Down-regulation of p21-activated kinase 1 by progestin and its increased expression in the eutopic endometrium of women with endometriosis

Sung Hoon Kim¹,³, Hoi Woul Lee¹, Young Hoon Kim², Yun Hee Koo¹, Hee Dong Chae¹, Chung Hoon Kim¹, Pil Ryang Lee¹, and Byung Moon Kang¹

¹Department of Obstetrics and Gynecology, University of Ulsan College of Medicine, Asan Medical Center, 388-1, Pungnap-2dong, Songpa-gu, Seoul 138-736, South Korea ²Department of Pharmacology, University of Ulsan College of Medicine, Asan Medical Center, Songpa-gu, Seoul 138-736, South Korea ³Correspondence address. Tel: +82-2-3010-3647; Fax: +82-2-3010-6944; E-mail: kimsung@amc.seoul.kr

BACKGROUND: P21-activated kinase 1 (Pak1) integrates various signaling pathways that are vital to cell survival and function. This study was performed to evaluate whether sex steroids may regulate the expression of Pak1 in endometrial cells as well as whether its expression is increased in the eutopic endometrium of women with endometriosis.

METHODS: Following in vitro estradiol (E2) and/or medroxyprogesterone acetate (MPA) treatment of Ishikawa cells and endometrial stromal cells (ESCs), Pak1 protein was analyzed utilizing western blot analysis and immunocytochemistry. Immunohistochemistry was performed to evaluate Pak1 immunoreactivity semiquantitatively in women with endometriosis and in controls. To assess the role of Pak1 on endometrial cell viability, crystal violet assay was performed following transfection of Ishikawa cells with Pak1 small interfering RNA (siRNA).

RESULTS: In vitro treatment with E2 plus MPA or MPA alone led to a significant decrease of Pak1 protein in Ishikawa cells and ESCs (both \( P < 0.05 \) versus control). Immunohistochemistry also revealed that Pak1 protein is significantly decreased during the secretory phase in both epithelial and stromal cells in the control subjects (\( P < 0.001 \) and \( P < 0.01 \), respectively). The immunoreactivity of Pak1 in glandular cells was significantly increased in the eutopic endometrium of women with endometriosis compared with the controls during the secretory phase (\( P < 0.01 \)). Crystal violet assay has shown that transfection of Ishikawa cells with Pak1 siRNA led to a significant decrease of cellular viability (\( P < 0.05 \)).

CONCLUSIONS: These findings suggest that Pak1 is down-regulated by progesterone during the secretory phase in normal endometrium and increased Pak1 activity during the secretory phase might lead to establishment of endometriosis.

Key words: P21-activated kinase 1 / endometrium / endometriosis / progestin

Introduction

P21-activated kinases (Paks) are a family of well characterized serine/threonine kinases integrating various signaling pathways that are vital to normal cell survival and function (Kumar et al., 2006). Paks have been shown to regulate diverse cellular activities including growth factor and steroid receptor signaling, energy homeostasis, and transcription and mitosis (Bokoch, 2003). Originally identified as a protein interacting with cell division cycle 42 (Cdc42) and ras-related C3 botulinum toxin substrate (Rac), p21-activated kinase 1 (Pak1) is the first and the best-characterized member of the Pak family (Manser et al., 1994). Studies have demonstrated that Pak1 is essential for cell transformation induced by various oncogenes and its expression is up-regulated in some human tumor types (Osada et al., 1997; Tang et al., 1997; Vadlamudi et al., 2000; Kumar et al., 2006).

