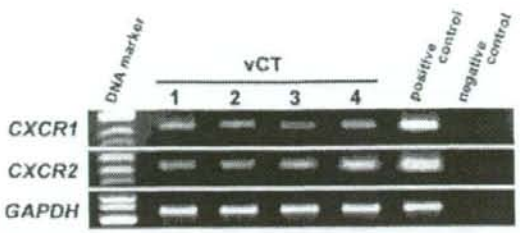

FIG. 4. Expression of IL-8 receptors *CXCR1* and *CXCR2* mRNA in human first-trimester vCTs. Total RNA isolated from human first-trimester vCTs was reverse transcribed and amplified by PCR using primers for CXCR1 and CXCR2. Amplification of the internal control GAPDH was used to ensure RNA quality and 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.

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.[6] 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 × 10^5 cells/well for reverse transcription and polymerase chain reaction (RT-PCR) and Western blotting, or 96-well plates at 0.5 × 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-

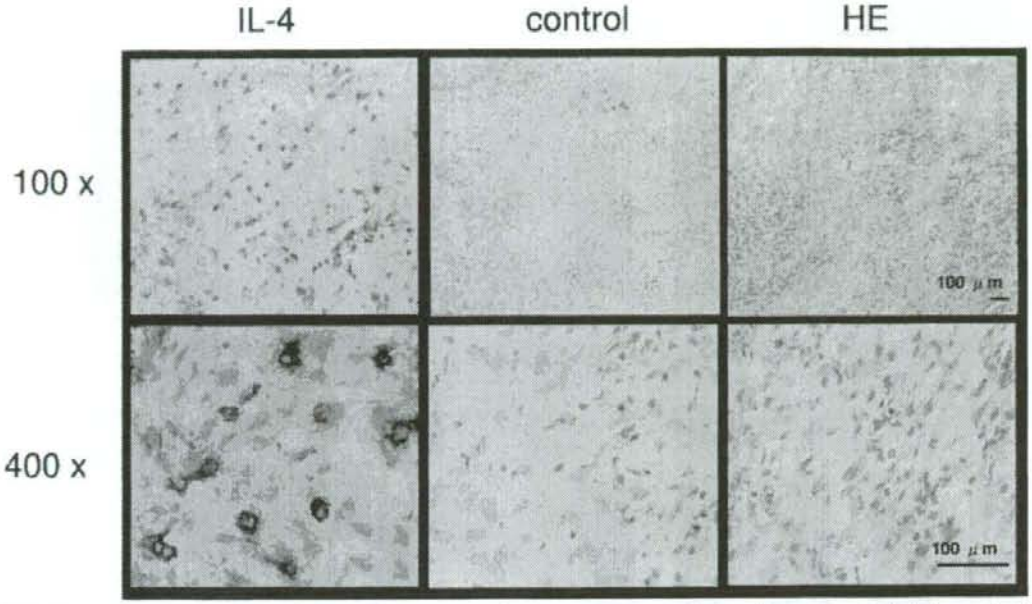

Figure 1. Immunohistochemistry of IL-4 in the human endometriotic tissue. Sections were immunostained with anti-human IL-4 antibody (IL-4), mouse IgG1 (control), and H&E. The result is representative of four separate experiments using samples from four different patients. Original magnifications: ×100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

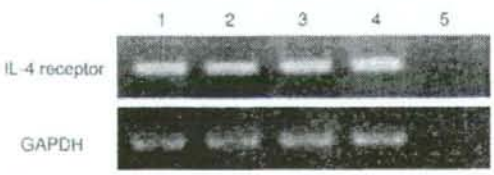

Figure 2. Gene expression of IL-4 receptor in ESCs. The samples were randomly selected out of the study population. Total RNA isolated from ESCs of four women with endometriosis was reverse-transcribed and amplified by PCR using primers for IL-4 receptor. Amplification of GAPDH was used to ensure RNA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

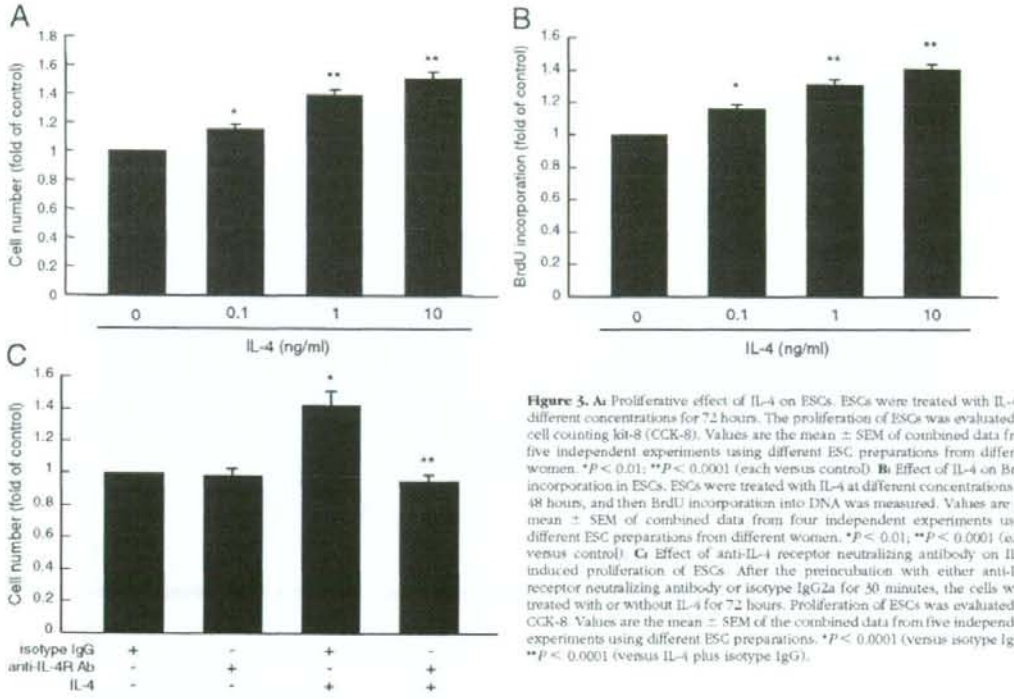

Figure 3. A: Proliferative effect of IL-4 on ESCs. ESCs were treated with IL-4 at different concentrations for 72 hours. The proliferation of ESCs was evaluated by cell counting kit-8 (CCK-8). Values are the mean ± SEM of combined data from five independent experiments using different ESC preparations 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

