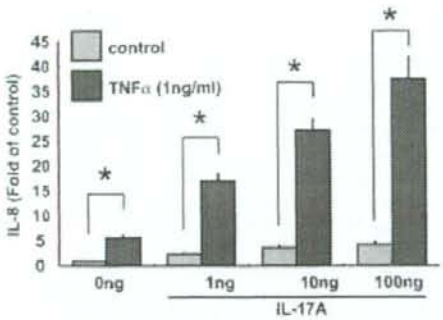

FIG. 5. Effect of IL-17A on TNFα-mediated IL-8 secretion from ESCs. ESCs were treated with IL-17A or TNFα or in combination for 24 h. The conditioned medium was collected and assayed for IL-8 concentration using a specific ELISA. All values are expressed as the mean ± SEM of pentaplicate cultures. *, $P < 0.0001$ *vs.* each control. The data are representative of four independent experiments.

ysis demonstrated that IL-17A up-regulated IL-8 and COX2 mRNA. Maximal increases in IL-8 and COX2 mRNA were observed at 4 h, followed by a decrease with time up to 24 h (Fig. 6, B and C). The maximal increase of IL-8 mRNA was 6.2-fold of the control, and that of COX2 mRNA was 13.6-fold of the control.

Effect of IL-17A on cell proliferation of ESCs

The effect of IL-17A on cell proliferation was determined in ESCs (Fig. 7A). IL-17A at 10 and 100 ng/ml significantly increased cell number by 106 and 111%, respectively, after exposure for 48 h. As shown in Fig. 7B, IL-17A at 1–100 ng/ml significantly increased BrdU incorporation into DNA in ESCs. The maximal effect (3.98-fold of control) was observed at 10 ng/ml.

Discussion

In the present study, we first demonstrated that presence of IL-17A-positive cells in the endometriotic tissue. In addition, the presence of Th17 cells in PFMCs was clearly shown by flow cytometric analysis. These findings instigated us to examine possible roles of IL-17A in endometriosis. We then showed that IL-17A stimulated the secretion of IL-8 from ESCs. ESCs expressed IL-17RA, and the anti-IL-17RA antibody inhibited IL-17A-induced IL-8 secretion. IL-17A stimulated the activation of p38 MAPK, p42/44 MAPK, and SAPK/JNK, and inhibitors of these kinases suppressed IL-17A-induced IL-8 secretion. TNFα synergistically enhanced IL-17A-induced IL-8 secretion. IL-17A also stimulated ESC proliferation and COX2 expression in ESCs.

A recent study on the Th1/ Th2 concept of T cell immunology revealed that endometriosis is an inflammatory disease with a Th2 immune response component (20, 21). The emerging concept of the Th17 pathway has challenged the conventional paradigm of Th1/Th2 hypothesis (4, 5). Together with the recent discovery of Treg, our understanding of the mechanisms underlying T cell immunology has advanced into a new era. In this context, the presence of Th17 cells in PFMCs demonstrated in our study might lead the

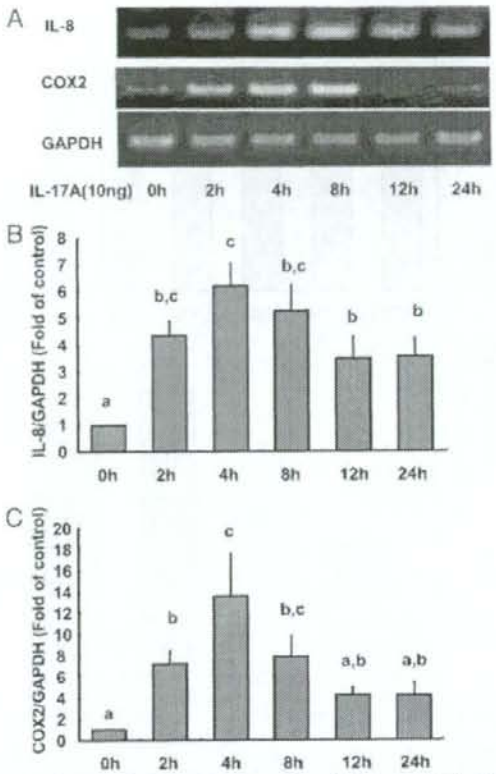

FIG. 6. Effect of IL-17A on the expression of IL-8 and COX2 mRNA in ESCs. ESCs were incubated with IL-17A (10 ng/ml) for the indicated duration. A, Expression of IL-8 and COX2 mRNA in ESCs was examined by RT-PCR. The data shown are representative of four separate experiments using samples from different women. B, Expression of IL-8 mRNA in ESCs was examined by real-time quantitative PCR. The data shown are the relative ratio (IL-8 to GAPDH) measures by real-time quantitative PCR. Data are the mean ± SEM of five independent experiments using samples from five different women. *Different letters* denote significant differences between groups ($P < 0.05$). C, Expression of COX2 mRNA in ESCs was examined by real-time quantitative PCR. The data shown are the relative ratios (COX2 to GAPDH) measured by real-time quantitative PCR. Data are the mean ± SEM of five independent experiments using samples from 5 different women. *Different letters* denote significant differences between groups ($P < 0.05$).

concept of immune response in endometriosis to a novel direction. In particular, abundant IL-17A-positive cells in the endometriotic tissue imply possible Th17 immune response therein. The present study has demonstrated multiple functions of IL-17A in ESCs. Given that IL-17A is a key effector molecule of Th17 cells, our findings form the foundation for understanding the etiology of endometriosis under the novel concept of T cell differentiation and regulation.

Substantial evidence points to IL-8 as a pivotal factor involved in the progression of endometriosis. IL-8 exerts pleiotropic functions, such as chemoattraction and activation of neutrophils, angiogenesis, stimulation of proliferation, and

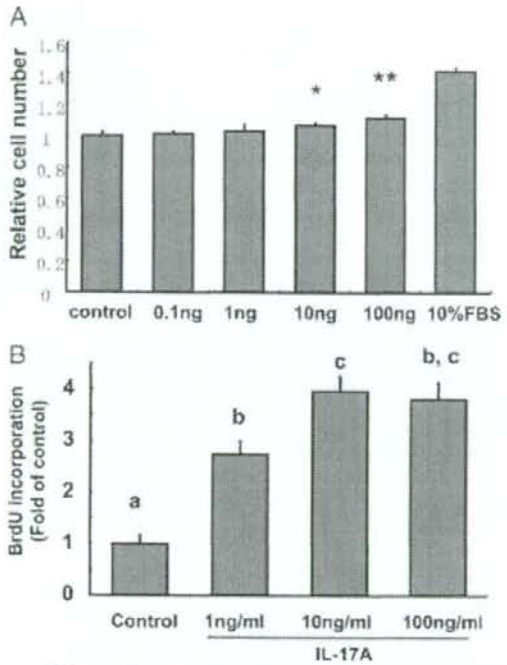

FIG. 7. Effect of IL-17A on proliferation of ESCs. A, The effect of IL-17A on proliferation of ESCs was examined by measuring cell number with cell-counting kit. ESCs were cultured in 1% FBS with different doses of IL-17A for 48 h. Values are the mean ± SEM of pentaplicate cultures. *, $P < 0.05$; **, $P < 0.01$ *vs.* control. The result is representative of four separate experiments using samples from different patients. B, The effect of IL-17A on the proliferation of ESCs was examined by measuring BrdU incorporation into DNA by using a cell proliferation ELISA. ESCs were treated with IL-17A at different concentrations for 48 h. Values are the mean ± SEM of the pentaplicate cultures. *Different letters* denote significant differences between groups ($P < 0.05$). The data shown are representative of four independent experiments using samples from four different patients.

survival of endometrial cells (1, 9). Our previous studies suggest that expression of IL-8 in endometriotic cells is regulated by various cytokines and enzymes (15, 16, 22). Notably, these IL-8-inducing molecules are derived from macrophages, neutrophils, and mast cells, which are suggested to play important roles in the development of the disease. The present study provides evidence that IL-17A is an additional stimulant of IL-8 secretion from ESCs and strongly suggests that Th17 cells participate in the development of endometriosis.

The synergistic effect of IL-17A and TNFα in stimulating secretion of IL-8 from ESCs was remarkable. TNFα is a proinflammatory cytokine that plays multiple roles in the progression of endometriosis. The importance of TNFα in endometriosis is underpinned by recent findings that TNFα-targeted suppression by specific drugs inhibits the development of endometriosis in baboons (23, 24). During the inflammatory response TNFα is secreted from various cells type, such as peritoneal macrophage, endometrial epithelial, and stromal cells. IL-17A may be an accelerator of endometriosis during chronic pelvic inflammation accompanied by increased TNFα production.

IL-17A-induced phosphorylation of p42/44MAPK, p38MAPK, and SAPK/JNK in ESCs, and the IL-17A-induced increase in IL-8 secretion from ESCs was suppressed by inhibitors of these kinases. This finding suggests that these enzymes are involved in the pathway of IL-17A-induced IL-8 secretion. A similar finding was reported for human airway smooth muscle cells (25). Interestingly, our previous study showed that IL-1β stimulated phosphorylation of these MAPKs, and IL-1β-induced IL-8 secretion from ESCs was inhibited by the same MAPK inhibitors (22). IL-1β is a well-known central player of the inflammatory condition of endometriosis, and thus, it can be speculated that cytokines promoting endometriosis, such as IL-1β and IL-17A, share similar pathways to induce IL-8 secretion from ESCs.

