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 child-bearing 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*^{-/-} mice⁸ 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-

Supported in part by National Institutes of Health grants HD12304, DA06668 and P01CA077839. S. K. Dey is the recipient of Method to Extend Research in Time (MERIT) Awards from the NICHD and the National Institute on Drug Abuse (NIDA). Y. Hirota is supported by a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists. S. Tranguch was supported by an NICHD Training Grant 2THD007043.

Accepted for publication August 20, 2008.

Supplementary material for this article can be found on <http://ajp.amjpathol.org>.

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tients is sustained despite progestin therapy.^{3,6} The prevailing hypothesis is that an enhanced estrogenic influence and/or reduced P₄ responsiveness leads to endometriosis.^{3,12,14} Therefore, an animal model presenting more than normal estrogenic activity with suboptimal P₄ 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 P₄ 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 P₄ 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 P₄ 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 P₄ supplementation, depending on the genetic background of mice.¹⁷ Thus, FKBP52 deficiency confers endometrial P₄ 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 P₄-PR signaling, rather than reduced P₄ levels, is a major contributing factor for P₄ resistance in endometriosis. Unlike *PR*^{-/-} mice with no P₄-PR signaling, *Fkbp52*^{-/-} mice with basal uterine P₄-PR responsiveness are perhaps a more physiologically relevant model to study the role of P₄ 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 P₄ responsiveness in furthering the development of endometriosis.

Materials and Methods

Mice

Fkbp52 null mice were originally established on a C57BL/6J/129SvJ background¹⁸ 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 P₄ 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 $\pm$ 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 μ m) 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 ER α (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 P₄-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 endo-

metrial 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 ER α 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 chemoattractant 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.³⁸ 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'-AGATGACAGCCGAG-GAGATG-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 μ m. 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 P₄ 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 P₄-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 μ m.

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 μ m. **C**: Microvessel density in endometriotic lesions of WT-Flk1^{WT} and KO-Flk1^{KO} group. Values are presented as mean $\pm$ 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 ER α , 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 & Sup-

plemental 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 P₄ resistance and the pathogenesis of endometriosis. ER α expression in ectopic lesions of KO-KO group was nearly identical to those of WT-WT, and the intensity of stromal ER α 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 P₄-PR

Figure 4. Reduced PR and FKBP52 expression with unaltered ER α expression is observed in mouse model of endometriosis. Immunohistochemistry of PR (**A**), FKBP52 (**B**), and ER α (**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 μ m.

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 pathogenesises⁴⁹ 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 exam-

ined 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 μ m.

Figure 7. Reduced Fkbp52 mRNA levels in eutopic and ectopic human endometria. Fkbp52 mRNA levels in endometriosis-free, eutopic and ectopic human endometrium (A), and their relation to the phase of the menstrual cycle (B). Boxes represent the distance between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes denote the median. Whiskers represent the 10th percentile at the lower limit and the 90th percentile at the upper limit. Ectopic endometrium is derived from ovarian endometriosis. A: *P < 0.05 and **P < 0.01 compared with control endometrium. B: *P < 0.05 compared with endometriosis-free endometrium of the same menstrual phase; Mann-Whitney U-test.

endometria compared to endometriosis-free endometria, the signal intensity was even lower in ectopic lesions (Figure 8, A). As expected, PR immunostaining in endometriosis-free PP endometria was higher than SP, but the signal intensity was remarkably low in eutopic endometria or in ectopic lesions regardless of the cycle stage (Figure 8B).

COX-2 expression was low in endometriosis-free endometria in both PP and SP, but appeared higher in eutopic endometria primarily at SP. Ectopic lesions also had higher levels of COX-2 (Figure 8C). In contrast, VEGF immunostaining appeared to be of similar intensity in control, eutopic endometria, and ectopic lesions regard-

Figure 8. Reduced PR expression with increased COX-2 and VEGF expression is observed in human ectopic endometria. Representative immunostaining of FKBP52 (A), PR (B), COX-2 (C), and VEGF (D) in endometriosis-free, eutopic and ectopic human endometria at proliferative (PP) and secretory (SP) phases. Serial sections of the same sample are displayed in each horizontal row. e, epithelium; s, stroma. Scale bar = 200 μm.

less of the cycle stage (Figure 8D). These results show that our genetic mouse model of endometriosis recapitulates similar read-outs as those found in human endometriosis, specifically reduced levels of FKBP52 and PR expression and increased levels of COX-2.