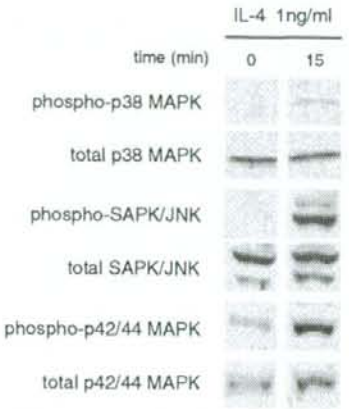

Figure 4. IL-4 induced MAPK activation. ESCs were incubated with 1 ng/ml of IL-4 for 15 minutes. Cell extracts were prepared and assayed for phospho-/total p38 MAPK, phospho-/total SAPK/JNK and phospho-/total p42/44 MAPK by Western blotting. The result is representative of three separate experiments.

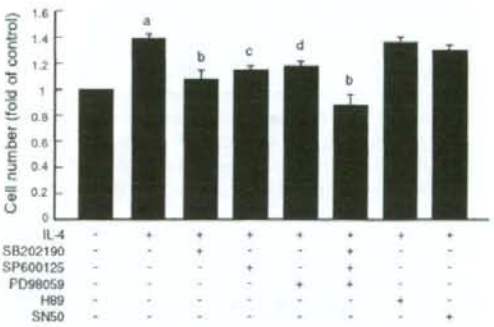

Figure 5. After the preincubation with SB202190 (p38 MAPK inhibitor, 10 μmol/L), SP600125 (SAPK/JNK inhibitor, 10 μmol/L), PD98059 (p42/44 MAPK inhibitor, 25 μmol/L), mixture of all of the MAPK inhibitors, H89 (PKA inhibitor, 5 μmol/L), or SN50 (NF-κB inhibitor, 50 μmol/L) for 1 hour, the cells were 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).

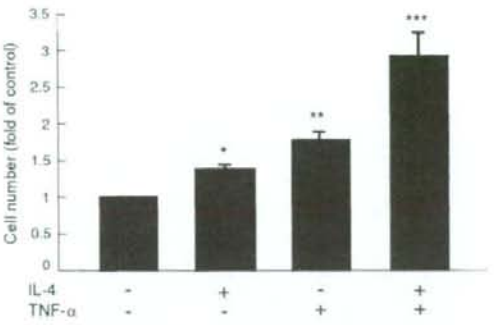

Figure 6. Synergistic effect of IL-4 and TNF-α on ESC proliferation. The cells were treated with 1 ng/ml of IL-4 and/or 0.1 ng/ml of TNF-α for 72 hours. The proliferation of ESCs was evaluated by CCK-8. Values are the mean ± SEM of the combined data from five independent experiments using different ESC preparations from different women. $^{*}P < 0.05$ (versus control); $^{**}P < 0.005$ (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.

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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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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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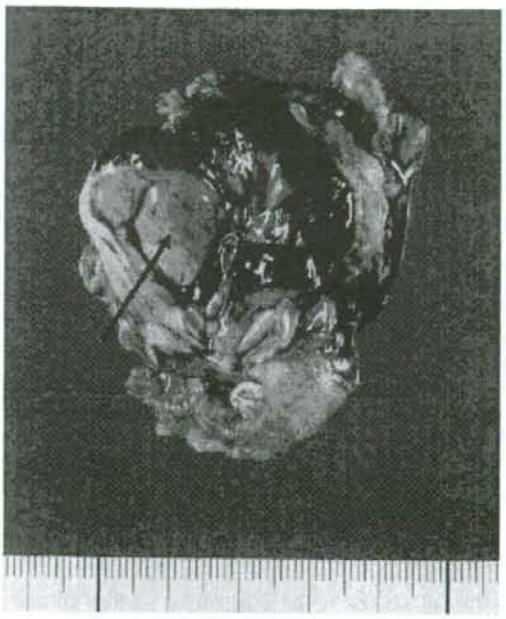
FIGURE 2

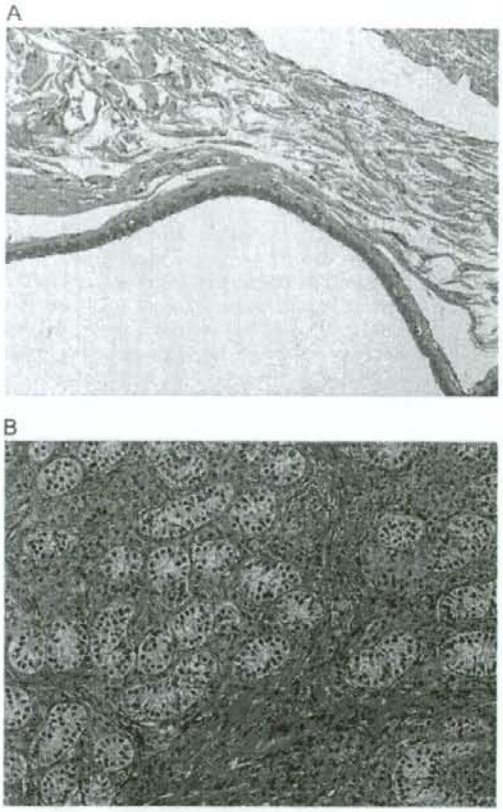
Gross appearance of right gonad. The right testis was swollen with a cyst (indicated by the arrow). The solid lesion of the right testis appeared to be a normal testis.