COX2, a key enzyme in prostaglandin biosynthesis, is up-regulated in endometriotic stromal cells (26–28). COX2 plays an important role in the inflammatory response associated with endometriosis and appears to function in the pathogenesis of endometriosis (29, 30). The present finding of IL-17A-induced COX2 expression in ESCs provides further evidence for a key role of IL-17A in endometriosis.

In the present study, IL-17A stimulated ESC proliferation. Human tumors show increased expression of IL-17A (31), and IL-17A promotes angiogenesis and tumor growth (32). IL-17A also enhances the proliferation of normal airway epithelial cells (33). Because IL-17A appears to exert a mitogenic effect on various cell types, IL-17A may stimulate progression of endometriosis as a result of this mitogenic effect, possibly in addition to other functions. Interestingly, IL-8 has been shown to stimulate the proliferation of endometrial stromal cells (34). Therefore, the proliferative effect of IL-17A on ESCs may be partially attributable to the increased production of IL-8 induced by IL-17A.

The proinflammatory and mitogenic action of IL-17A in ESCs demonstrated in the present study revealed possible roles of the molecule in the development of endometriosis. The prevailing medical treatment for endometriosis is suppression of ovarian hormones by GnRH analogs. The adverse effects of this treatment induced by the hypoestrogenic status often lead to a lack of compliance. IL-17A is well placed as a candidate target molecule for novel treatment strategies of endometriosis. Further investigation would benefit the development of an improved treatment option involving IL-17A.

In summary, the present study demonstrated that IL-17A stimulates inflammatory responses and proliferation of ESCs, suggesting a role for IL-17A in the pathogenesis of endometriosis.

Acknowledgments

Received June 6, 2007. Accepted December 3, 2007.

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This work was partially supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor, and Welfare.

Disclosure Statement: The authors of this manuscript have nothing to declare.

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Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

Dienogest inhibits BrdU uptake with G_0/G_1 arrest in cultured endometriotic stromal cells

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Objective: To investigate the effect of dienogest on the proliferation of endometriotic stromal cells.
Design: Comparative and laboratory study.
Setting: University of Tokyo Hospital.
Patient(s): Endometriotic stromal cells were isolated and cultured from ovarian endometriomas of patients undergoing surgery.
Intervention(s): Dienogest was added to the cultured endometriotic stromal cells.
Main Outcome Measure(s): 5-Bromo-2′-deoxyuridine (BrdU) incorporation into DNA of the endometriotic stromal cells was measured by ELISA. Cell cycle analysis of the cultured endometriotic stromal cells was performed by flow cytometry.
Result(s): Dienogest at concentration of 10^{-7} M and 10^{-6} M significantly inhibited BrdU incorporation into DNA at 24 and 48 hours. Dienogest significantly increased the cells in G_0/G_1 phase and reduced the cells in S phase and G_2/M phase in 24 and 48 hours.
Conclusion(s): The present study indicates that dienogest can inhibit the proliferation of the endometriotic stromal cells with G_0/G_1 arrest, suggesting a possible direct effect of dienogest in the treatment of endometriosis. (Fertil Steril® 2008;89:1344–7. ©2008 by American Society for Reproductive Medicine.)

Key Words: Dienogest, endometriosis, proliferation, cell cycle

Endometriosis, defined by the presence of viable endometriotic tissue outside the uterus, is an enigmatic disease. Infertility and pain are the most common symptoms that debilitate women affected with endometriosis (1, 2). Although a most prevailing medical treatment for the disease is suppression of ovarian hormones by GnRH analogue (GnRH-a), its adverse effects caused by hypoestrogen often dismiss the compliance. Accordingly, development of better drugs is anticipated.

Dienogest (17α-cyanomethyl-17β-hydroxy-estra-4,9-dien-3-one) is a synthetic progestogen derived from 19-norsteroids. It has high progestational and significant antiandrogenic activity, but only moderate antigonadotrophic activity (3). Dienogest significantly decreased the volume of the endometrial implants in a rat experimental model, suggesting its effect on endometriosis (4). In humans, dienogest was highly effective on endometriotic lesions and symptoms, showing an objective endoscopic and subjective symptomatic improvement in 80% and 83%, respectively (5, 6). Furthermore, efficacy of dienogest was confirmed to be comparable with triptorelin in the treatment of endometriosis after laparoscopic surgery (7). A recent pilot study demonstrated an efficacy of long-term high-dose dienogest treatment for endometriosis (8).

The main antiendometriotic effect of dienogest has been suggested to be attributable to a central inhibition of ovulation. However, its peripheral effects are also indicated. The direct antiproliferative effect of dienogest has been shown in human eutopic endometrial stromal cells (9). In contrast, its effect on ectopic endometriotic cells remains to be elucidated. It is well known that ectopic endometrium is different from eutopic endometrium in its gene expression (10, 11). Likewise, the expression of steroid receptors differ between eutopic and ectopic endometrial tissues (12, 13). In view of these findings, the effects of dienogest may differ between eutopic and ectopic endometrial tissues.

In the present study, therefore, we examined the effect of dienogest on the proliferation of endometriotic stromal cells from ovarian endometrioma. We also examined the effect of dienogest on the cell cycle by flow cytometric analysis.

MATERIALS AND METHODS

Tissue Collection

Endometriotic tissues were collected from the walls of ovarian endometriomas during surgery. A total of 14 patients (aged 37.6 ± 4.4 years, mean ± SD) with endometriosis were recruited in this study. All the patients had regular menstrual cycles, and none had received hormonal treatment at

Received November 18, 2006; revised January 29, 2007; accepted March 13, 2007.
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0015-0282/08/$34.00
doi:10.1016/j.fertnstert.2007.03.042

least 6 months before surgery. Informed consent in writing was obtained from each patient for use of the ovarian endometriotic lesion; consent forms and experimental protocols were approved by the institutional review board (IRB) of the University of Tokyo. Tissues were placed in Dulbecco's minimum essential medium (DMEM)/F12 medium (GIBCO, Grand Island, NY) on ice and transported immediately to the laboratory for separation and culture of endometriotic stromal cells. Cells obtained from each patient were used for separate experiments.

Isolation and Culture of Human Endometriotic Stromal Cells

Endometriotic stromal cells (ESC) were purified from ovarian endometriotic tissue and cultured as described previously (14, 15). The fresh endometriotic lesion collected in sterile medium was dissected free from underlying parenchyma, minced into small pieces, incubated in DMEM/F12 with 2.5 mg/mL type 1 collagenase (Sigma, St. Louis, MO) and 15 U/mL deoxyribonuclease 1 (Takara, Tokyo, Japan) for 2 hours at 37°C with agitation. The resultant suspension was separated by serial filtration. Debris was removed with a 100-μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ), and some epithelial glands were eliminated with a 70-μm nylon cell strainer (Becton Dickinson). Stromal cells remaining in the filtrate were collected by centrifugation, resuspended in DMEM/F12, and plated onto 100-mm dishes (Iwaki, Chiba, Japan) and allowed to adhere at 37°C for 30 minutes, after which nonadhering epithelial cells and blood cells were removed with phosphate-buffered saline (PBS) rinses. The cells were cultured in DMEM/F12 reconstituted with 10% charcoal-stripped fetal bovine serum (FBS) (Hyclone, Logan, UT) and antibiotics (Sigma). The purity of ESC population was more than 95%, as confirmed by positive immunocytochemical staining for vimentin. When the cells reached confluence, they were passed into a 60- by 15-mm dish and 96-well culture plates (Becton Dickinson) at the densities of 10^6 cells/dish and 3×10^3 cells/well, respectively, in medium supplemented with 10% charcoal-stripped FBS. After 48 hours of culture, the cells were sustained with serum starvation for 24 hours and then used for experiments with 1.5% charcoal-stripped FBS in the medium.

5-Bromo-2′-Deoxyuridine Incorporation

The effect of dienogest on the proliferation of ESC was evaluated by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA. The BrdU incorporation was detected by using the Biotrak cell proliferation ELISA system (Amersham Pharmacia Biotech), as we reported previously (16, 17). Briefly, ESC were seeded into Falcon 96-multiwell plates at a density of 3×10^3 cells per well in 100 μL of culture medium containing 10% FBS and cultured for 48 hours. After 24 hours of serum starvation, the medium were replaced with fresh medium supplemented with 1.5% charcoal-stripped FBS containing the control vehicle or dienogest, which was dissolved in dimethyl sulfoxide and then diluted with the medium. The final concentration of dimethyl sulfoxide in the medium never exceeded 0.1%. During the last 4 hours of 24-hour and 48-hour of culture, 10 μL of BrdU solution was added and incubated at 37°C. 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 (ASYS Hitech GmbH, Eugendorf, Austria).

