± SEM of the combined data from five independent experiments using different ESC preparations from different women. $^{*}P < 0.05$ (versus control); $^{**}P < 0.0005$ (versus control); $^{***}P < 0.0001$ (versus all of the others).

suggest that IL-4 is one of the cytokines in the immune network in endometriosis that promote the progress of the disease.

The present finding also has an implication in immunology of endometriosis. Multiple lines of evidence have shown that development of endometriosis is accompanied by the activation of a Th2 immune response.[12,13,33] Because IL-4 is a typical Th2 cytokine, our findings extend the notion that a Th2 immune response may directly develop the disease through local IL-4 production. Interestingly, our recent study suggested that IL-17 stimulates the progress of endometriosis.[25] IL-17 is a typical cytokine of Th17 cells, a novel member of helper T lymphocytes that has been believed to be only Th1 and Th2 for a long time. Therefore, both Th2 and Th17 immune response could contribute to the pathogenesis of the disease. Further study is warranted to elucidate the precise mechanism.

Anti-IL-4 receptor antibody abandoned the proliferation of ESCs induced by IL-4 in the present study. This finding clearly demonstrates that the proliferative effect of IL-4 on ESCs is exerted through its ligation to IL-4 receptor. It also implies that blocking IL-4 action on ESCs would be a possible treatment of endometriosis. Asthma is a disease in which IL-4 contributes to inflammation and airway obstruction. Aiming to develop a new drug for asthma therapy, drugs that prevent IL-4 binding to its receptor has been explored.[34] A promising IL-4 receptor antagonist is under clinical trials, and the drug also might be useful in the treatment of endometriosis.

Downstream signal transduction of IL-4 receptor has been known to be diverse. In this study, we have shown that IL-4 activated p38 MAPK, SAPK/JNK, and p42/44 MAPK in ESCs. Moreover, inhibitors of p38 MAPK, SAPK/JNK, and p42/44 MAPK suppressed IL-4-induced proliferation of ESCs. We have observed similar findings in the previous study that activation of p38 MAPK, SAPK/JNK, and p42/44 MAPK were involved in the proliferation of ESCs by PAR2 activation.[21] We have also reported that activation of p38 MAPK is higher in endometriotic tissues as compared to eutopic endometrium, and p38 MAPK inhibitor reduces endometriotic tissue in the experimental mouse model of endometriosis.[35,36] Taken together, it is plausible that MAPKs are mediators used in common with various pro-endometriotic molecules and play a pivotal role in the development of endometriosis.

It is generally conceptualized that pelvic inflammation is a promoting factor for endometriosis. Increased activated macrophages in the peritoneal cavity of endometriotic women are suggested to produce proinflammatory cytokines and sustain self-perpetuating inflammation. TNF-α is a typical proinflammatory cytokine that plays multiple roles in the progression of endometriosis,[30,37,38] and TNF-α-targeted suppression by specific drugs has been shown to inhibit the development of endometriosis in baboons.[39,40] The present study demonstrated the synergistic effect of IL-4 and TNF-α to stimulate the proliferation of ESCs. The finding is interesting in that Th2 immune response may accelerate the progress of the disease in synergy with another inflammatory mediator.

In the present study, we used the tissues from ovarian endometrioma. It is suggested that endometriosis of ovarian, peritoneal, or deep pelvic lesions has different characteristics. In particular, a high steroid environment may affect ovarian lesions. It is well known that ovarian steroids have influence on various immune cells including T cells.[41] Circulating IL-4 levels are demonstrated to be increased by estrogen.[42,43] Therefore, a degree of contribution of IL-4 to the development of endometriosis might somehow be different in endometriotic lesions outside the ovary. Lastly, we would like to remark that the present study demonstrated the IL-4 effects on the proliferation and intracellular signaling in ESCs by the *in vitro* experiments. Further studies using *in vivo* animal models would be warranted to elucidate a definitive role of IL-4 in endometriosis.

In summary, the present study demonstrated that IL-4-immunoreactive cells are present in endometriotic tissues and that IL-4 stimulates the proliferation of ESCs. These findings suggest that locally produced IL-4 may be involved in the development of endometriosis.