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

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.



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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tients is sustained despite progestin therapy.[3,6] The prevailing hypothesis is that an enhanced estrogenic influence and/or reduced P_4 responsiveness leads to endometriosis.[3,12,14] Therefore, an animal model presenting more than normal estrogenic activity with suboptimal P_4 responsiveness will be valuable for delineating the etiology of endometriosis, because all human studies focus on this disease when it is already in progress or established, precluding studies on its initiation. Mice missing FKBP52 fulfill this purpose, because these null females show uterine specific P_4 resistance as described below.

The immunophilin cochaperone FK506-binding protein 4 (FKBP52) is a key component of the mature PR complex. Functionally mature steroid hormone receptors including that of PR consist of a receptor monomer, a 90-kDa heat shock protein (Hsp90) dimer, the cochaperone p23, and one of the four cochaperones containing a tetratricopeptide repeat that binds to Hsp90.[15] FKBP52 is one such cochaperone that binds both Hsp90 and PR to stabilize the complex for optimal P_4 binding to PR and subsequent transcriptional activation.[15] Basal PR responsiveness, however, persists in the absence of FKBP52.[16] We have recently shown that *Fkbp52* deficient female mice with normal PR expression and P_4 levels show reduced uterine PR responsiveness with more than normal estrogenic influence, leading to implantation failure.[16] Implantation and full-term pregnancy, however, can be rescued with excess P_4 supplementation, depending on the genetic background of mice.[17] Thus, FKBP52 deficiency confers endometrial P_4 resistance during pregnancy.

Because serum ovarian hormone levels during the menstrual cycle in women with endometriosis are similar to those without endometriosis, it is possible that reduced P_4-PR signaling, rather than reduced P_4 levels, is a major contributing factor for P_4 resistance in endometriosis. Unlike $PR^{-/-}$ mice with no P_4-PR signaling, $Fkbp52^{-/-}$ mice with basal uterine P_4-PR responsiveness are perhaps a more physiologically relevant model to study the role of P_4 resistance in endometriosis.[16] Using $Fkbp52^{-/-}$ females, we show here that FKBP52 deficiency promotes the growth of endometriotic lesions with increased inflammation, cell proliferation and angiogenesis. These findings in mice corroborate our observations of down-regulation of FKBP52 expression in eutopic endometria and ectopic lesions of women with endometriosis compared to endometria of women without endometriosis. Together, these findings provide evidence that reduced levels of FKBP52 contribute to decreased P_4 responsiveness in furthering the development of endometriosis.

Materials and Methods

Mice

Fkbp52 null mice were originally established on a C57BL/6/129SvJ background[18] and then backcrossed with CD1 mice to the F10 generation.[17] $Flk1^{lacZ+/-}$ transgenic mice were originally generated on a C57BL/6J/Sv129 background and backcrossed to CD1 background to the F10 generation.[19] CD1 $Fkbp52^{-/-}/Flk1^{lacZ+/-}$ (knockout [KO]; Flk^{KO}) mice were generated by crossing $Fkbp52^{+/-}/Flk1^{lacZ+/-}$ males and $Fkbp52^{+/-}$ females. $Fkbp52^{+/+}/Flk1^{lacZ+/-}$ (wild-type [WT]; $Flk1^{WT}$) littermates were used as control. Mice were housed and used in the present investigation in accordance with the National Institutes of Health and institutional guidelines on the care and use of laboratory animals.

Mouse Endometriosis Model

There is evidence that estrogen promotes growth of endometriotic lesions in ovariectomized mice.[20] However, estrogen doses used in the study were much higher than physiological levels. *Fkbp52* null mice have normal estrous cycles and the status of ovarian hormones during early pregnancy is comparable to WT littermates (data not shown). Because we have shown that $Fkbp52^{-/-}$ uteri during pregnancy have more than normal estrogenic influence due to uterine P_4 resistance,[17] we were able to circumvent ovariectomy or estrogen treatment in our current study of endometriosis. Induction of endometriosis was performed according to the method previously published with some modifications.[20,21] Seven to ten-week-old female mice were used for endometriosis induction. Vaginal smears of all mice were examined daily at least 7 days before inoculation. Mice in diestrus were selected to be used for donor and recipient mice. Donor mice were sacrificed, and 0.8 ml PBS was injected into the peritoneal cavity, and peritoneal fluid (donor/control) was collected. Then, uterine horns were removed and weighed. One piece of uterine tissue was kept in 10% neutral-buffered formalin as a control (donor) for immunostaining. The remaining uterine tissue was placed into a dish and minced using a surgical knife. Fragments suspended in 0.6 ml PBS were injected with an 18-gauge needle through the abdominal wall just below the umbilicus into the peritoneal cavity of recipient mice with the ratio of 1 donor to 2 recipients. All procedures were performed under aseptic conditions.

Fourteen days postinjection, recipient mice were sacrificed, their peritoneal cavities washed with 0.8 ml PBS, and peritoneal fluid collected. Then, uteri (eutopic) and endometriotic implants (ectopic) were removed and weighed. Tissues were fixed in 10% neutral-buffered formalin for histological analysis and immunostaining, or in 0.2% paraformaldehyde solution for lacZ staining. Each sample of peritoneal fluid was centrifuged, and the supernatant was kept at −80°C until enzyme-linked immunosorbent assay.