Flow Cytometry

The ESC were seeded into a 60- by 15-mm culture dish (Becton Dickinson) at a density of 1×10^6 cells/dish in 2 mL of the culture medium. After 48 hours of culture and later 24 hours of serum starvation, the medium was replaced with fresh medium containing 1.5% FBS and 10^{-6} M dienogest or control vehicle, and the culture was continued for an additional 24 or 48 hours. The cells were then harvested with trypsin (0.05%)/EDTA (0.02%), washed twice with ice-cold PBS (pH 7.4), and fixed with 70% ethanol at -20°C overnight. After washing twice with ice-cold PBS, the cells were incubated in 0.25 mg/mL ribonuclease solution (QIAGEN GmbH, Hilden, Germany) for 30 minutes at 37°C and stained with 50 μL/mL propidium iodide for 30 minutes, on ice, in the dark, followed by filtration through a 40-μm nylon mesh (Becton Dickinson) to remove cell clumps. A total of 2×10^5 stained cells per treatment were analyzed in the EPICS XL Flow Cytometry (Beckman Coulter, Inc., Fullerton, CA).

Statistical Analysis

The data were expressed as the mean ± SEM. Data were analyzed by Student's *t*-test for paired comparison of cell cycle analysis and one-way ANOVA with post hoc test for multiple comparisons of BrdU incorporation experiments using StatView software (SAS Institute Inc., Cary, NC). A P value less than .05 was accepted as statistically significant.

RESULTS

Effect of Dienogest on the Proliferation of ESC

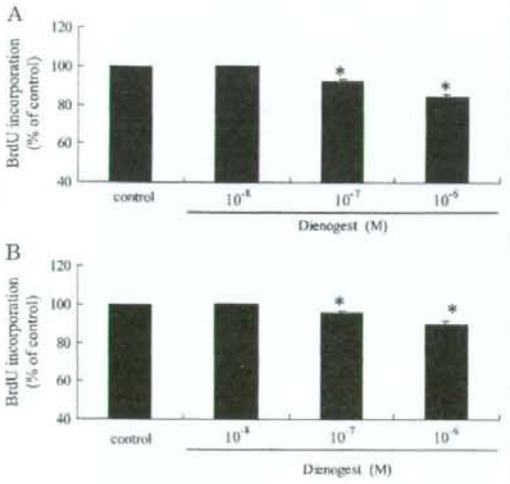
The effect of dienogest on the proliferation of ESC was examined by measuring BrdU uptake in cultured ESC. As shown in Figure 1, dienogest at concentration of 10^{-7} M and 10^{-6} M induced significant suppression of BrdU incorporation into DNA at 24 and 48 hours of treatment.

Effect of Dienogest on Cell Cycle in ESC

The effect of 10^{-6} M dienogest on the cell cycle in ESC was determined by flow cytometric analysis. Table 1 listed the combined data from four independent experiments. Dienogest significantly increased the cells in G_0/G_1 phase and reduced the cells in S phase and G_2/M phase in 24 and 48 hours.

FIGURE 1

Effect of dienogest on the proliferation of endometriotic stromal cells. The effect was examined by 5-bromo-2′-deoxyuridine (BrdU) incorporation using cell proliferation ELISA system. (**A**) Treatment with dienogest at concentrations between 10^{-8} M and 10^{-6} M for 24 hours. (**B**) Treatment with dienogest at concentrations between 10^{-8} M and 10^{-6} M for 48 hours. Results shown are the mean percentage of the untreated control ± SEM (bars) of hexaplicate cultures of four independent experiments. *$P<.05$ vs. control.



Fu. Antiendometriotic effect of dienogest. Fertil Steril 2008.

DISCUSSION

In the present study, we demonstrated that dienogest inhibited the proliferation of endometriotic stromal cells in vitro. The inhibitory effect was significant from a dose of 10^{-7}, which was similar to that reported in eutopic endometrial stromal cells. It has been shown that maximum serum dienogest concentrations were reached within ~2 hours and the mean maximum serum concentrations were 0.9 x 10^{-7}, 1.7 x 10^{-7}, 3.2 x 10^{-7}, and 6.8 x 10^{-7} M after therapeutic doses of 1, 2, 4, and 8 mg, respectively (3). In addition to our finding that dienogest inhibited ESC proliferation at a dose of 10^{-7} M, dienogest at therapeutic doses conceptually yields direct suppression of the growth of endometriosis.

In recent framework of concept for etiology of endometriosis, a theory of P resistance is advocated (18). Progestins, such as medroxyprogesterone acetate (MPA) and dienogest, has been shown to inhibit the proliferation in eutopic endometrial stromal cells (9, 19, 20). However, it has also been reported that P-induced molecular changes in eutopic endometrium and ectopic endometriotic tissues of women with endometriosis are either blunted or undetectable (21–23). These observations, indicative of P resistance, were partially explained by an overall reduction of P receptors and the absence of P receptor B in the endometriotic tissue (24). In this context, it is remarkable that dienogest directly inhibited the proliferation of endometriotic cells in the present study. The present finding underscores the specific property of dienogest among progestins for endometriosis treatment.

Although antiproliferative effect of dienogest on eutopic endometrial cells has been reported, the mechanism has not been understood. Concerning this, a notable finding in the present study was that antiproliferative effect of dienogest on endometriotic stromal cells could be attributable to arrest of cell cycle in G_0/G_1. This finding seems to be similar to that P-inhibited proliferation of rat aortic smooth muscle cells with arrest of cell cycle in G_0/G_1 (25). Interestingly, P suppresses E_2-induced cell proliferation, increasing p27 protein expression in endometrial glandular cells (26). It can be thus speculated that the inhibition of the proliferation of eutopic endometrial stromal cells is also associated with G_0/G_1 arrest. Recent studies have shown that G_0/G_1 arrest of ESC was also induced by other drugs (27, 28).

Except for the antiproliferative effect demonstrated in the present study, direct effect of dienogest on endometriotic stromal cell has been hitherto poorly understood. There is only one report that dienogest attenuated tumor necrosis factor-α-induced interleukin-8 production (29). Further study would be warranted to understand additional direct effects of dienogest on endometriotic lesion.

TABLE 1

Effect of dienogest on the number of endometriotic stromal cells in the respective phase of the cell cycle.

	G_0/G_1	S	G_2/M
24 h	102.04% ± 0.89%[a]	92.90% ± 3.21%[a]	89.39% ± 3.91%[a]
48 h	101.88% ± 0.75%[a]	89.51% ± 5.64%[a]	88.69% ± 3.55%[a]

Note: Values are the mean ± SEM of the control from four independent experiments using samples from different women.
[a] $P<.05$ vs. control.

Fu. Antiendometriotic effect of dienogest. Fertil Steril 2008.

In summary, we demonstrated that dienogest inhibited proliferation of endometriotic stromal cells in vitro with G_0/G_1 cell cycle arrest. The finding subserves the understanding of the antiendometriotic effect of dienogest, a new therapeutic alternative to GnRH-a and conventional progestins.

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High soluble CD44 concentration in peritoneal fluid in endometriosis

The concentration of the soluble form of CD44, a competitive inhibitor for CD44 and hyaluronic acid binding, is higher in the peritoneal fluid of patients with endometriosis than in the peritoneal fluid of patients without endometriosis. (Fertil Steril® 2008;89:1267–8. ©2008 by American Society for Reproductive Medicine.)

Endometriosis is an enigmatic disease that affects women of reproductive age, causing a decline in health and often resulting in infertility (1). Implantation of endometrial tissues in retrograde menstrual flux is a widely accepted etiology of the disease. From this perspective, adhesion molecules that form connections between endometrial and mesothelial cells may play a pivotal role in the pathogenesis of endometriosis. The adhesion molecule CD44 is a type I transmembrane protein that interacts with hyaluronic acid (HA) in a wide variety of physiological and pathological processes including lymphocyte homing, cell migration, and cancer cell metastasis (2). The soluble form of CD44 (sCD44), which consists of the ectodomain of CD44, has recently emerged as a key regulator of CD44 action. Soluble CD44 can also bind to HA, acting as a competitive inhibitor for membrane-bound CD44. Although there is a growing body of evidence that CD44 is involved in endometriosis (3, 4), the importance of sCD44 is still unknown. As a first step, we investigated the sCD44 expression in the peritoneal fluid (PF) of patients with endometriosis.

A total of 63 women of reproductive age with (n = 46; age, 34.4 ± 5.4 years) and without (n = 17; age, 33.9 ± 6.0 years) endometriosis participated in this study. Informed consent was obtained from each woman before laparoscopy. All of the women had regular menstrual cycles. None of the women took medications that affect hormonal and/or immunological status in the 3 months before laparoscopy, and no patient had undergone surgical treatment for endometriosis within 1 year. At the time of surgery, 35 women were in the proliferative phase, and 28 were in the secretory phase. The stage of endometriosis was evaluated according to the revised American Society for Reproductive Medicine (r-ASRM) classification (5). The distribution of the stage of endometriosis was as follows: stage I, n = 12; stage II, n = 5; stage III, n = 12; stage IV, n = 17. PF was collected via a laparoscopic cannula introduced into the cul-de-sac before any manipulative procedure. The fluid was centrifuged at 400 *g* for 10 minutes, and the supernatants were frozen and stored at −80°C until assay. Soluble CD44 concentrations in the PF were measured using a specific enzyme-linked immunosorbent assay for sCD44 (Kamiya Biomedical, Seattle, WA). The data were described as median and interquartile range (IQR). The Mann-Whitney test was used for the statistical analysis.