Discussion

Progestin therapy is commonly used to treat endometriosis-related symptoms including pain. In fact, progestin treatment is considered one of the most useful therapies for alleviating endometriosis-related pain. Still, some women are unresponsive to the treatment.^{3,6} Therefore, it has long been suspected that some patients with endometriosis have P₄ resistance. The recent development of genetic and molecular approaches allows for the examination of underlying causes of P₄ resistance. Effects of P₄ are primarily mediated by PR, which has two isoforms, PR-A and PR-B. Studies in PR-A and PR-B null mice have shown that these two isoforms are expressed in a cell-specific manner and function differently.^{50,51} PR-A is essential for normal ovarian and uterine functions including ovulation, implantation and decidualization.⁵⁰ In contrast, PR-B is critical for normal mammary gland development.⁵¹ Given that stromal cells of human endometria contain predominantly PR-A,⁵² it is assumed that functional FKBP52-PR-A signaling is crucial for normal endometrial events. PR-A expression is substantially decreased in endometriotic lesions compared with eutopic endometria with non-detectable PR-B expression.¹¹ In our present study, we also found reduced PR expression in endometriosis of both mice and humans. These findings suggest that decreased PR expression is one reason for P₄ resistance. However, our results of reduced FKBP52 expression in ectopic lesions also suggest that FKBP52 deficiency contributes to attenuated PR signaling in endometriosis.

Molecular and cellular interactions between the ectopic endometria and peritoneal surface are crucial for the development of ectopic lesions. Existing endometriotic models are heterologous, using human endometrial tissues transplanted in immunocompromised mice. Our homologous mouse model provides the opportunity to study immunological aspects of endometriosis. Because immunophilin FKBP52 has properties of an immunological factor, our homologous model could also be a valuable system to study the immunological functions of FKBP52 in endometriosis.

In our *Fkbp52* null mouse model, we transplanted minced endometria from donor mice into the peritoneal cavity of recipient mice to simulate the aspect of human endometriosis that results from retrograde endometrial shedding into the peritoneal cavity during menstruation. The ability of transplanted endometrial tissues to form ectopic lesions depends not only on the state of the donor endometrium, but also on the peritoneal environment of the recipient. We evaluated the effects of loss of *Fkbp52* on cooperation of endometrial tissues with peritoneal receptivity. Our results show that loss of *Fkbp52* in both the donor and recipient mice is required to

significantly increase the number and growth of lesions, suggesting that FKBP52 deficiency in both the donor endometrium and recipient peritoneal cavity is involved for optimal development of endometriosis. It is not yet understood why and how the lack of FKBP52 in the peritoneum bolsters endometriotic growth, but it is possible that FKBP52 has other functions independent of PR.^{18,53–55}

Many studies have shown anti-proliferative roles of PR in the uterus.^{7,56} In fact, a previous study using PR null mice found that estrogen is a primary factor that determines the size of ectopic lesions.⁸ This study also found that the anti-proliferative effects of P₄ on estrogen-mediated lesion growth are mediated by PR. Our findings are consistent with this study, because deletion of *Fkbp52* that reduces P₄-PR signaling, leads to enhanced endometriotic growth with increased proliferation. However, the novelty of our present study is that we provide genetic evidence in mice that even a reduced responsiveness to P₄-PR signaling encourages endometriotic growth; the complete absence of uterine P₄-PR signaling is not an absolute requirement.

Endometrial angiogenesis is normally regulated by estrogen and P₄. Estrogen stimulates proliferation of human endometrial endothelial cells *in vitro*,^{57,58} but inhibits angiogenesis in mouse uteri *in vivo*.³⁰ In contrast, P₄ inhibits estrogen-induced proliferation of human endometrial endothelial cells *in vitro*,^{57,58} but stimulates angiogenesis in mouse endometria *in vivo*.³⁰ These findings suggest that uterine endothelial cells respond to ovarian steroids differently in extrauterine sites, and/or that heterogeneous cell types of the uterus respond differently to these hormones in a dynamic manner. Here we present genetic evidence that more Flk1-positive blood vessels enter *Fkbp52* null ectopic lesions than WT lesions. Because these lesions are mainly composed of stromal cells with minimal contribution from the epithelial component, we propose that this change in cell composition may alter the angiogenic response to ovarian hormones. This may explain then why reduced P₄ responsiveness due to FKBP52 deficiency promotes migration of endothelial cells into endometriotic lesions.