Human Tissue

The following tissues were obtained from 80 women undergoing laparoscopy: 1) endometrial tissues of women without endometriosis (endometriosis-free endometrium, $n = 40$), 2) endometrial tissues of women with endometriosis (eutopic endometrium, $n = 40$), 3) endometriotic tissues of women with endometriosis (ectopic endometrium; ovarian endometriosis, $n = 32$: peritoneal endometriosis, $n = 8$: deep-infiltrating endometriosis, $n = 4$). All women underwent laparoscopy for pain, infertility or other benign gynecological disorders during the period of

2006 to 2007. Endometriosis was diagnosed laparoscopically and confirmed histologically. Lesions of deep-infiltrating endometriosis were defined as those deeper than 5 mm beneath the peritoneal surface according to the previous study by Cornillie et al.[22] Forty women (aged 35.8 ± 6.2 years; mean ± SD) were diagnosed with endometriosis, and 40 women (aged 37.2 ± 6.5 years) had no endometriosis. Severity of endometriosis was determined according to the revised American Society for Reproductive Medicine classification. Among 40 women with endometriosis, 17 and 23 women were classified as stage 3 and stage 4, respectively. Forty endometriosis-free endometria and 40 eutopic endometria were collected from 40 different women without and with endometriosis, respectively. Eight of 44 endometriotic lesions were obtained simultaneously from four women, and rest of them from 36 different individuals. All subjects had regular menstrual cycles without any hormonal treatment for at least 6 months before surgery. Endometrial samples were dated according to the women's menstrual history and standard histological criteria by Noyes et al.[23] Tissues were fixed for histology and immunohistochemistry and snap-frozen for RNA isolation. The experimental procedures were approved by the institutional review board of University of Tokyo (IRB number 324), and signed informed consent for use of tissues was obtained from each woman.

Immunohistochemistry

Immunostaining of Ki-67, cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), PR, FKBP52, and estrogen receptor (ER) α was performed in 10% neutral-buffered formalin-fixed and paraffin-embedded sections (5 μm) of human and mouse tissues as previously described.[24] Antibodies specific to Ki-67 (Thermo Fisher Scientific, Fremont, CA), COX-2 (Cayman Chemical, Ann Arbor, MI), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), PR (Zymed Laboratories, Carlsbad, CA), FKBP52 (kindly given by David F Smith, Mayo Clinic), and ERα (Santa Cruz Biotechnology) were used. A Histostain-Plus kit (Zymed Laboratories) was used to visualize specific antigens. Brown deposits indicate sites of positive immunostaining.

Aberrant proliferation in ectopic lesions is a characteristic of endometriosis, with ectopic cells growing outside the normal hormonal regulation. In fact, the pattern of cell proliferation in ectopic lesions differs from estrogen- and P_4-governed proliferation of eutopic endometria.[25,26] The proliferative status of mouse ectopic lesions due to FKBP52 deficiency was assessed by Ki-67 immunohistochemistry. For Ki-67 quantification, five high-powered fields per respective section were analyzed microscopically. The percentage of the total cells staining for Ki-67 was calculated.

COX-2 and VEGF are widely accepted markers of endometriosis and are associated with its pathophysiology.[27,28] To assess whether there is any similarity between our mouse model of endometriosis with that of humans, immunostaining of COX-2 and VEGF was performed in uterine sections from donors (controls), as well as in sections from eutopic endometria and ectopic lesions retrieved from reciprocal transplantation of endometrial tissue in mice. Estrogen is a key factor in development of endometriosis, and the levels of ER and PR expression modulate estrogenic effects on various cells and tissues. To evaluate the contribution of these hormone receptors to our mouse model of endometriosis, immunostaining of PR and ERα was also performed. In addition, immunostaining of COX-2, VEGF, and PR in human endometriosis was performed to compare with the results of those in our mouse model of endometriosis.

FKBP52 immunostaining was performed in both human and mouse tissues. The intensity of staining was analyzed by a semiquantitative method, H-scoring.[29] H-score was calculated by the following equation: H-score = $\sum P_i(i + 1)$ where i is the intensity of staining with a value of 0, 1, 2, or 3 (negative, weak, moderate, or strong, respectively) and P_i is the corresponding percentage of the cells. Five high-powered fields per respective section were analyzed microscopically. Stromal cells in mice and both epithelial and stromal cells in humans were evaluated.

LacZ Staining and Quantification of Microvessel Density

LacZ staining and quantification of vessel density were performed as previously described.[30,31] Briefly, tissues were fixed in 0.2% paraformaldehyde for 24 hours followed by infusion in 30% sucrose at 4°C overnight. Tissues were then embedded in optimal cutting temperature compound and snap-frozen. Frozen sections were mounted onto glass slides and stained overnight at 37°C using β-galactosidase as a substrate. Sections were counterstained with eosin. Blue deposits indicate sites of positive staining. Endometriotic lesion areas occupied by lacZ-stained blood vessels were quantified. Six sections per lesion were randomly selected, digital images were obtained, and measurements were made using the Scion Image (Scion Corporation, Frederick, MD). The percentage of area occupied by lacZ-positive vessels was measured for each section.

Enzyme-Linked Immunosorbent Assay

There is evidence that several cytokines and growth factors are increased in peritoneal fluids of women with endometriosis and associated with its pathophysiology.[32,33] Among the up-regulated factors, levels of monocyte chemotactic protein-1 (MCP-1), regulated on activation, normal T-cell expressed and secreted (RANTES) and VEGF are elevated by estrogen.[34–37] To assess the contribution of FKBP52 deficiency to excessive estrogenic effects, concentrations of MCP-1, RANTES, and VEGF in mouse peritoneal fluid were measured by respective enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, MN) according to the manufacturer's protocol. Absorbance was read at 450 nm with an ELx800 automated microplate reader (BIO-TEK, Winooski, VT).