The sCD44 concentrations in PF showed no significant difference between the proliferative phase and the secretory phase (data not shown). The data were then combined and analyzed regardless of the menstrual phases.The sCD44 concentrations in the PF of the women with endometriosis (median, 423ng/mL; IQR, 292–638) were significantly higher than those of the women without endometriosis (257 ng/mL, 196–337; P=.0004). As shown in Figure 1A, a positive correlation between the sCD44 concentrations of PF and r-ASRM score was observed (r = 0.536; P<.0001). The women with endometriosis were then subdivided into those with stage I/II and those with stage III/IV endometriosis. As shown in Figure 1B, the concentrations of sCD44 in stage III/IV endometriosis (460 ng/mL, 311–758) were significantly higher than those in stage I/II endometriosis (310 ng/mL, 261–442; P=.0144) and those in non-endometriosis (257 ng/mL, 196–337; P<.0001). The sCD44 concentrations in stage I/II endometriosis were also significantly higher than those in non-endometriosis (P=.0439).

This study is the first to demonstrate the presence of sCD44 in the PF of patients with endometriosis. We found a significant increase in sCD44 concentration in the PF of patients with endometriosis compared with patients without endometriosis. Furthermore, the expression of sCD44 was positively correlated with the stage of endometriosis.

It has been reported that CD44 is expressed not only in endometrial cells but also in endometriosis lesions (3, 4). Moreover, Dechaud et al., using the in vitro adhesion model, reported that interaction between endometrial cell–associated CD44 and mesothelial cell–associated HA might be crucial for the initiation of endometriosis (6).

In the light of recent evidence on the importance of soluble receptors in various physiological settings and in the pathophysiology of various diseases (7–9), it is tempting to speculate that sCD44 in PF may have a role in the pathogenesis of endometriosis. CD44 expressing endometrial cells is delivered into peritoneal cavity via retrograde

Received May 7, 2007; revised and accepted May 24, 2007.
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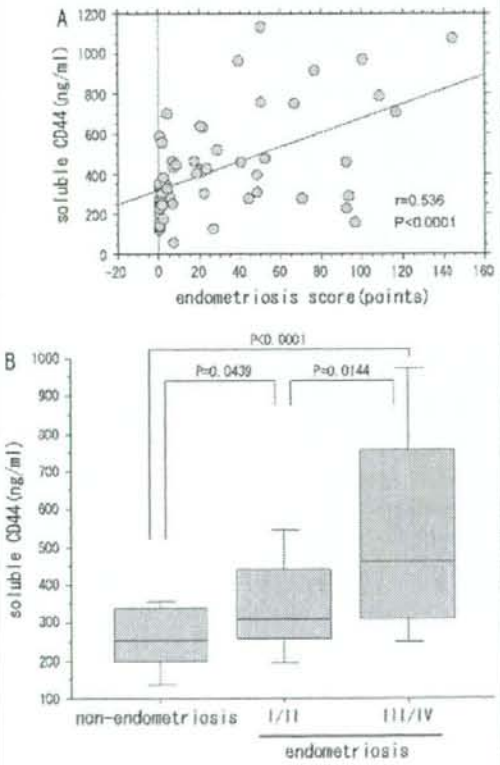
0015-0282/08/$34.00
doi:10.1016/j.fertnstert.2007.05.051

FIGURE 1

(**A**) Correlation between endometriosis score and soluble CD44 concentration in PF. Simple regression analysis showed that endometriosis score was positively correlated with the soluble CD44 concentration; $r = 0.536$, $P < .0001$, N = 63 (n = 17 patients without endometriosis; n = 46 patients with endometriosis). (**B**) Concentration of soluble CD44 in PF of women without endometriosis (n = 17), women with I/II endometriosis (n = 17), and women with III/IV endometriosis (n = 29). Boxes represent the distance between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent medians.



Hasegawa. Soluble CD44 in endometriosis. Fertil Steril 2008.

menstruation. Given that sCD44 can act as a competitive inhibitor for the interaction of membrane-bound CD44 and HA, up-regulation of sCD44 in women with endometriosis may inhibit attachment of influxed endometrial cells to the peritoneal mesothelial cells as a defense mechanism against disease progression. Because CD44 is expressed in most human cell types (2), the source of sCD44 in PF is unclear. Nevertheless, in the PF, which reflects the peritoneal environment, sCD44 is expressed at a high concentration in patients with endometriosis. In view of this finding, one can hypothesize that the relative proportion of CD44 and sCD44 in PF might be important for initiation and progression of the disease. Further study is needed to elucidate the mechanism of sCD44 up-regulation in patients with endometriosis and to understand the action of sCD44 in the pathogenesis of endometriosis.

Acknowledgments: We thank Dr. Heather E. McMahon for her helpful discussion and critical reading of this manuscript.

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CASE REPORT

A case of hydrosalpinx associated with the menstrual cycle

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Objective: To describe a case report of hydrosalpinx that changed dramatically in size during the menstrual cycle.
Design: Case report.
Setting: University teaching hospital reproductive endocrinology and infertility practice.
Patient(s): A 32-year-old woman with a history of medical and surgical treatments of endometriosis who sought infertility treatment.
Intervention(s): Transvaginal ultrasonography performed sequentially during menstrual cycles.
Main Outcome Measure(s): Size of hydrosalpinx-like image.
Result(s): The size of the hydrosalpinx-like image in the left adnexal region varied; it peaked during the ovulatory period and then remarkably diminished in a cyclic manner. Laparoscopy revealed a dense adhesion between the left tubal fimbriated end and the posterior uterine wall, which led to terminal obstruction of the tube.
Conclusion(s): Change in the volume of the hydrosalpinx in this case was speculated to reflect the normal tubal fluid production regulated by ovarian hormones. (Fertil Steril® 2008;90:199.e9–e11. ©2008 by American Society for Reproductive Medicine.)

Key Words: Hydrosalpinx, ultrasound, endometriosis, menstrual cycle, tubal fluid

Hydrosalpinx is usually found as a consequence of chlamydia or other infections in the fallopian tube (1). Cases of hydrosalpinx that appear during ovarian stimulation, though very rare, have also been reported (2, 3). Here we report a unique case of hydrosalpinx, which developed in the absence of infection and hormonal treatment and changed size dramatically with the menstrual cycle. No specific institutional review board approval was sought because the patient was not enrolled in a research protocol.

CASE REPORT

The patient was a 32-year-old woman who had had endometriosis since the age of 24 years. She underwent ethanol sclerotherapy of bilateral ovarian endometrioma at age 26 years and laparoscopic cystectomy for the right endometrioma at age 30 years. She received GnRH analogue treatments at 24 and 26 years of age. She was seen for infertility treatment after 1 year of attempting to get pregnant. Her basal body temperature was biphasic, with adequate duration of high phase. Serum anti-*Chlamydia trachomatis* IgA and IgG antibodies were negative. Hysterosalpingography conducted just after menstruation showed terminal obstruction of the left fallopian tube. During ultrasonography workups to check follicular growth and ovulation, we noticed a hydrosalpinx-like structure at the left adnexal area, which varied in size and appearance with each observation. We then observed this structure sequentially by ultrasonography during menstrual cycles. Interestingly, the size of the structure was minimal during the menstrual period but increased and reached a maximum size at ovulation, to then shrink again (Fig. 1A and B). Laparoscopy revealed a dense adhesion between the left tubal fimbriated end and the posterior uterine wall, presumably caused by previous surgery and endometriosis, which subsequently led to terminal obstruction of that tube (Fig. 2). Salpingectomy was undertaken with the hope of improving IVF-ET outcome. Histologically, the structural integrity of tubal epithelium was maintained, and endometriosis was found at the serosa of the tubal wall.

DISCUSSION

This is a unique case of hydrosalpinx with menstrual cycle–dependent change during normal menstrual cycles. Although there are several reports of hydrosalpinx that enlarge during ovarian stimulation (2, 3), there has been no report of hydrosalpinx with enlargement and shrinking during menstrual cycles. Another unique aspect of this case is that no evidence of pathological damage was found within the tubal epithelium.