Specifically, it has been well documented that Pak1 is involved in the development and progression of breast cancer. Balasenthil et al. (2004a) have shown that Pak1 is overexpressed in 57% (34 of 60)
of human breast cancer sections in concordance with increased cyclin D1 expression. They also found that transgenic mice overexpressing Pak1 in mammary tissue developed mammary hyperplasia in accordance with enhanced cyclin D1 expression. Moreover, a recent study utilizing a transgenic mice model has suggested that Pak1 hyperactivation may be sufficient for the formation of mammary gland tumors (Wang et al., 2006). Paks are regulated by many upstream signaling pathways, which include those that involve membrane-bound growth factor receptors, integrin cell adhesion complexes and G protein-coupled receptors (Kumar et al., 2006). It has been shown in breast cancer cells that estradiol (E2) can also activate Pak1 signaling in an estrogen receptor (ER)-dependent manner, providing new evidence that Pak1 is a signaling component of estrogen action and Pak1 acts as an important mediator of the cell survival function of estrogen (Mazumdar and Kumar, 2003).

Endometrium exhibits rapid cyclical growth and shedding throughout female reproductive life, and estrogen is responsible for promotion of its proliferation. Defined as the presence of endometrial tissue outside the uterus, endometriosis causes diverse diseases, including infertility, pelvic pain and dysmenorrhea. Although the mechanisms responsible for its pathogenesis and progression remain poorly understood, it is well established that endometriosis grows and regresses in an estrogen-dependent fashion. Our hypothesis is that sex steroids may regulate the expression of Pak1 in endometrial cells and that Pak1 expression changes according to the menstrual cycle as well as the presence of endometriosis. We aimed to identify the in vitro effects of sex steroids on the level of Pak1 protein in endometrial cells, to evaluate its in vivo expression in endometrium of women with and without histological evidence of endometriosis and to assess the functional activity of Pak1 in endometrial cells by transfecting with small interfering RNA (siRNA).

Materials and Methods

Tissue collection

For endometrial stromal cell (ESC) cultures, endometrial samples (n = 5) were obtained from fertile women with carcinoma in situ (CIS) of the uterine cervix at the time of hysterectomy, who had no evidence of endometrial abnormalities, adenomyosis or pelvic endometriosis and had not received any hormonal medications in the preceding 3 months. The endometrial samples were placed in Hank’s balanced salt solution (HBSS) and transported to the laboratory for ESC isolation and culture. Written informed consents were obtained from each patient using consent forms and protocols approved by the Review Board for Human Research of Asan Medical Center.

For immunohistochemical staining, endometrial sections were obtained from 31 women with CIS (control) as well as 29 women with histological evidence of endometriosis. All of the recruited women had regular menstrual cycles and had undergone hysterectomies by transabdominal or laparoscopic methods. Women with endometrial abnormality, adenomyosis or pelvic endometriosis were excluded from the control group, and all of the women in the endometriosis group were confirmed as having advanced stage endometriosis, staged according to the American Society for Reproductive Medicine (1997). The phase of the menstrual cycle was classified as proliferative (Days 1–13), early secretory (Days 14–19), mid-secretory (Days 20–23) and late secretory (Days 24–28) phases by endometrial histology using the criteria of Noyes et al. (1975). The clinical characteristics, menstrual phases of each group and stages of endometriosis are summarized in Table I. There was no significant difference in age between the two groups, but the average number of deliveries was significantly lower in the endometriosis group (P < 0.05). We did not need additional informed consent to use the specimens in this study, because only archived material was used. The Review Board for Human Research in our hospital approved this project.

Chemicals and cell lines

E2, medroxyprogesterone acetate (MPA) and the Ishikawa cell line (a well-differentiated endometrial adenocarcinoma cell line) were purchased from Sigma-Aldrich (St Louise, MO, USA).

Isolation and culture of human ESC

ESCs were separated and maintained in monolayer culture as described previously (Arici et al., 1993). Briefly, endometrial tissue was minced with a sterile surgical blade and digested in HBSS (Sigma-Aldrich) containing collagenase B (1 mg/ml, 15 U/mg, Roche, Indianapolis, IN, USA), deoxyribonuclease I (0.1 mg/ml, 1500 U/mg, Roche), penicillin (200 U/ml) and streptomycin (200 mg/ml) for 60 min at 37 °C with gentle shaking. The dispersed endometrial cells were separated by filtration through a wire sieve (73-μm diameter pore, Sigma-Aldrich) and were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) Ham’s F-12 (1:1 v/v; Sigma-Aldrich) containing fetal bovine serum (10% v/v; Gibco BRL, Rockville, MD, USA). The cultures were maintained in a standard 95% air; 5% CO2 incubator at 37 °C and allowed to replicate to confluence. Thereafter, ESCs were passed by standard methods of trypsinization, plated in culture dishes and grown in DMEM supplemented with 10% charcoal-stripped calf serum (Flow Laboratories, Rockville, MD, USA).