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CASE REPORT

# Androgen insensitivity syndrome with serous gonadal cyst

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**Objective:** To present a patient with androgen insensitivity syndrome with serous gonadal cyst who underwent laparoscopic surgery.
**Design:** Case report.
**Setting:** University hospital.
**Patient(s):** An 18-year-old female with a history of primary amenorrhea.
**Intervention(s):** Laparoscopic gonadectomy.
**Main Outcome Measure(s):** Diagnosis and surgical approach to gonadal cyst.
**Result(s):** Ultrasound and magnetic resonance imaging revealed the presence of a 4-cm cystic smooth mass close to the right external iliac vein and artery. We performed laparoscopic bilateral gonadectomy. The pathological findings suggested that the serous gonadal cyst was formed by occlusion of the glandular duct in the right gonad.
**Conclusion(s):** We reported a case of laparoscopic gonadectomy for cystic mass in the gonad of a patient with androgen insensitivity syndrome. (Fertil Steril® 2008;90:2018.e9–e11. ©2008 by American Society for Reproductive Medicine.)

**Key Words:** Androgen insensitivity syndrome, gonadal cyst, laparoscopic surgery

Androgen insensitivity syndrome (AIS, previously termed "testicular feminization syndrome") is caused by a mutation in the androgen receptor gene (1). AIS patients exhibit female phenotype because of insensitivity to the androgen receptor. Differentiation of the gonads is normal, and serum androgen level is comparable with that of a normal male. We report a case of a patient with AIS with a gonadal serous cyst who underwent laparoscopic surgery.

FIGURE 1

Images by ultrasound and MRI. Ultrasound revealed a cyctic, smooth mass of dimension 41×35×39 mm close to the right external iliac vein and artery (**A**). MRI showed 40 mm high signal intensity in T2-weighted (**B**) and low intensity in T1-weighted (**C**) right gonadal cyst, with thin cystic wall.

*Yanai. AIS with serous gonadal cyst. Fertil Steril 2008.*

## CASE REPORT

An 18-year-old female presented to our clinic with a complaint of primary amenorrhea. Examination revealed normal bilateral breast development; however, axillary and pubic

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Y.Y. has nothing to disclose. H.H. has nothing to disclose. Y.O. has nothing to disclose. A.F. has nothing to disclose. M.M. has nothing to disclose. T.Y. has nothing to disclose. Y.T. has nothing to disclose.
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FIGURE 2

Gross appearance of right gonad. The right testis was swollen with a cyst (indicated by the arrow). The solid lesion of the right testis appeared to be a normal testis.