Received May 22, 2007; revised and accepted June 2, 2007.
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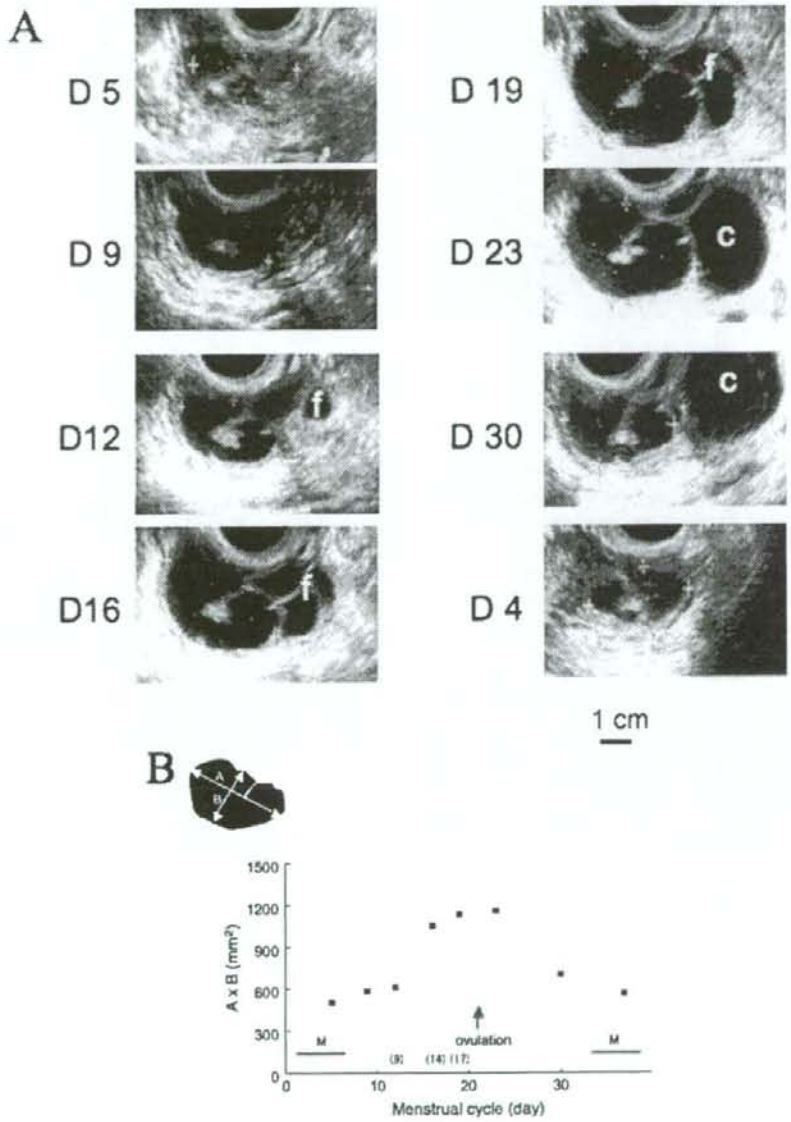
0015-0282/08/$34.00
doi:10.1016/j.fertnstert.2007.06.047

FIGURE 1

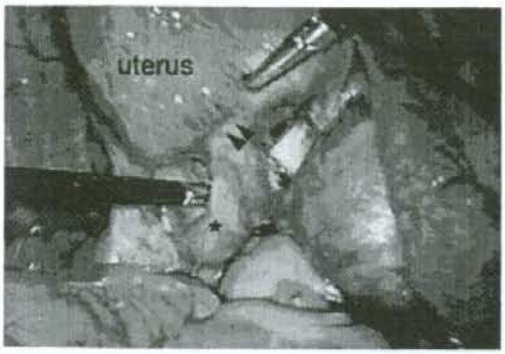
Sequential change of ultrasonographic images of the left fallopian tube in a representative menstrual cycle. (**A**) The left adnexal area was scanned by transvaginal ultrasonography on various menstrual days. A cystic part on the right side of the hydrosalpinx at day (D)12 and D16 are follicles (f), whereas D23 and D30 are corpus luteum (c). Ovulation occurred at D20, confirmed by basal body temperature. (**B**) The size of the hydrosalpinx area imaged by ultrasonography was plotted. M = menstruation. Values in parenthesis show the mean diameter (in millimeters) of the dominant follicle.



Osuga. Cyclic enlargement of hydrosalpinx. Fertil Steril 2008.

FIGURE 2

Laparoscopic finding of the left tube. A dense adhesion between the left tubal fimbriated end and the posterior uterine wall was found (*arrowheads*), which subsequently led to terminal obstruction of the left tube (*asterisk*).



Osuga. Cyclic enlargement of hydrosalpinx. Fertil Steril 2008.

Hydrosalpinx is often a sequel of chlamydial infection, which affects fallopian tube epithelial transporters and ion channels, particularly the cystic fibrosis transmembrane conductance regulator, resulting in increased epithelial secretion and decreased fluid absorption, hence an accumulation of hydrosalpinx fluid (4). In our case, however, the serologic chlamydial test was negative and the microscopic structure of the tubal epithelium was normal, suggesting that the capacity of fluid secretion and absorption in the affected tube was maintained (5). Given these observations, what was the underlying cause of the tubal fluid retention and its cyclic volume change?

It is well established in animal models that the fluid production by the fallopian tube is controlled by ovarian hormones and that the volume is highest during the ovulatory period. A recent study suggests that an ovarian hormone–regulated water channel, aquaporin 9, located in the oviductal epithelium, is involved in tubal fluid balance (6). In the human, the tubal fluid may also be regulated hormonally, reaching maximum levels during the ovulatory period. However, the fluid constantly empties into the peritoneal cavity, so that changes are not detected by ultrasonography. Only in patients undergoing ovarian stimulations, in whom ovarian hormone levels are superphysiological, fluid production is excessive and detectable by ultrasonography, when it is not effectively discharged from the tube. The resulting fluid retention could explain the etiology of hydrosalpinx during ovarian stimulation, as reported previously. On the other hand, in the present case, obstruction of the tubal terminal end would lead to fluid accumulation or a hydrosalpinx as seen by ultrasonography. Indeed, increased volume during the ovulatory period may reflect normal tubal fluid production, regulated by ovarian hormone.

In summary, we have reported a unique case of hydrosalpinx that changed size during menstrual cycles. This change is proposed to reflect a normal tubal epithelial function, producing fluid under ovarian hormone regulation.

Acknowledgements: The authors thank Dr. Kate Hale for editing the manuscript.

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Endocrine Journal 2008, **55** (5), 795–810

REVIEW

Molecular and Cellular Mechanisms for Differentiation and Regeneration of the Uterine Endometrium

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Abstract. The human endometrium undergoes cyclical changes including proliferation, differentiation, tissue breakdown, and shedding (menstruation) throughout a woman's reproductive life. The postovulatory rise in ovarian progesterone induces profound remodeling and differentiation of the estradiol-primed endometrium. This change, termed decidualization, is crucial for embryo implantation and maintenance of the pregnancy. To date, activation and crosstalk of cAMP- and progesterone-mediated signaling pathways have emerged as key cellular events to drive integrated changes at both the transcriptome and the proteome levels. This results in the induction and maintenance of the decidual phenotype and function. Our recent series of studies highlights the critical role of SRC kinase activation (v-src sarcoma viral oncogene homolog) and STAT5 (signal transducer and activator of transcription 5) phosphorylation in decidualization. After separation of the functional layer of the differentiated endometrium that follows progesterone withdrawal, *i.e.*, menstruation, the basal layer of the endometrium, under the influence of estradiol, regrows and initiates a unique form of angiogenesis and regenerates a new functional layer. The molecular and cellular mechanisms for this process remain elusive, mainly because of difficulties in reproducing menstrual tissue breakdown, shedding, and subsequent tissue regeneration *in vitro*. We have recently developed a "humanized" mouse model in which a functional human endometrium is reconstituted. It may be used as an *in vivo* experimental tool for the study of endometrial angiogenesis and regeneration. This model may also be used to identify and test new therapeutic strategies for endometriosis, endometrial cancer, implantation failure, and infertility related to endometrial dysfunction.

Key words: Endometrium, Decidualization, Regeneration, SRC, Progesterone, Animal model

(*Endocrine Journal* **55**: 795–810, 2008)

THE maternal decidua intertwines with the invading fetal trophoblast at the fetomaternal interface, crucially regulating placental function and the growth and development of the conceptus. Decidualization can be defined as the postovulatory process of endometrial remodeling in preparation for pregnancy, which includes secretory changes of the uterine glands, differentiation of stromal cells, accumulation of specialized uterine natural killer cells, and vascular remodeling. In the humans, decidualization, which starts to take place in the luteal phase of the menstrual cycle, is more strictly defined. It denotes the progesterone-induced differentiation of fibroblast-like endometrial stromal cells (ESCs), located in the proliferative estrogen-primed endometrium, into decidual cells. Decidualization is characterized histologically by the appearance of larger and rounder cells surrounding the spiral arteries and eventually spreading through most of the endometrium. Following embryo implantation, decidualization persists and extends throughout the endometrium, leading to the formation of the pregnancy decidua. This morphological change is accompanied by integrated changes at both the transcriptome and the proteome levels. As a consequence, decidualizing ESCs acquire the unique ability to regulate trophoblast invasion, to resist inflammatory and oxidative insults, and to diminish local maternal immune responses. This process is accomplished through local production of numerous biological sub-

Received: March 2, 2008
Accepted: March 9, 2008
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stances including growth factors, cytokines, neuropeptides, free radical scavengers, and extracellular matrix components [1–6]. Major secretory products are PRL and IGF-binding protein 1 (IGFBP-1), two proteins that have been used widely as phenotypic markers of decidual cells [7]. Thus, decidualization is crucial for successful embryo implantation and maintenance of the pregnancy. Impaired decidual responses may cause a variety of endometrial and pregnancy disorders including infertility, recurrent miscarriages, uteroplacental dysfunction, endometriosis, and endometrial cancer.