Experimental setup

Each experiment with Ishikawa cells was repeated at least three times using cells prepared separately at five different times. Each experiment with ESCs was also repeated at least three times using cells prepared from endometrial tissue specimens obtained from five different patients. When Ishikawa cells and ESCs were grown to 70% confluence, they were treated with serum-free, phenol red-free media (Sigma-Aldrich) for 24 h before treatment with steroids. Cell cultures were treated with

| Table I Clinical characteristics of patients and controls |
|---------------------------------|-----------------|
| Control group                  | Endometriosis group |
| (n = 31)                       | (n = 29)         |
| Agea                          | 37.2 ± 3.6       | 39.2 ± 5.6       |
| No. of deliveriesa             | 1.9 ± 0.7        | 1.2 ± 0.9b       |
| Menstrual phases               |                 |                 |
| Proliferative                 | 13               | 12              |
| Early secretory               | 4                | 4               |
| Mid-secretory                 | 6                | 6               |
| Late secretory                | 8                | 7               |
| American Fertility Society Classification of endometriosis |       |
| Stage III                     | 7                |                 |
| Stage IV                      | 22               |                 |

aValues are mean ± SD.

bP < 0.05, Student’s t-test.
either vehicle (control) or E2 (10⁻⁹, 10⁻⁸ or 10⁻⁷ M), E2 10⁻⁸ M plus MPA 10⁻⁷ M and MPA 10⁻⁷ M for 24 and 48 h.

**Western blot analysis**
Total proteins from cultured Ishikawa cells and ESCs were extracted with cell extraction buffer (Pierce, Rockford, IL, USA) containing 3 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich). The protein concentration was determined by a detergent compatible protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples (5 μg) were loaded on 7.5% Tris–HCl Ready Gels (Bio-Rad Laboratories), electrophoretically separated and electroblotted onto polyvinylidene fluoride membrane (Qbiogene Molecular Biology, Solon, OH, USA). The membrane was blocked with 5% non-fat dry milk in tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 1 h to reduce non-specific binding. Subsequently, the membrane was incubated for 1 h with primary antibody against Pak1 [rabbit monoclonal anti-human Pak1 antibody (Abcam, Cambridge, MA, USA)]. The membrane was washed with TBS-T for 1 h and incubated with horseradish peroxidase conjugated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h. The protein was visualized by light emission on film (Agfa-Gevaert, Septestraat, Mortsel, Belgium) with enhanced chemiluminescence substrate (Bio-Rad Laboratories). Immunoblot bands for Pak1 were quantified using a laser densitometer.

**Immunocytochemistry**
ESCs after the first passage were assayed immunocytochemically using specific cell-surface markers for evaluating the purity of ESCs. Cells were plated on cover glass coated with polyethyleneimine (Sigma-Aldrich) and grown at 37°C in a humidified CO₂ incubator until 70% cell confluence was reached. The cover glasses were fixed in 3% hydrogen peroxide for 15 min and permeabilized in 0.1% Triton X-100 for 15 min. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide for 5 min and followed by rinsing in TBS. Following exposure to 5% donkey serum (Jackson Immunoresearch Laboratories) for 1 h and incubated with horseradish peroxidase conjugated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h. The protein was visualized by light emission on film (Fujifilm, Tokyo, Japan) with enhanced chemiluminescence substrate (Bio-Rad Laboratories). Immunoactivity bands for Pak1 were quantified using a laser densitometer.