Reverse Transcription and Quantitative PCR

Total RNA was isolated from human tissues using Isogen (Nippongene, Toyama, Japan). Reverse transcription (RT)

and quantitative PCR were performed as previously described.[28] The RT reaction was performed using ReverTra Ace-α (Toyobo, Osaka, Japan). Quantitative PCR was performed in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using FastStart DNA Master Plus SYBR Green (Roche Diagnostics GmbH). The following primers were used: *Fkbp52*, sense, 5′-AGATGACAGCCGAGGAGATG-3′; antisense, 5′-AATTTGTCCTTGCGATCCAG-3′; *Gapdh*, sense, 5′-ACCACAGTCCATGCCATCAC-3′, antisense, 5′-TCCACCACCCTGTTGCTGTA-3′. Fkbp52 expression was normalized to RNA loading for each sample using Gapdh mRNA as an internal standard. Standardization of data was performed by subtracting the signal threshold cycles of Gapdh from that of Fkbp52. Each PCR product was purified with a QIAEX II gel extraction kit (Qiagen, Hilden, Germany), and identities were confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Statistical Analysis

Mann-Whitney *U*-test was used to compare expression levels of *Fkbp52* mRNA in human tissues. All other data were analyzed using unpaired Student's *t*-test and analysis of variance with posthoc analysis. $P < 0.05$ was accepted as statistically significant.

Results

FKBP52 Deficiency Promotes Endometriotic Growth and Cell Proliferation in a Newly Established Mouse Model

Using *Fkbp52*$^{-/-}$ mice, we examined the effects of FKBP52 deficiency on endometriosis and the relative roles of donor versus recipient FKBP52 in this disease using reciprocal transplantation of endometrial tissue minces within the peritoneum. All donor and recipient mice were synchronized at the diestrus stage to have their hormonal milieu comparable. Our reciprocal transplantation protocol was as follows: WT recipient to WT donor (WT-WT), *Fkbp52*$^{-/-}$ donor to *Fkbp52*$^{-/-}$ recipient (KO-KO), WT donor to *Fkbp52*$^{-/-}$ recipient (WT-KO), and *Fkbp52*$^{-/-}$ donor to WT recipient (KO-WT). We used age-matched groups, and there were no significant dif-

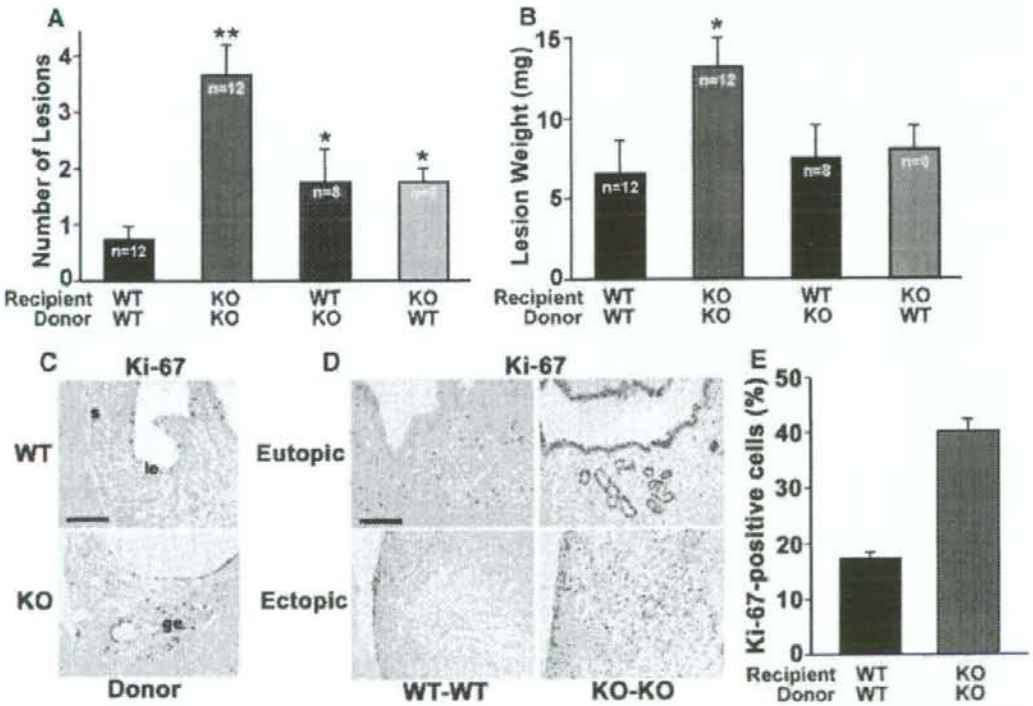

Figure 1. FKBP52 deficiency promotes growth of ectopic endometrial lesions in mouse model of endometriosis. The number (**A**) and total weight (**B**) of ectopic lesions in mouse endometriosis is shown. Both the number and weight of ectopic lesions in *Fkbp52*$^{-/-}$ recipient mouse with injection of *Fkbp52*$^{-/-}$ endometrium (KO-KO) is significantly higher than those in WT recipient mouse with injection of WT donor endometrium (WT-WT). Values are mean ± SEM. $^{*}P < 0.05$ compared with WT-WT group, $^{**}P < 0.0001$ compared with WT-WT group, analysis of variance. Immunohistochemistry of Ki-67 in (**C**) WT and KO donor endometrium (control) and (**D**) eutopic and ectopic endometrium of WT-WT (left column) and KO-KO (right column). ge, glandular epithelium; le, luminal epithelium; s, stroma. Scale bar = 200 μm. **E** The percentage of Ki-67-positive stromal cells in ectopic endometrium of WT-WT and KO-KO group. The values (Ki-67-positive stromal cells/total stromal cells, %) are presented as mean ± SEM of five different lesions. $^{*}P < 0.05$, unpaired Student's *t*-test.