ESCs isolated from human endometrium and cultured in the presence of progesterone in combination with cAMP or estradiol (E_2), exhibit morphological and functional changes *in vitro* that mimic *in vivo* decidual transformation [8]. With the development of this *in vitro* model of decidualization, many studies have addressed the molecular mechanisms underlying decidual transformation. Abundant clinical and experimental evidence substantiates that this differentiation process largely depends on the convergence of the progesterone and cAMP signaling pathways [8]. These pathways, alone, however, cannot completely account for functional and morphological characteristics of the decidual phenotype. Decidualized human ESCs (hESCs) produce many bioactive substances, including growth factors and cytokines, whose downstream signaling pathways also may contribute to decidual transformation in a paracrine/autocrine manner [1–3, 6].

The first half of this review summarizes the molecular mechanisms responsible for the initiation and maintenance of decidualization. The second half briefly addresses what is currently understood regarding the cellular mechanisms underlying endometrial regeneration and presents our recently developed experimental model for the study of the process.

Hormone signals for decidualization

Progesterone signaling

Progesterone and its receptor action

i. Genomic action

It is generally accepted that progesterone initiates and drives decidualization. In endometrial cells, progesterone exerts its actions predominantly through activation of the progesterone receptor (PR), which is a member of the nuclear receptor family of ligand-dependent transcription activators.

The general pathway of progesterone-inducible PR-mediated gene transcription is well characterized. Progesterone binding induces a conformational change(s) in the PR that promotes dissociation from a multi-protein chaperone complex. This is followed by homodimerization and binding to specific progesterone response elements (PREs) within the promoter of target genes [9]. Indeed, there are two putative progesterone responsive elements in the promoter region of IGFBP-1 [10], one of the representative decidual markers. DNA bound receptors increase rates of gene transcription by influencing recruitment of RNA polymerase II to the initiation site. Through protein-protein interactions, the hormone activated PR recruits coactivators that serve as essential intermediates for transmitting signals from the receptor to the transcription initiation complex. Coactivators facilitate transcription initiation through protein interactions with components of the general transcription machinery, and by promoting local remodeling of chromatin at specific promoters. Nuclear receptor-associated coactivators possess intrinsic enzyme activity for acetylation (histone acetyltransferase activity, HAT) or methylation of core histone proteins. These modifications of core histones relieve the repressive effects of chromatin on transcription and facilitate access of the general transcription machinery [11].

The PR is expressed as two different sized proteins from a single gene; PR-A and PR-B. PR-A lacks the 164 N-terminal amino acids of PR-B, is transcriptionally less active, and can transrepress PR-B on palindromic progesterone-responsive elements [12]. PR-A is the dominant isoform involved in decidualizing hESC *in vivo* and *in vitro*. In mice, PR-A null mice exhibit a defective decidual response to the implanting blastocyst [13–15]. In hESCs, the relative level of expression of these two PR isoforms may partially account for the escape of many decidual genes from the strict and direct transcriptional control of progestins. Furthermore, emerging evidence suggests a major role for the activated PR, specifically the PR-A isoform, as a scaffold for the both the direct and indirect recruitment of other activated transcription factors in response to cAMP signaling [8]. Direct physical interaction has indeed been demonstrated between the PR and the signal transducer and activator

of transcription 5 (STAT5), CCAAT enhancer-binding proteins (C/EBPs), and forkhead box O (FOXO1) [16–18]. By hijacking these transcription factors, the activated PR acquires control of the diverse gene families involved in decidualization.

ii. Non-genomic action

Progesterone, like other steroid hormones, can trigger rapid cytoplasmic events that are independent of its genomic actions [19]. The molecular basis of this was unknown until the recent discovery of a family of membrane progestin receptors (mPR-α, mPR-β, mPR-γ) [20, 21]. Though expression of these mPRs has been identified in the human endometrium [22], their precise role in hESC differentiation is unclear. Alternatively, it has been suggested that the rapid, non-genomic effects of progesterone are mediated by binding of a cytoplasmic PR to the SH3 domain of SRC, a non-receptor type tyrosine kinase. Binding results in phosphorylation and activation of the p42/44 MAPK/ERK signal transduction pathway downstream of SRC [23]. The ability of the PR to activate kinase cascades shows that the PR is not only capable of acting as a transcription factor, but also may directly activate signaling pathways from the cytoplasm. The role of SRC signaling in decidualization will be addressed later in this article.

PR-associated decidual proteins

There is growing evidence suggesting a role for the PR as a platform for the formation of a decidua-specific transcriptional complex involving such diverse transcription factors as FOXO, C/EBPβ, STAT5, and co-activators [8].

i. PR and C/EBPβ

Since PRL is one of the major products of decidualized hESCs, the decidual PRL (dPRL) promoter has been exploited as a tool to identify transcription factors relevant to decidualization. Analysis of this promoter reveals that the dPRL-332/-270 promoter element contains a PR binding half-site adjacent to the C/EBP binding sites [8]. Among the C/EBPs, C/EBPβ is the predominant form in decidualized stromal cells [24]. C/EBPβ is essential for female reproduction; its absence hampers ovulation, breast development and function [25–27]. Taking advantage of this information, Christian *et al.* demonstrated that the PR can physically associate with the two C/EBPβ isoforms [17]: the full-length liver-enriched activating protein (LAP) and the truncated liver-enriched inhibitory protein (LIP). LIP lacks the N-terminal transactivation domains of LAP and acts as a potent repressor of C/EBP-dependent transcription [28].

The functional consequences of this interaction are dependent upon the relative ratios of PR and C/EBPβ isoforms in the cell [17]. Transfection studies demonstrate that PR-A, but not PR-B, greatly enhances LAP-dependent activation of the dPRL –332/–270 promoter region in a ligand-dependent manner. Conversely, overexpression of LIP, but not LAP, enhances PR-B transactivation of single and complex progesterone response element-dependent promoters [17]. Western blot analysis studies show that only LAP is present in normal non-pregnant human endometrium [29]. Intriguingly, C/EBPβ is also involved in the transcriptional regulation of the IGFBP-1 promoter [30], another representative decidual marker.

ii. PR and FOXO1

The FOXO proteins constitute a subclass of the winged helix/Forkhead box class of transcription factors. FOXO transcription factors are critical mediators in cell fate decisions in response to growth factor, hormonal and environmental cues [31]. Of the three human FOXO proteins (FOXO1, FOXO3a, and FOXO4), FOXO1 is markedly induced upon decidualization both *in vivo* and *in vitro*, and is involved in regulating the expression of decidual marker genes, such as PRL and IGFBP-1 [8, 18, 29, 32]. Indeed, FOXO1 enhances the activity of the dPRL promoter cooperatively with C/EBPβ through the discrete –332/–270 region, which also harbors the imperfect PR binding site [29]. FOXO1 also stimulates the IGFBP-1 promoter through direct interaction with HOXA10, a homeobox transcription factor [32].

iii. PR and STAT5

Members of the STAT family are activated by phosphorylation within the cytoplasm by diverse cell signaling pathways, including receptor-associated Janus kinases (JAKs) [33]. Phosphorylation of a conserved tyrosine residue in all STAT family members induces their dimerization and translocation to the nucleus. Within the nucleus, they regulate genes involved in the growth and differentiation of many tissues including adipocytes, hepatocytes, and mammary epithelial cells [33]. In the human endometrium, STAT5 is selectively expressed in the glandular epithelium. It is also expressed in a subset of stromal cells that also express the PRL receptor during the secretory phase, suggesting a potential role for STAT5 in differentiation [34].

STAT5 enhances the activity of the –332/–270 dPRL promoter region in hESCs [35]. Like C/EBP and FOXO1, STAT5 has also been shown to interact with the PR [16], which might contribute to the STAT5-mediated activation of the dPRL promoter region.

iv. Coactivators

Coactivators promote transcription initiation through protein interactions with components of the general transcription machinery and by promoting local remodeling of chromatin at specific promoters. The transcriptional coactivator CBP (CREB-binding protein) or its paralogue p300, was identified based on its ability to bind to CREB (cAMP-response element binding protein) [36]. It is now recognized as an integrator for a large number of transcriptional signals. It simultaneously interacts with diverse transcription factors and RNA polymerase II complexes, thus establishing contact between specific inputs and the basal transcription machinery [37, 38]. CBP/p300 interacts with C/EBPβ and enhances its activity [39]. C/EBPβ is also an important mediator of cAMP signaling in hESC, as will be outlined below. [24]. CBP is recruited to pre-initiation complexes containing steroid hormone receptors through the 160 kDa steroid receptor coactivator proteins (SRC-1/p160) [40]. The expression profiles of these coactivators and corepressors have been demonstrated in the endometrium throughout the menstrual cycle [41–43].

Nuclear receptor-associated coactivators including CBP/p300 and SRC-1 possess histone acetyltransferase activity (HAT), and histone acetylation has been implicated in decidualization [44]. Histone deacetylase inhibitors (HDACIs), which induce hyperacetylation of chromatin, facilitate the transcription of several genes. In this manner, they stimulate decidualization of human endometrial stromal cells *in vitro* [45]. In addition, HDACIs, through up-regulation of glycodelin, an implantation-related protein, also stimulate differentiation and cell motility of the endometrial epithelial cell line, Ishikawa [46, 47]. Thus, the increased levels of histone acetylation may contribute to implantation, one of the most essential functions of the endometrium. In support of this, we have recently demonstrated that glycodelin induction, following treatment with ovarian steroid hormones or an HDAC inhibitor, enhances implantation, as determined by an *in vitro* implantation assay using Ishikawa cells and the choriocarcinoma cell line, JAR [48].