Our study also used *Flk1^{lacZ}+/–* mice to explore donor versus recipient induced angiogenesis. We found that ectopic lesions recruited blood vessels for angiogenesis from the recipient. We have previously shown that while estrogen attenuates angiogenesis in the mouse uterus, P₄ promotes this process in the uterus.³⁰ Here we found that while FKBP52 deficiency confers reduced angiogenesis in eutopic endometria due to enhanced estrogenic influence resulting from reduced P₄-PR signaling, loss of FKBP52 enhances angiogenesis in ectopic lesions. The question then is why does the loss of *Fkbp52*, associated with decreased P₄-PR signaling, promote angiogenesis in ectopic lesions? One possibility is that there is an angiogenic switch in endometrial tissues with regard to steroid hormone responsiveness depending on the site (eutopic versus ectopic). Further studies are warranted to address this issue.

The establishment of a new blood supply is essential for survival of endometriotic lesions and development of

endometriosis. VEGF is a heparin-binding angiogenic growth factor, the most potent mediator of angiogenesis.⁵⁹ It is a strong endothelial mitogen/survival factor and an inducer of vascular permeability. VEGF binds to a family of tyrosine kinase receptors, particularly Flt1 (VEGFR1) and Flk1 (VEGFR2). Flk1 is the major transducer of VEGF signals that induce migration and proliferation of endothelial cells.⁵⁹ To date, evidence indicates that VEGF is involved in the pathophysiology of endometriosis.^{28,46,47} We show here that VEGF is expressed in ectopic lesions in our mouse model of endometriosis, and that VEGF expression is increased in *Fkbp52* null ectopic lesions compared to WT. There is evidence that ER along with its cofactors binds to the VEGF promoter to promote its transcription in human endometrial cells.⁶⁰ Moreover, estrogen induces VEGF secretion in human endometrial stromal cells, an induction that is suppressed by progesterone.³⁶ We propose that in the absence of FKBP52, P₄-PR signaling is reduced and P₄ cannot inhibit estrogen-induced VEGF secretion, resulting in excess VEGF in ectopic lesions and increased angiogenesis.

Estrogen is also known to up-regulate expression of several cytokines in endometriosis. MCP-1 and RANTES are well-known chemokines whose concentrations are high in peritoneal fluid of women with endometriosis^{61,62} and whose secretions are promoted by estrogen.^{34,35} In human endometriotic stromal cells, inflammatory stimuli induce secretion of these chemokines, and estrogen enhances their secretion.^{34,35} *In vitro* studies in human endometrial stromal cells have shown that adding P₄ does not suppress estrogen-induced expression of these chemokines,^{34,35} suggesting P₄ resistance in endometriotic cells. Our present findings that *Fkbp52* null mice with endometriotic lesions have enhanced levels of chemokines in their peritoneal fluids corroborates well with reduced PR and FKBP52 expression and unchanged ER α expression in ectopic lesions.

COX-2, an inducible enzyme that synthesizes prostaglandins, is known to play an important role in both immunological and angiogenic responses during endometriosis.^{63–65} Because inflammatory stimuli, growth factors, and cytokines induce COX-2, it is possible that the immune environment imposed by the developing endometriotic lesions in the face of reduced P₄-PR signaling in the absence of FKBP52 induces COX-2 expression, which also could contribute to up-regulation of VEGF expression.

There is very limited information linking FKBP52 with disease processes. To our knowledge, this is the first report showing that down-regulation of FKBP52 is associated with a human disease. In contrast, FKBP52 overexpression is observed in breast and prostate cancer.^{66–68} In fact, the immunosuppressant drug FK506, which binds immunophilins FKBP51 and FKBP52, inhibits androgen receptor activity in prostate cancer cells.⁶⁷ Likely, FK506 is considered to affect other steroid receptor activities.^{69,70} Although there is no report that FK506 influences the incidence and development of endometriosis, it may be prudent to address this issue, because endometriosis is considered an immunological disorder.^{71,72}

In conclusion, our novel mouse model of endometriosis shows that the deletion of FKBP52 promotes the growth of endometriotic lesions with increased angiogenesis recruited from the recipient. Moreover, we demonstrate that FKBP52 expression is decreased in eutopic and ectopic endometria of women with endometriosis compared with endometria of women without endometriosis. After our genetic studies on the role of FKBP52 in uterine biology and pregnancy in mice were reported,^{16,17} a study reported that FKBP52 levels are reduced in eutopic endometria of baboons subjected to experimental endometriosis.⁷³ Collectively, these findings suggest that endometriosis induced by FKBP52 deficiency is a conserved phenomenon and FKBP52 is not only physiologically but also pathologically critical molecule for female reproductive functions.

Acknowledgments

The experiments using human samples were performed at University of Tokyo, and the design of the study, experiments on mice, data evaluation, and manuscript preparation were performed at Vanderbilt University.

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