ferences in uterine and body weights of donor and recipient mice (data not shown). In each group, ectopic endometrial lesions formed in the peritoneum, omentum and perivesical fat tissues, intestine, and liver. On gross examination, these lesions resembled red, yellow, or white inflamed tissues (Supplemental Figure 1, A–D, see *http://ajp.amjpathol.org*). They did not appear cystic as observed in previous studies that used estrogen administration to induce endometriosis.[20,21] Histologically, lesions were comprised primarily of stromal cells with a small number of epithelial cells; this is also the characteristic of human endometriosis. Hemorrhage and hemosiderin depositions, normally seen in human endometriosis,[39] were also found in these ectopic lesions (Supplemental Figure 1E, see *http://ajp.amjpathol.org*). The KO-KO group had a significantly higher number of lesions with significantly higher weights of the lesions than the other groups (Figure 1, A and B). While the WT-KO and KO-WT groups had a higher number of lesions than the WT-WT, weights of the lesions of these groups were comparable (Figure 1, A and B). These results show that deletion of *Fkbp52* in both the donor and recipient produces more robust endometriotic lesions.

Previous studies have shown that while proliferative activity is reduced in endometriotic lesions compared with eutopic endometria,[40] red endometriotic lesions, known to be relatively active, have high proliferative activity compared with black lesions, which are more established and matured.[25,41] These findings suggest that the status of lesions correlate with the mitogenic activity. While the proliferative status was comparable between WT and KO donor tissues (Figure 1C), we observed a remarkable increase in Ki-67-positive cells in ectopic lesions of the KO-KO group compared with those of the WT-WT group (Figure 1D). Indeed, quantification of Ki-67-positive cells showed that proliferation was significantly higher in the KO-KO ectopic lesions compared to those of WT-WT (Figure 1E). These findings suggest that FKBP52 deficiency aggravates endometriotic lesions with cell proliferation.

COX-2 and VEGF, known endometriosis markers, were expressed in ectopic lesions of both WT-WT and KO-KO groups, although their expression was more intense in the KO-KO group (Figure 2, A and B). These results are consistent with previous studies in both humans and mouse models.[42–45] Because COX-2 and VEGF are known regulators of uterine angiogenesis,[30,31] our observed increased stromal expression of COX-2 and VEGF primarily in KO-KO ectopic lesions led us to next question whether events of angiogenesis differ in the absence of FKBP52.

FKBP52 Deficiency Induces Angiogenesis in Murine Endometriotic Lesions

It is well-recognized that angiogenesis plays a key role in the development of endometriosis.[46,47] We have previously shown that ovarian estrogen and P_4 govern normal endometrial angiogenesis in mice.[30] This observation, together with our present finding of COX-2 and VEGF expression in ectopic lesions led us to hypothesize that decreased P_4-PR signaling observed in *Fkbp52* null uteri

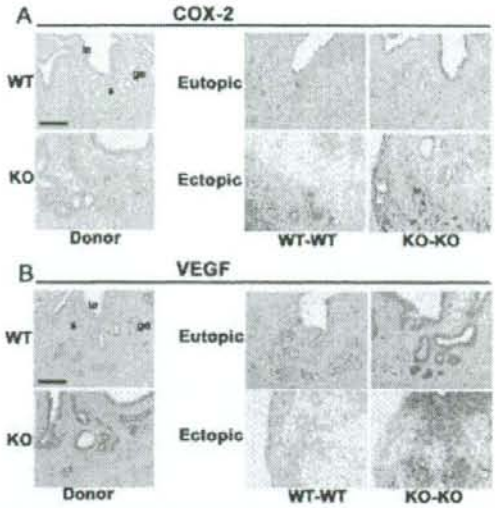

Figure 2. COX-2 and VEGF expression are increased in mouse endometriosis. **A:** COX-2 immunohistochemistry in donor (WT or KO), and eutopic and ectopic endometria of both WT-WT and KO-KO. **B:** VEGF immunohistochemistry in donor (WT or KO), and eutopic and ectopic endometria of both WT-WT and KO-KO. ge, glandular epithelium; le, luminal epithelium; s, stroma. Scale bar = 200 μm.

would lead to altered angiogenesis in ectopic lesions, contributing to the pathophysiology of the disease. To address this, we used *Fkbp52*$^{+/+}$/*Flk1*$^{lacZ+/-}$ (*Flk1*^{WT}) and *Fkbp52*$^{-/-}$/*Flk1*$^{lacZ+/-}$ (*Flk1*^{KO}) mice. *Flk1*$^{lacZ+/-}$ mice express β-galactosidase as a read-out for Flk1 promoter activity in newly formed endothelial cells.[48] Using this model, we were able to separately examine donor-derived and recipient-derived angiogenesis in ectopic lesions and evaluate the contribution of donor versus recipient derived blood vessels. We divided mice into the following four groups for reciprocal transplantation of minced endometrial tissues within the peritoneal cavity: WT donor to *Flk1*^{WT} recipient (WT-*Flk1*^{WT}), *Flk1*^{WT} donor to WT recipient (*Flk1*^{WT}-WT), *Flk1*^{KO} donor to *Fkbp52*$^{-/-}$ recipient (*Flk1*^{KO}-KO) and *Fkbp52*$^{-/-}$ donor to *Flk1*^{KO} recipient (KO-*Flk1*^{KO}). We found lacZ-stained Flk1-positive blood vessels in ectopic lesions of WT-*Flk1*^{WT} and KO-*Flk1*^{KO} groups, but not in those of *Flk1*^{WT}-WT or *Flk1*^{KO}-KO (Figure 3, A & B). These results show that endometriotic lesions arising from transplanted uterine tissues recruit blood vessels from the recipients for their growth. The microvessel density in ectopic lesions of the KO-*Flk1*^{KO} group was higher than those in WT-*Flk1*^{WT} group, showing that *Fkbp52* deletion also promotes angiogenesis (Figure 3C). The angiogenic status in eutopic endometria is shown in Supplemental Figure 2, (see *http://ajp.amjpathol.org*). *Fkbp52* null recipient endometria (*Flk1*^{KO}) with higher than normal estrogenic influence, had less new vessels (marked by lacZ staining) than that of WT with normal hormonal status. This is consistent with our previous findings showing that estrogen negatively regulates uterine angiogenesis in mice.[30] However, increased angiogenesis observed in ectopic lesions of *Fkbp52* null