**Immunohistochemistry**
Formalin-fixed paraffin-embedded samples of endometrium were cut into 5 μm sections. After deparaffinization, slides were boiled in citrate buffer (10 mM, pH 6.0) for 15 min for antigen retrieval. Then, sections were immersed in 3% hydrogen peroxide for 15 min to block endogenous peroxidase. Slides were then incubated in a humidified chamber with 5% blocking goat serum (Vector Laboratories) in TBS for 30 min at room temperature. Afterwards, excess serum was drained and sections were incubated with primary antibody [rabbit monoclonal anti-human Pak1 antibody (Abcam)] overnight at 4°C in a humidified chamber. Non-immune (normal) immunoglobulin G1 was used at the same concentration as the primary antibody for negative controls. The sections were washed three times for 5 min with TBS, and then biotinylated goat anti-rabbit antibody (Vector Laboratories) was added as secondary antibody for 30 min at room temperature. The antigen–antibody complex was detected using an avidin-biotin-peroxidase kit (Vector Laboratories). Diaminobenzidine (Vector Laboratories) was used as the chromogen and sections were counterstained with hematoxylin.

The intensity for Pak1 immunoreactivity was semiquantitatively evaluated using the following intensity categories: 0 (no staining), 1+ (weak but detectable staining), 2+ (moderate or distinct staining) and 3+ (intense staining). For each tissue, a HSCORE value was derived by summing the percentages of cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining, using the formula: HSCORE = Σ Pi(i + 1), where i represents the intensity scores and Pi is the corresponding percentage of the cells. In each slide, five randomly selected areas were evaluated under a light microscope (×40 magnification), and the percentage of cells for each intensity within these areas was determined at different times by three investigators blinded to the type and source of the tissues. The average score of three was used for final analysis.

**Small interfering RNA transfection**
and real-time reverse-transcription polymerase chain reaction
Pak1 siRNA and non-specific control siRNA were designed and produced by Genolution (Seoul, South Korea). Ishikawa cells, grown to 30% confluence, were transfected utilizing oligofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA) with Pak1 siRNA or non-specific control siRNA according to the manufacturer's instructions. The 21 mer sequence of Pak1 siRNA was 5’-CUCCAACCCAGAGAGAAGA-3’ (forward) and 3’-GGAGGUGGUUGCUCCUU-5’ (reverse). The final concentration of siRNA was 30 nmol/l.

Forty-eight hours after transfection, total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Quantitative real-time reverse-transcription polymerase chain reaction (RT–PCR) was performed using PrimeScript™ RT Enzyme Mix II, RNase-free water, 10 pmol each of forward and reverse primers of the target gene and 10 μl of total RNA. RT was carried out for 5 min at 37°C and 10 s at 95°C. PCR amplification was performed for 40 cycles of 95°C for 5 s and 60°C for 30 s. Pak1 gene expression was normalized against β-actin gene expression in each sample. Each experiment was repeated three times using cells prepared separately at different times.

**Crystal violet assay following Pak1 siRNA transfection**
Ishikawa cells were seeded into 24-well culture plates and grown to 10% confluence. After transfection with Pak1 siRNA or non-specific control siRNA, cells were incubated for 72 h. Then, the medium was aspirated off and cells washed with 500 μl of phosphate-buffered saline. After fixation with 250 μl of 10% formalin solution for 15 min, 200 μl of 1% crystal violet solution (Junsei Chemical, Tokyo, Japan) was added to each well and incubated for 1 h. To remove excess dye, cells were washed three times with distilled water and subsequently dried. Plates were shaken for 10 min after which 200 μl of 1% sodium dodecyl sulfate was added to each well to solubilize the stain. After transferring 100 μl of solubilized solution to a 96-well plate, absorbance was read at 570 nm. Results are expressed as percentage viability, where
untransfected cells are normalized to 100%. Each experiment was repeated three times using cells prepared separately at different times.

Statistical analysis
All of the data were normally distributed as assessed by Kolmogorov–Smirnov test. Analysis of variance and Fisher’s least significant difference post hoc test for pairwise comparisons were used for statistical analysis of the data from western blot analysis and the HSCOREs obtained from immunohistochemistry. Student’s t-test was performed to compare the data obtained from real-time PCR and crystal violet assays. Statistical analyses were performed using Statistical Programs for the Social Sciences (SPSS, version 14.0, Chicago, IL, USA) software programs. Statistical significance was defined as $P < 0.05$.