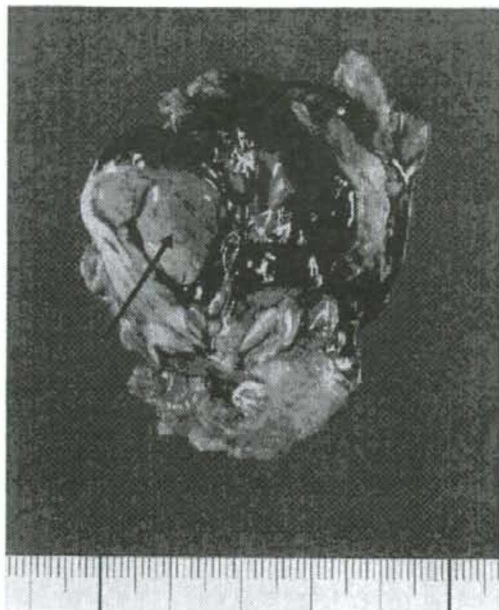

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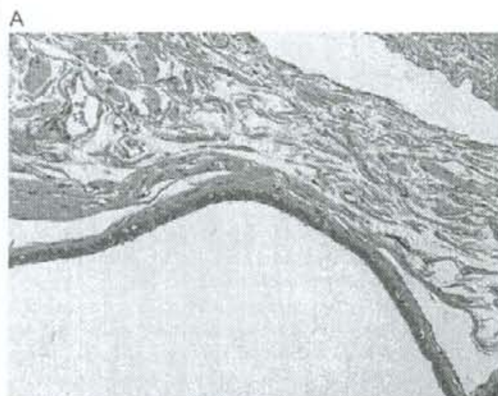
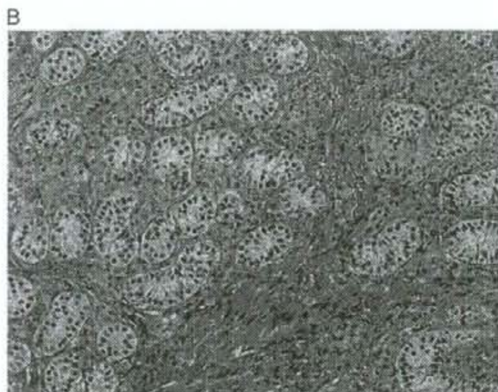
FIGURE 3

Microscopic appearance of the right gonad. Serous cyst in the right gonad was lined by ciliated cells (**A**). No signs of spermatogenesis and Leydig cell hyperplasia in the interstitial tissue are seen in right gonad (**B**). Hematoxylin-eosin stain; magnification ×100.

A



B



*Yanai. AIS with serous gonadal cyst. Fertil Steril 2008.*

hair was scarce. Gynecological examination revealed normal appearance of vulva and perineum. The distal vagina was 6 cm in length and ended blindly. Abdominal ultrasound revealed an absence of uterus and ovaries and the presence of a cystic, smooth mass of dimensions 41×35×39 mm close to the right external iliac vein and artery (Fig. 1A). A low echoic mass of dimensions 18.5×11.7×17.2 mm was observed near the left external iliac vein and artery; we suspected that the mass was a testis. The absence of uterus, a shortened vagina, left cryptorchidism, and right gonad with cystic change were revealed (Figs. 1B and 1C) on magnetic resonance imaging (MRI). Ultrasound and MRI revealed no signs of malignancy in the tumor. Hormonal analysis showed FSH, 24.7 mIU/mL; LH, 30.5 mIU/mL; $E_2$, 30.2 pg/mL; and T, 5.64 ng/mL. The chromosome test revealed a normal 46 X,Y male karyotype. The diagnosis of AIS was made based on these findings.

We performed laparoscopic surgery because the tumor showed no sign of malignancy. The patient was placed in low lithotomy position under general anesthesia during laparoscopic surgery. The first 12-mm trocar was placed just under the umbilicus level for laparoscope. Two 5-mm trocars were placed at the level of the right and left iliac fossae two fingerbreadths within the anterior superior iliac spine. One 12-mm trocar was placed four fingerbreadths above the symphysis pubis. The peritoneal cavity was inflated with carbon dioxide. Peritoneum overlying on the gonads was opened by monopolar electric scissors. Bilateral gonads were mobilized from their surrounding connective tissue using monopolar electric scissors and grasping forceps. Arteries and veins into gonads were identified and ligated with the use of bipolar forceps. After removal of bilateral gonads, Interceeds were attached to prevent adhesion (Gynecare, Somerville, NJ). Vaginoplasty was not performed because the vagina length was 6 cm. Internal female organs were absent. Both testes were extirpated. The left testis was of normal size, and the right testis was swollen with a cyst (40×35 mm). The right

testis had a cystic lesion and solid mass that appeared to be a normal testis (Fig. 2). The cyst wall was thin, containing serous fluid. Histopathological examination of the right gonadal tissue showed nodular proliferation composed entirely of immature seminiferous tubules lacking lumina and spermatocytes, which were lined by immature Sertoli cells (Fig. 3A). No sign of spermatogenesis was observed. Leydig cell hyperplasia in the interstitial tissue was evident and characterized by the presence of Leydig cell nodules (Fig. 3B). The cyst wall was lined by ciliated cells. Estrogen therapy was initiated postoperatively.

## DISCUSSION

In patients with AIS, the incidence of gonadal tumor (e.g., seminoma) is 3.6% and 33% at the age of 25 years and 50 years, respectively (2). Gonadectomy is recommended after puberty to eliminate the risk of gonadal malignancy and aid the development of feminine secondary sexual characteristics (3). Tere is no circulating specific reliable tumor marker for monitoring tumor development, and ultrasound imaging of intraabdominal gonads is not sufficiently sensitive. Because the patient was 18 years old, we performed bilateral gonadectomy.