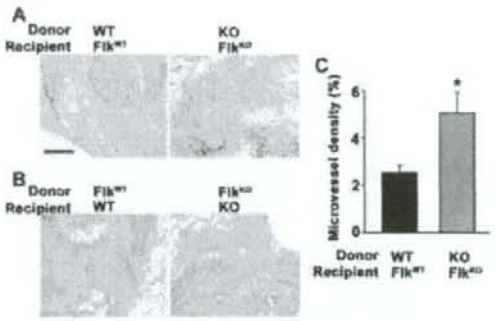

Figure 3. FKBP52 deficiency promotes angiogenesis in ectopic lesions in mouse models of endometriosis. **A** and **B**: LacZ staining of Flk1-expressing blood vessels in mouse endometriotic lesions. Observed blood vessels are derived from the recipient (**A**) but not from the donor (**B**). Scale bar = 200 μm. **C**: Microvessel density in endometriotic lesions of WT-*Flk1*^{WT} and KO-*Flk1*^{KO} group. Values are presented as mean ± SEM of five different lesions. *$P < 0.05$, unpaired Student's *t*-test.

mice suggests that the angiogenic environment in ectopic lesions is different from that of eutopic endometria.

PR and FKBP52, but Not ERα, Expression Is Reduced in Mouse Endometriosis

Immunohistochemistry results show reduced PR expression in both WT-WT and KO-KO ectopic lesions compared with eutopic and donor (control) endometria (Figure 4A). These results in mice are in accord with previous findings of reduced PR expression in human endometriosis.[11,25,26] We also observed that FKBP52 levels were reduced in ectopic lesions of WT-WT samples with only a few stromal cells showing expression (Figure 4B & Supplemental Figure 3A, see *http://ajp.amjpathol.org*). Our results showing exacerbated endometriotic lesion growth in *Fkbp52*$^{-/-}$ mice and decreased FKBP52 expression in WT endometriotic lesions suggest that down-regulation of PR or FKBP52 potentially contributes to P_4 resistance and the pathogenesis of endometriosis. ERα expression in ectopic lesions of KO-KO group was nearly identical to those of WT-WT, and the intensity of stromal ERα expression in ectopic lesions appeared similar to that of eutopic and donor endometria (Figure 4C).

Peritoneal Levels of MCP-1 Are Increased in Mice with Endometriotic Lesions

Because our findings suggest that FKBP52 deficiency confers more than normal estrogenic effects, we hypothesized that concentrations of MCP-1, RANTES, and VEGF in mouse peritoneal fluids would increase. Indeed, enzyme-linked immunosorbent assay results show that levels of MCP-1, RANTES, and VEGF in endometriotic peritoneal fluids (recipients) were significantly increased compared with those found in controls (donors) (Figure 5A–C). MCP-1 levels in KO-KO endometriotic peritoneal fluid were also significantly higher than those of WT-WT (Figure 5A). While the levels of RANTES and VEGF in KO-KO endometriotic samples also increased compared to WT-WT, these levels did not reach statistical significance ($P = 0.081$ and 0.138, respectively) (Figure 5, B and C).

FKBP52 Expression Is Reduced in Human Endometriosis

The results described above using *Fkbp52* null mice as an endometriosis model demonstrate that reduced P_4-PR

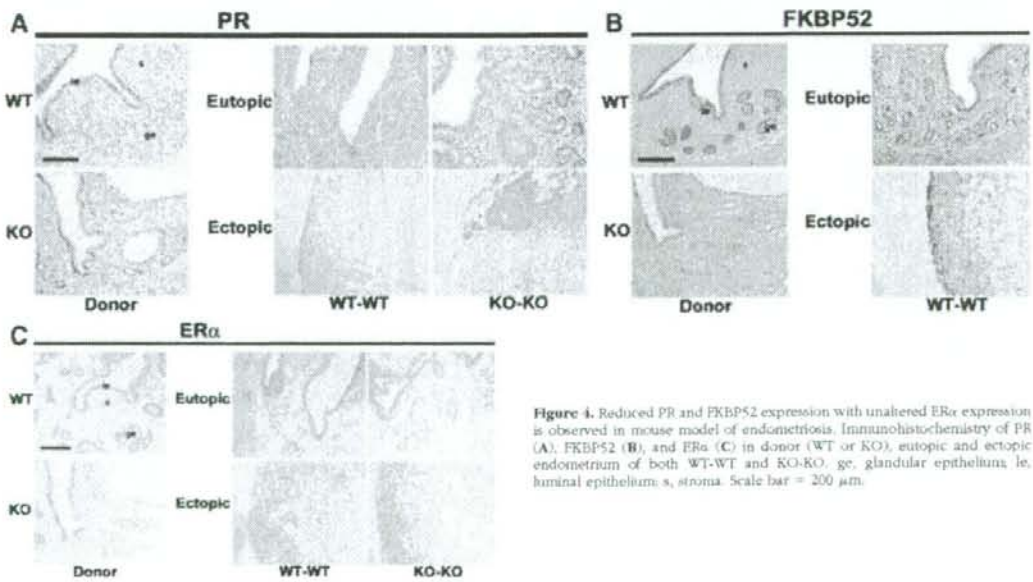

Figure 4. Reduced PR and FKBP52 expression with unaltered ERα expression is observed in mouse model of endometriosis. Immunohistochemistry of PR (**A**), FKBP52 (**B**), and ERα (**C**) in donor (WT or KO), eutopic and ectopic endometrium of both WT-WT and KO-KO. ge, glandular epithelium; le, luminal epithelium; s, stroma. Scale bar = 200 μm.