Upstream signaling pathways regulate and cross-talk with PR signaling

Recently, it has become apparent that cAMP signaling regulates, modifies, and engages in cross-talk with the progesterone signaling pathway. cAMP signaling sensitizes human endometrial stromal cells to progesterone and eventually controls the expression and activity of a large number of transcription factors involved in decidualization [8].

cAMP signal transduction

cAMP is a ubiquitous second messenger molecule that is generated from adenosine triphosphate by adenylate cyclase. This enzyme is activated upon ligands binding to members of the family of G protein-coupled receptors (GPCRs) that are coupled with a stimulatory heterodimeric guanine nucleotide-binding protein (G protein). cAMP signaling is controlled at many levels. These include the receptor level, catabolism of cAMP by phosphodiesterases, modified composition of the PKA holoenzyme, expression of CREB and CREM (cAMP-response element modulator) isoforms with altered transcriptional activity, or a change in the expression level of coactivators or corepressors.

After ovulation, the endometrium is increasingly exposed to a variety of local and endocrine factors including prostaglandin E2, relaxin (RLX), CRH, LH, and FSH that are capable of stimulating cAMP production in hESCs. Activation of the cAMP pathway is the well established, initial and obligatory event that starts the decidual process *in vitro* [8]. In agreement, adenylate cyclase activity in the human endometrium increases during the menstrual cycle, and the cAMP content in biopsies obtained from patients during the secretory phase is higher than that in the proliferative phase [49, 50]. In pregnancy, the decidua is further exposed to a high level of hCG, which signals predominantly through the cAMP pathway [51].

In hESCs, RLX has the potential to modify the composition of the PKA holoenzyme, presumably resulting in a net increase in free, activated C protein and an increase in target protein phosphorylation [52]. Additionally, CREM isoforms and ICER (inducible cAMP early repressor) are involved in decidualization [53].

Downstream events of cAMP signaling

cAMP induces the expression or activation of several transcription factors, including FOXO1, STAT5,

and C/EBPβ, all of which are capable of interacting directly with the PR [16, 18, 29, 35, 54]. In addition, p53 is also up-regulated upon cAMP-induced differentiation of human endometrial stromal cells [55]. The promoters of dPRL and IGFBP-1 genes are activated by multimeric transcription factor complexes which assemble in response to an interplay of cAMP- and progesterone-dependent signals [8].

i. C/EBPβ

The C/EBP binding sites in the dPRL promoter are crucial for cAMP-induced activation, implicating C/EBPβ protein as a central mediator of the cAMP signal towards decidualization [24]. In agreement, C/EBPβ is not only induced by cAMP in cultured hESCs but also up-regulated *in vivo* in late secretory phase stromal cell nuclei [29].

ii. FOXO1

The expression and activity of FOXO1 itself is subject to intricate control mechanisms involving both the PKA pathway and the ligand-activated nuclear PR. Within three days of cAMP treatment, cultured hESCs up-regulate FOXO1 mRNA and protein. This response is markedly enhanced by progestin, although treatment with progestin alone does not induce FOXO1 expression [54]. In a manner strikingly parallel to C/EBPβ, FOXO protein accumulates in the nuclei of decidualized stromal cells *in vivo* [8]. Eventually, FOXO1 and C/EBPβ physically interact and cooperatively activate the dPRL promoter [29].

iii. STAT5

STAT5 is also a cAMP-induced transcription factor in decidualizing ESCs that has also been shown to interact with PR [35]. Treatment of primary ESC cultures with cAMP, with or without progestin, for two or more days results in induction, phosphorylation, dimerization, and nuclear translocation of STAT5 [35]. Induction of the dPRL promoter by cAMP plus progestin is markedly enhanced by STAT5 through its nuclear translocation and interaction with PR. This is abolished by coexpression of a dominant negative mutant of STAT5 [35].

iv. p53

The tumor suppressor protein p53 is a transcription factor that is present at extremely low levels in normal cells. In response to genotoxic stress, p53 protein is stabilized and rapidly accumulates. This ultimately leads to cell cycle arrest and DNA repair or to induction of apoptosis in damaged cells [56]. Thus, p53 exerts its biological function as the cellular gatekeeper for growth and division by transactivating cell cycle genes [56]. A massive and sustained up-regulation of p53 occurs during cAMP-induced decidualization of cultured hESCs [55]. Furthermore, a direct physical association with transrepression occurs between p53 and C/EBPβ [57]. Although the precise role of p53 in human decidualization remains elusive, in mice, p53 has recently been shown to be essential for regulating maternal reproduction, in particular, implantation, through leukemia-inhibitory factor (LIF) production [58].

v. Coactivators and Corepressors

cAMP activation of the PKA pathway disrupts the interaction of the PR with the corepressors NCoR and SMRT [59], thereby facilitating recruitment of the coactivator SRC-1 [60]. These corepressors and SRC-1 are all present in the human endometrium [61]; however, how cAMP regulates their behavior remains to be elucidated.

vi. PR and the SUMO pathway

Like many other transcription factors and cofactors, PR-A and PR-B are rapidly modified by small ubiquitin-like modifier (SUMO) -1 upon ligand binding [62].

Sumoylation denotes a process whereby SUMO covalently binds to target proteins, mostly transcription factors. Sumoylation profoundly changes the subnuclear localization, interactions, and activities of transcription factors [63]. Sumoylation often confers repressive properties. Intriguingly, cAMP signaling in ESCs alters the expression of many SUMO enzymes, resulting in a gradual loss of PR-A sumoylation and increased PR activity [64].

Other signaling pathways

i. SRC signal transduction

a. SRC activation and its essential role in decidualization

Many surface receptors for growth factors and cytokines possess tyrosine kinase activity and/or associate with non-receptor type tyrosine kinases [65]. As decidualized endometrial stromal cells produce a myriad of cytokines and growth factors [1–3, 6], it is likely that tyrosine phosphorylation signaling may be deeply involved in decidualization.

To address this, we performed an immunoblot analysis using a phosphotyrosine antibody and found that there were several differences in the profiles of the phosphotyrosinyl proteins between non-decidualized

and decidualized stromal cells [66]. Furthermore, we found that tyrosine kinase activity of SRC was increased during *in vitro* decidualization [66]. Subsequent immunohistochemical studies of the human endometrium and pregnancy decidua revealed that the kinase-active form of SRC was strongly expressed in decidual cells in humans and mice [67, 68].

SRC is a non-receptor tyrosine kinase that associates with integrins and many surface receptors including those for growth factors, cytokines, and G-protein coupled receptors (GPCR) [69]. SRC becomes activated upon ligand binding, and converts the extracellular stimuli to intracellular signals [69]. SRC is tethered to perinuclear membranes, endosomes, and secretory vesicles, and the cytoplasmic face of the plasma membrane by an N-terminal myristoyl group [69]. The kinase activity of SRC is up-regulated by dephosphorylation of its negative regulatory tyrosine residue, tyrosine 527 (Y527, corresponding to Y530 in humans), located at the carboxyl terminus. Its kinase activity is enhanced by autophosphorylation of tyrosine 416 [69]. C-terminal SRC kinase (CSK) phosphorylates Y527 thereby inactivating SRC; whereas a number of phosphatases (PTPs) dephosphorylate Y527 thereby activating SRC [69]. hESCs produce several bioactive substances including PDGF, EGF, CSF-1, IGF, IL-11, angiotensin II, bradykinin, PAF, prolactin, and oncostatin M during decidualization [1–3, 6]. Interestingly, all of these soluble factors have the potential to function as ligands for the transmembrane receptors that can couple with and activate SRC [70, 71]; therefore, it seems reasonable that SRC activation is accompanied by decidualization.

To address the essential role of SRC in decidualization of hESCs, knockdown experiments were subsequently performed using specific inhibitors of the SRC family of kinases: 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1) and 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) [72]. These inhibitors, however, unexpectedly promoted decidualization together with paradoxical SRC activation [72] by a mechanism that remains to be elucidated. We therefore examined the role of SRC in murine decidualization. Src null mice showed no apparent decidual response in their uteri [68]. This result clearly demonstrates that Src activity is indispensable for an appropriate progesterone induced decidualization response in mice [68]; however, it remains unclear whether SRC and its kinase activity are essential for decidualization in humans. To clarify this point, we recently conducted experiments in which an adenovirus was used to introduce the dominant negative mutant of SRC into hESCs [73]. The elimination of SRC kinase activity by overexpression of the mutant almost completely inhibited *in vitro* decidualization. This indicates that SRC kinase activation is also essential for decidualization in humans [73]. These results together corroborate the phenotype in the mouse and establish the importance of hormone-mediated SRC kinase activation in decidualization across species.