Results

Immunocytochemical characterization for the purity of ESC
Expression of vimentin and cytokeratin on the cultured ESC was evaluated by immunocytochemistry. Almost all of the cells were stained for vimentin (Fig. 1A), whereas no cells were stained by anti-cytokeratin antibody (Fig. 1B). The purity of isolated ESCs was >95%.

Effect of sex steroids on the expression of Pak1 in Ishikawa cell and ESC
Although in vitro E2 treatment had no effect on Pak1 protein level in Ishikawa cells, E2 plus MPA and MPA treatment for 24 h led to a significant decrease of Pak1 protein in Ishikawa cells (Fig. 2A) (both $P < 0.05$ versus control) compared with the vehicle as well as E2 ($10^{-8}$ M) treatment. There were no significant differences of Pak1 protein in Ishikawa cells among different treatment groups after 48 h (Fig. 2B).

In ESCs, we could find no differences among different treatment groups after 24 h (Fig. 3A). In vitro E2 treatment for 48 h also had no effect on Pak1 protein level in ESCs (Fig. 3B). However, in vitro E2 plus MPA and MPA treatment for 48 h led to a significant decrease of Pak1 in ESCs compared with the vehicle treatment (both $P < 0.05$ versus vehicle) (Fig. 3B).

Immunocytochemistry results also revealed that Pak1 expression was obviously decreased in Ishikawa cells following E2 plus MPA and MPA treatment for 24 h as well as in ESCs after E2 plus MPA and MPA treatment for 48 h (Fig. 4), which was consistent with the western blot results.

Expression of Pak1 in the endometrium of control subjects throughout the menstrual cycle
Pak1 immunoreactivity was cytoplasmic and intramembranous in endometrial glandular and stromal cells (Fig. 5A). Pak1 expression was significantly decreased during the secretory phase in the glandular cells and the stromal cells within the control subjects ($P < 0.001$ and $P < 0.01$, respectively; Fig. 5B).

Expression of Pak1 in the eutopic endometrium of women with endometriosis
At all menstrual cycle phases, the immunoreactivity of Pak1 was significantly increased in the glandular cells of eutopic endometrium of women with endometriosis compared with the control subjects ($P < 0.01$, Fig. 6). Although there was no significant difference between the two groups in the proliferative phase, the expression of Pak1 in the glandular cells was significantly higher during the secretory phase in the endometriosis group ($P < 0.01$) and the main increase was obvious during the mid-secretory phase ($P < 0.01$, Fig. 6). The immunoreactivity of Pak1 in the stromal cells was not different between the two groups for the combined menstrual phases, the proliferative phase and the secretory phase. However, Pak1 was also increased during the mid-secretory phase in the stromal cells of women with endometriosis compared with the control subjects ($P < 0.01$, Fig. 6).

Pak1 mRNA level and cell viability following transfection of Ishikawa cells with Pak1 siRNA
Real-time PCR demonstrated that relative mRNA level of Pak1 was decreased to $21.54 \pm 3.37\%$ (mean $\pm$ SE, $P < 0.001$) (Fig. 7A) in cells transfected with Pak siRNA compared with non-specific control siRNA. Cell viability, assessed by crystal violet assay, was significantly decreased in cells transfected with Pak siRNA compared with non-specific control siRNA ($69.71 \pm 0.56$ versus $89.51 \pm 5.11\%$, respectively, mean $\pm$ SE, $P < 0.05$) (Fig. 7B).

Figure 1 | Immunocytochemical characterization of vimentin (A), cytokeratin (B) and negative control (C) in endometrial stromal cells (ESCs) after primary culture ($\times 100$).
Discussion

Human endometrium undergoes various physiological processes including implantation, trophoblast invasion, immune surveillance and menstruation throughout female reproductive life. These processes involve proliferation, differentiation and shedding of endometrial tissue and are mainly regulated by E2 and progesterone, the timing and concentrations of which dictate the balance between endometrial growth and differentiation (Kayisli et al., 2002). Given the role of Pak1 in a variety of cellular processes which occur in the endometrium under the influence of sex steroids, we hypothesized that sex steroids may regulate the expression of Pak1 in endometrial cells and that its expression changes according to the menstrual cycle.