We report a patient with AIS with a serous gonadal cyst. A literature search revealed only one report of a patient with AIS with a serous gonadal cyst (3); the clinical features of the previous case are almost identical to our case. The pathological findings of these cases suggest that the serous gonadal cyst was formed by occlusion of the glandular duct in the right testis. The differentiation of the spermatic duct in testis occurs during adolescence. It is possible that occlusion might occur in the gland of testis together with development of secondary sexual characters. Thus, it is more likely that gonadal serous cysts in patients with AIS at a young age may not be malignant neoplasm. For patients with AIS with a serous gonadal cyst, gonadectomy can be delayed until the end of puberty, and laparoscopic gonadectomy can be safely performed.

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*Metabolic, Endocrine and Genitourinary Pathobiology*

# Deficiency of Immunophilin FKBP52 Promotes Endometriosis

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**Endometriosis is a common gynecological disease that affects approximately 10% of women of childbearing age. It is characterized by endometrial growth outside the uterus and often results in inflamed lesions, pain, and reduced fertility. Although heightened estrogenic activity and/or reduced progesterone responsiveness are considered to be involved in the etiology of endometriosis, neither the extent of their participation nor the underlying mechanisms are clearly understood. Heterogeneous uterine cell types differentially respond to estrogen and progesterone ($P_4$). $P_4$, primarily acting via its nuclear receptor (PR), activates gene transcription and impacts many reproductive processes. Deletion of *Fkbp52*, an immunophilin cochaperone for PR, results in uterine-specific $P_4$ resistance in mice, creating an opportunity to study the unique aspects of $P_4$ signaling in endometriosis. Here we explored the roles of FKBP52 in this disease using *Fkbp52*$^{-/-}$ mice. We found that the loss of FKBP52 encourages the growth of endometriotic lesions with increased inflammation, cell proliferation, and angiogenesis. We also found remarkable down-regulation of FKBP52 in cases of human endometriosis. Our results provide the first evidence corroborated by genetic studies in mice for a potential role of an immunophilin cochaperone in the etiology of human endometriosis. This investigation is highly relevant for clinical application, particularly because $P_4$ resistance is favorably indicated in endometriosis and other gynecological diseases.** *(Am J Pathol 2008, 173:1747–1757; DOI: 10.2353/ajpath.2008.080527)*

Endometriosis, the growth of endometrium-like tissues outside the uterus that differentially respond to reproductive hormones, is a common gynecological disease often associated with pelvic pain and infertility, affecting about 10% of women of reproductive age.[1–3] Although the etiology of endometriosis remains elusive, implantation and growth of endometrial tissues within the peritoneal cavity after retrograde menstruation is a widely accepted pathogenesis.[4] Estrogen, a potent mitogen that affects both eutopic endometria and ectopic lesions, is thought to be a major player in the development of endometriosis.[5] The basis of this tenet is that endometriotic lesions regress in low-estrogen environments, eg, in menopausal women, in patients after ovariectomy or in women undergoing hormonal therapy with gonadotropin-releasing hormone agonists.[6]

Progesterone ($P_4$) is also considered an important contributor to this disease, because it inhibits the mitogenic action of estrogen and promotes endometrial cell differentiation.[7] Regression of endometriosis often occurs in women under high progesterone dominance, ie, during pregnancy or those undergoing progestogen therapy.[6] There is also evidence that endometriosis is aggravated in *PR*$^{-/-}$ mice[8] and that $P_4$ responsiveness in eutopic and ectopic endometria is reduced compared to disease-free endometria in humans.[9,10] Furthermore, progesterone receptor (PR) expression and the antiproliferative effects imposed by $P_4$-PR signaling are suppressed in endometriosis.[11,12] In addition, many $P_4$-responsive genes are aberrantly expressed in eutopic endometria of women with endometriosis.[13,14] However, clinical studies have shown that endometriosis-related pain in select pa-

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