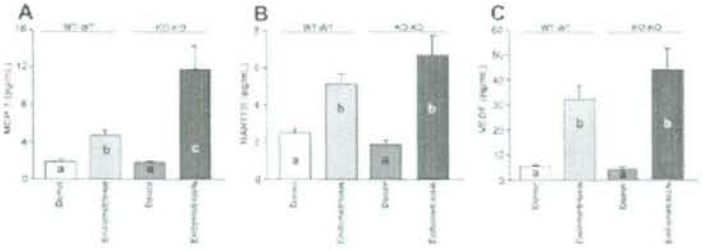

Figure 5. Increased cytokine levels in endometriotic peritoneal fluid of *Fkbp52*$^{-/-}$ mice with endometriosis. Concentrations of MCP-1 (**A**), RANTES (**B**), and VEGF (**C**) in peritoneal fluid of mouse endometriosis. Control peritoneal fluid (donor), endometriotic peritoneal fluid (endometriosis) is from the recipient. Letters within the bars indicate statistical significance (*a* vs *b*, $P < 0.05$; *b* vs *c*, $P < 0.0005$; analysis of variance).

signaling facilitates the development of endometriosis. We speculated that FKBP52, because of its conserved role as a PR cochaperone, is also critical in the pathogenesis of human endometriosis. Thus, we examined its expression in human endometria of women with and without endometriosis (eutopic and endometriosis-free endometria, respectively) and in human endometriotic lesions ($n = 5$ separate individuals in each stage of the menstrual phase). Results from three representative individuals in each menstrual phase are shown (Figure 6, A–C). Immunostaining detected FKBP52 in stromal and epithelial components of both endometriosis-free and eutopic endometria, although immunoreactive FKBP52 was less intense in eutopic than endometriosis-free endometria, especially in stromal cells (Figure 6, A–B and Supplemental Figure 3B, see *http://ajp.amjpathol.org*). Interestingly, levels of FKBP52 were also remarkably low in ectopic lesions, showing only weak staining in epithelial cells (Figure 6C). There were no striking differences in FKBP52 expression between tissues in proliferative (PP) or secretory phases (SP) among the three groups. Because there is evidence that ovarian, peritoneal and deep-infiltrating endometriotic lesions each have different pathogeneses[49] and because our samples were of ovarian endometriosis, we also confirmed reduced FKBP52 expression in ectopic tissues of peritoneal and deep-infiltrating endometriosis (Supplemental Figure 4, see *http://ajp.amjpathol.org*).

The immunohistochemical studies were complemented by quantitative expression levels of Fkbp52 mRNA in human endometrial and endometriotic samples. Fkbp52 mRNA levels in eutopic endometria and ectopic lesions were significantly lower than endometriosis-free endometria, confirming our immunostaining results (Figure 7, A). We subdivided the data to compare differences in Fkbp52 mRNA levels between PP and SP of each group. We found that Fkbp52 mRNA levels in eutopic endometria and ectopic lesions were significantly decreased in PP, but only moderately in SP compared with endometriosis-free endometria at these phases (Figure 7B).

Reduced PR and FKBP52 Levels with Increased COX-2 and VEGF Expression Is Observed in Human Endometriosis

Our *Fkbp52* null mouse model of endometriosis shows decreased PR expression with increased COX-2 and VEGF expression in ectopic lesions. We therefore examined whether similar expression patterns occur in human endometriosis. Immunostaining of PR, COX-2 and VEGF in human tissues showed that these expression patterns are conserved between mice and humans. While the intensity of FKBP52 immunostaining was lower in eutopic

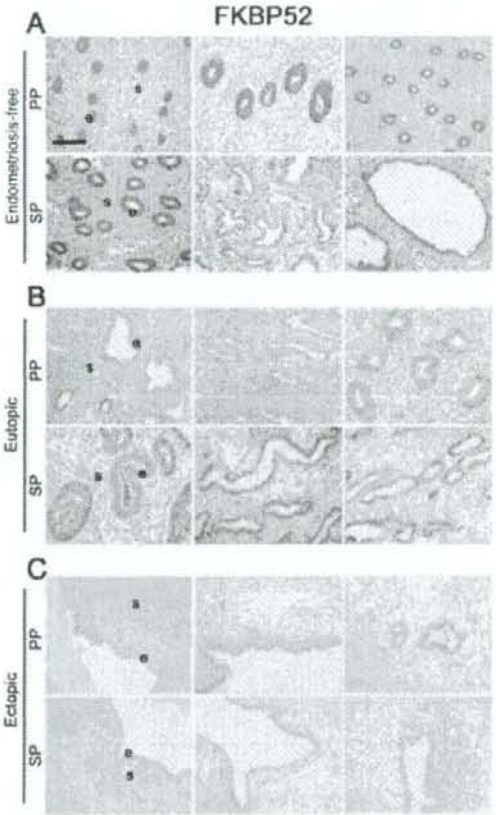

Figure 6. FKBP52 expression is reduced in human eutopic and ectopic endometria. Representative FKBP52 immunostaining in human endometria of women without endometriosis (endometriosis-free) (**A**), or of women with endometriosis: eutopic (**B**) and ectopic (**C**). Each column represents a different individual. Each panel is a representative photograph of five samples of endometriosis-free, eutopic and ectopic human endometria in each menstrual phase. Ectopic endometrium is derived from ovarian endometriosis. PP, proliferative phase; SP, secretory phase; e, epithelium; s, stroma. Scale bar = 200 μm.