The present study has shown that progesterin (MPA) can lead to a significant decrease of Pak1 protein in Ishikawa cells and ESCs, whereas E2 has no effect. Down-regulation of Pak1 by progesterin was demonstrated by western blot analysis as well as immunocytochemistry using our in vitro treatment model. Moreover, in vivo immunocytochemistry results have also shown that level of Pak1 protein is markedly decreased during the secretory phase, when the serum progesterone level is elevated. To the best of our knowledge, this is the first report that has revealed a possible effect of progesterone on Pak1 expression. There has been only one study (Mazumdar and Kumar, 2003) on regulation of Pak1 by estrogen, in which estrogen was shown to activate Pak1 activity in breast cancer cells utilizing an in vitro kinase assay. Since our findings were based only on semiquantitative comparisons of Pak1 protein, further studies are necessary to...
Figure 4  Effect of sex steroids on the expression of Pak1 in Ishikawa cells and ESCs. Immunocytochemical staining of Ishikawa cells treated with either vehicle (C), estradiol $10^{-8}$ M plus progesterone (medroxyprogesterone acetate) $10^{-7}$ M (EP), and progesterone $10^{-7}$ M (P) for 24 h (A). Immunocytochemical staining of ESCs treated with either vehicle (C), estradiol $10^{-8}$ M plus progesterone $10^{-7}$ M (EP), or progesterone $10^{-7}$ M (P) for 48 h (B) ($\times$ 200).

Figure 5  Representative micrographs (A) ($\times$ 200; inset $\times$ 400) and HSCORES (B) of Pak1 immunostaining in the endometrium of control subjects. Arrows indicate cytoplasmic immunostaining. N, negative control; EP, early proliferative phase; LP, late proliferative phase; ES, early secretory phase; MS, mid-secretory phase; LS, late secretory phase. HSCORES are expressed as mean $\pm$ SEM. $^{*}P < 0.001$ and $^{**}P < 0.01$ versus proliferative phase.
clarify whether estrogen also activates Pak1 activity in endometrial cells. It might be speculated that estrogen could activate Pak1 without an obvious quantitative increase in total Pak1 protein in endometrial cells, considering the presence of multiple phosphorylated forms of Pak1 with different functional activities (King et al., 2000; Banerjee et al., 2002; Thiel et al., 2002).

Progesterone induces the differentiation of both ESCs and glandular cells exemplified by pseudodecidual and secretory changes, and reduction in mitotic figures and cell proliferation (Bulun et al., 2006). Progesterone can be regarded as a growth limiting hormone since it can inhibit and even reverse estrogen-induced growth, hyperplasia or adenocarcinoma of endometrium. Taking into account the biological action of Pak1 stimulating estrogen-inducible genes through direct phosphorylation of ERα (Wang et al., 2002; Balasenthil et al., 2004b), the findings of the present study suggest that progesterone could not only induce a decrease in the synthesis of ERα but also suppress the activation of ERα by decreasing Pak1 in endometrial cells.

Although the development of endometriosis has historically been viewed as an estrogen-dependent disease, some investigators have suggested that a failure of progesterone to appropriately regulate the expression of genes during endometrial differentiation might be a critical component of the disease process (Osteen et al., 2005).

Specifically, Kao et al. (2003) demonstrated that a group of target genes in the eutopic endometrium are normally down-regulated during the window of implantation but are significantly increased in women with endometriosis. They suggested that the action of progesterone to appropriately mediate the expression of key differentiation-related genes might be altered in the eutopic endometrium of endometriosis patients. In a recent review, Bulun et al. (2006) also suggested that progesterone-induced molecular changes in the eutopic endometrium of women with endometriosis are either blunted or undetectable, and progesterone resistance and failure to metabolize E2 can lead to establishment of endometriosis.