b. Downstream events of SRC signaling in hESCs

Though signaling pathways downstream of SRC are well elucidated in various types of cells, they are not clearly described in hESCs. We previously reported that despite the activation of decidual SRC, focal adhesion kinase (FAK) and paxillin, both well established substrates of SRC and components of the focal adhesion complex [69], remain hypophosphorylated in decidualized hESCs [74]. These results indicate that FAK and paxillin may not be substrates of SRC in decidualizing hESCs. Treatment of primary hESC cultures with cAMP (with or without progestin) leads to induction, phosphorylation, dimerization, and nuclear translocation of STAT5, eventually enhancing the activity of the –332/–270 decidual PRL promoter region [8, 35]. Members of the STAT family including STAT5 are activated by phosphorylation within the cytoplasm by diverse cell signaling pathways, including receptor-associated Janus kinases (JAK) [33, 75]. However, the nuclear accumulation of phosphorylated STAT5 in hESCs is independent of JAK activity, suggesting that other activating kinase(s) may regulate decidual STAT5 [8, 35]. Very recently, we have demonstrated that STAT5 was phosphorylated on tyrosine 694, a well-known SRC phosphorylation site during decidualization [73]. Knockdown of SRC signaling by the SRC dominant negative mutant markedly attenuates phosphorylation of STAT5 [73]. These results collectively indicate that the SRC-STAT5 pathway is essential for the decidualization of hESCs.

c. Possible regulatory mechanism of SRC activation in decidualized ESCs

The upstream regulatory mechanisms of SRC activity have been well elucidated in a variety of cells [69]. SRC couples with cell surface receptors for many bioactive substances including cytokines and growth

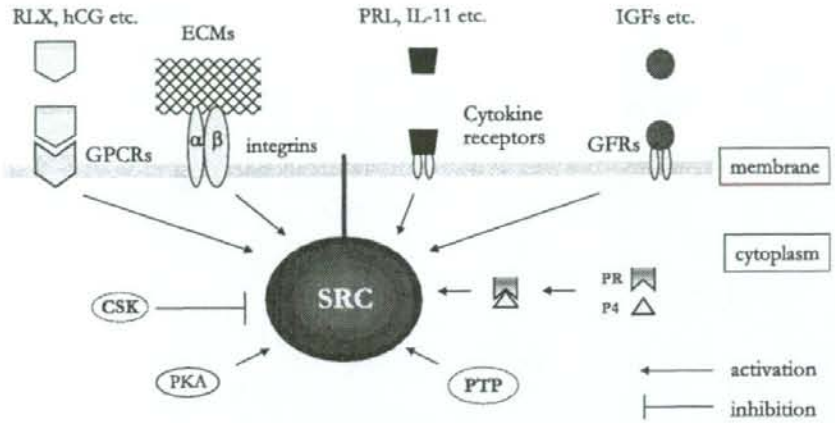

Fig. 1. Possible regulators of SRC activity in endometrial and decidual cells.
RLX, relaxin; hCG, human chorionic gonadotropin; GPCR, G protein-coupled receptors; ECMs, extracellular matrices; PRL, prolactin; IL-11, interleukin 11; IGFs, insulin-like growth factors; GFRs, growth factor receptors; CSK, c-terminal SRC kinase; PKA, protein kinase A; PR, progesterone receptor; PTP, protein tyrosine phoshphatase.

factors [69]; and these locally produced factors may activate SRC in an autocrine/paracrine manner. Indeed, IGF-I activates SRC in mouse endometrial cells [68]. In addition, SRC is activated by the cAMP/PKA signaling pathway in hESCs [76].

Progestins also positively regulate SRC activity [77]. They stimulate the SRC/MAPK pathway through indirect or direct interaction of ligand-bound progesterone receptors with SRC [23, 78]. This interaction may be facilitated when SRC becomes conformationally open upon dephosphorylation of tyrosine 527 (530 in human). In agreement, with this, we previously reported that decidual SRC becomes activated together with its dephosphorylation on tyrosine 530 [67, 72]. Furthermore, it is likely that SRC activation is hormone dependent in decidual hESCs, as withdrawal of E2 and progesterone reduces SRC kinase activity to its basal level and also changes the pattern of tyrosine phosphorylation to that of the unstimulated state [66]. Possible regulators of decidual SRC are illustrated in Fig. 1.

ii. PKB/AKT signal transduction

a. PKB/AKT signaling

The serine/threonine kinase (AKT), also known as protein kinase B (PKB), is the cellular homologue of the viral oncogene, v-Akt. It is phosphorylated and activated by multiple growth factors and functions as a downstream regulator of phosphoinositide 3-kinase (PI3K) signaling. Phosphorylated PKB/AKT is an important regulator of apoptosis and other multiple biological processes, including cell survival, the cell cycle, and glucose uptake [79, 80].

E_2 can directly and rapidly affect the PI3K-related signaling pathway by increasing the phosphorylation of PKB/AKT in endometrial cells [81]. This suggests that E_2 may exert part of its proliferative and anti-apoptotic effects by a non-genomic manner through the PKB/AKT signaling pathway. Progesterone/progestins counteract E_2 action at various molecular levels. In agreement, PKB/AKT becomes hypophosphorylated during progesterone-induced decidualization of hESCs *in vitro* [82, 83]. In contrast, phosphorylated AKT is strongly expressed in predecidual and decidual cells *in vivo* [83]. The discrepancy of the *in vivo* and *in vitro* results may be partly due to the production of decidualization-associated growth factors such as IGF-I that may activate PKB/AKT via PI3K.

b. Downstream events of PKB/AKT signaling

The transcriptional activity of FOXO proteins is critically regulated by their subcellular localization. Growth factor signaling through the PI3K pathway leads to phosphorylation of PKB/AKT that in turn phosphorylates downstream target proteins, including the FOXO transcription factors. Akt-dependent phosphorylation of nuclear FOXO results in its nuclear exclusion and inactivation [84, 85]. The observation that FOXO1 accumulates in the nuclei of cAMP-

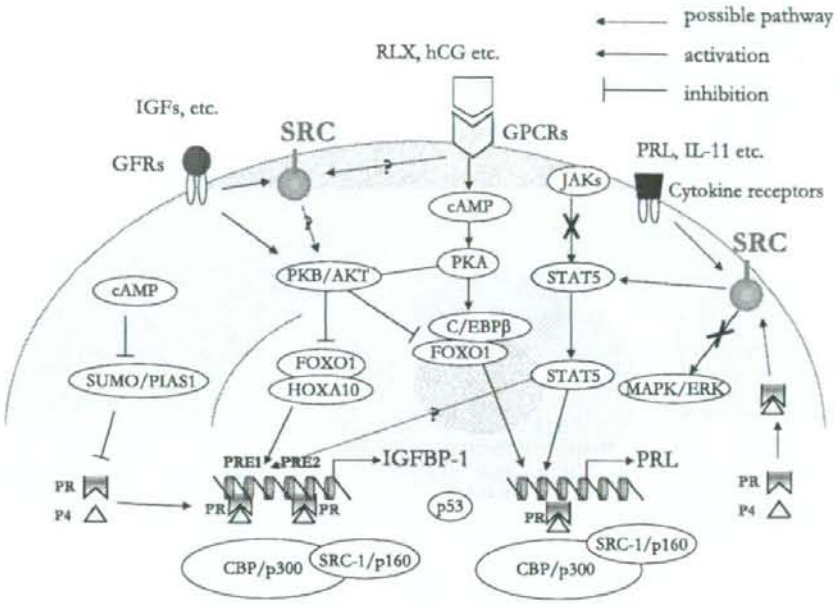

Fig. 2. Signaling events associated with decidualization.

treated ESC [8] suggests that the PI3K/PKB signaling pathway is suppressed upon decidualization. This is in agreement with previous reports that hypophosphorylation of PKB/AKT is tightly associated with *in vitro* decidualization of ESCs [82].

The PKB/AKT pathway also regulates the expression and localization of p53 [86]. Nuclear accumulation of p53 is the result of stabilization of p53 protein, rather than increased mRNA expression [86]. Proteasomal degradation of p53 is mediated by nuclear Mdm2 [86]. Nuclear translocation of Mdm2, in turn, is dependent on phosphorylation by PKB/AKT [86]. As expected, in cAMP-treated decidualized cells, p53 accumulation is associated with decreased nuclear Mdm2 and cytoplasmic PKB/Akt levels [55].

Thus, in addition to cAMP- and progestin-mediated signal transduction, other signaling pathways involving SRC and PKB/AKT, which are located downstream of the surface receptors for growth factors and cytokines, may serve to amplify and propagate the decidualization process in an autocrine or paracrine fashion. Signaling pathways responsible for decidualization are depicted in Fig. 2.

Regeneration of the human endometrium

After tissue breakdown and shedding of the differentiated endometrium (menstruation), the endometrium is programmed to regrow under the influence of E_2. The restructuring of the functional layer is critical to the development of a tissue ready for implantation or for menstruation. Vessel growth is particularly important in the endometrium of menstruating species where the spiral arterioles are a characteristic feature. These regeneration processes are comprised of endometrial epithelial regrowth, angiogenesis, and proliferation of endometrial stromal cells.

Cellular mechanisms of endometrial regeneration

Epithelial growth

After menstruation, the regeneration of all cell types, epithelial, endothelial, and stromal, occurs rapidly. The remaining basal layer acts as a germinal compartment from which the different cell types grow and differentiate [87]. Regrowth is estrogen dominated; and for epithelial cells EGF, TGFα, and EGF receptor are all likely to be involved. Both TGFα and EGF compete for the EGF receptor; and both, along