Based on the role of Pak1 in cellular growth, survival, invasiveness and apoptosis (Kumar et al., 2006), as well as possible regulation of its expression by sex steroids, the second hypothesis of the present study was that the expression of Pak1 may be increased in the eutopic endometrium of women with endometriosis. We have shown that the immunoreactivity of Pak1 was obviously increased in the eutopic endometrium of women with endometriosis compared with the control subjects during mid-secretory phase, in the glandular cells as well as stromal cells. These findings, along with the marked decrease of Pak1 expression in the control group during the secretory phase, strongly suggest that progesterone-induced down-regulation of Pak1 may be blunted in the eutopic endometrium of women with...
endometriosis. Although we do not have supporting evidence, resistance against the action of progesterone possibly plays a critical role in persisting Pak1 expression in the eutopic endometrium of women with endometriosis.

At present, it is unclear whether the increased level of Pak1 in the secretory phase is inherent to the eutopic endometrium itself or a secondary consequence of other factors associated with ovarian endometrioma. Utilizing crystal violet assay following transfection with Pak1 siRNA, we demonstrated that inhibition of Pak1 expression can lead to obvious decreased cellular viability in Ishikawa cells. It may be suggested from these findings that an abnormally high level of Pak1 expression itself could cause the endometrial cells to survive and grow at ectopic loci after retrograde menstruation and eventually lead to establishment of endometriotic lesions as well as invasion to adjacent tissues. Regarding a previous review by Osteen et al. (1997), it is also possible that increased proinflammatory cytokines due to the presence of endometrioma reduces the expression of progesterone receptor-B in endometrial cells, leading to diminished progesterone response and failure to suppress Pak1 expression.

Kitawaki et al. (1997) have shown that aromatase cytochrome P450 mRNA and protein are expressed in the eutopic endometria of patients with endometriosis, adenomyosis and/or leiomyomas, whereas neither are expressed in endometrial specimens obtained from normally menstruating women with CIS but no other gynecologic disease. Based on these findings, they suggested that eutopic endometria obtained from women with leiomyoma and adenomyosis are not necessarily normal endometria, and they utilized only the eutopic endometria from normally menstruating women with CIS but no other gynecologic disease-free control (Kitawaki et al., 1999; Koshiba et al., 2005). Although women who are confirmed as disease-free during laparoscopic tubal ligation seem to be better for comparison purposes, we recruited the control subjects just as Kitawaki et al. (1997, 1999) and Koshiba et al. (2005) had done since we do not perform laparoscopic tubal ligation in our institute.

In conclusion, we have shown that treatment of Ishikawa cells and ESCs with MPA in vitro leads to a significant decrease in Pak1 protein and that the in vivo immunoreactivity of Pak1 is markedly decreased during the secretory phase. Comparing the immunoreactivity of Pak1 in the eutopic endometrium between the control subjects and women with advanced endometriosis, we have also found that Pak1 is obviously increased in the endometriosis group. To the best of our knowledge, this is the first study evaluating the effect of sex steroid on Pak1 expression in endometrial cells with and without endometriosis. The findings of the present study strongly suggest that Pak1 is down-regulated by progesterone during the secretory phase in normal endometrium and that persisting high expression of Pak1 during the secretory phase in the eutopic endometrium might lead to establishment and progression of endometriosis. Further studies are necessary to evaluate the effects of proinflammatory cytokines on Pak1 expression in endometrial cells as well as the effects of sex steroids on the actual function of Pak1.

Figure 7  Relative Pak1 mRNA levels (A) and cell viability (B) after transfection of Ishikawa cells with Pak1 small interfering RNA (siRNA) and non-specific control siRNA. Data are expressed as mean ± SEM. *P < 0.001 and **P < 0.05 versus cells transfected with non-specific control siRNA.

**Funding**

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2007-311-E00331).

**References**


Submitted on May 30, 2008; resubmitted on December 6, 2008; accepted on December 15, 2008