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endometriosis, eutopic endometrial PKR2 transcript level was 4-fold higher ($p<0.05$) in the proliferative than in the secretory phase. No differences in EG-VEGF nor PKR1 in proliferative versus secretory endometrium in these patients were found. There were no significant differences in the expression of EG-VEGF in eutopic endometrium of normal women and those with endometriosis. In the paired laser captured micro-dissected eutopic endometrial and ectopic endometriotic samples, a significantly higher ($p<0.01$) EG-VEGF but not VEGF transcript level was detected in the ectopic when compared to eutopic samples; while the expressions of PKR1 and PKR2 were barely detectable.....

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Running Title: EG-VEGF/PK1 in Human Endometriotic Tissues

Up-regulation of Endocrine Gland-derived Vascular Endothelial Growth Factor (EG-VEGF/PK1) but not VEGF in Human Ectopic Endometriotic Tissue

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Capsule: High levels of EG-VEGF expression may play an important role in angiogenesis in endometriotic tissues.

Abstract

Objective: To study the expression of vascular endothelial growth factor (VEGF), endocrine gland-derived VEGF (EG-VEGF/PK1) and its receptors (PKR1 and PKR2) in eutopic and ectopic endometrial tissues. **Design:** A case control study. **Setting:** University reproduction unit. **Patients:** Infertile women undergoing diagnostic laparoscopy for tubal patency. **Intervention:** Endometrial and endometriotic tissue sampling from women with and without endometriosis. **Main Outcome Measure(s):** Quantitative PCR analysis of genes in eutopic and ectopic endometrial tissues. EG-VEGF protein was studied by immunohistochemistry. **Result(s):** In normal endometrium, EG-VEGF mRNA expression was 50-fold higher ($p<0.05$) in the secretory than in the proliferative phase, but that of PKR1 was 6-fold higher in the latter than in the former. PKR2 transcript was detected in the proliferative but not the secretory endometrium. In patients with endometriosis, eutopic endometrial PKR2 transcript level was 4-fold higher ($p<0.05$) in the proliferative than in the secretory phase. No differences in EG-VEGF nor PKR1 in proliferative versus secretory endometrium in these patients were found. There were no significant differences in the expression of EG-VEGF in eutopic endometrium of normal women and those with endometriosis. In the paired laser captured micro-dissected eutopic endometrial and ectopic endometriotic samples, a significantly higher ($p<0.01$) EG-VEGF but not VEGF transcript level was detected in the ectopic when compared to eutopic samples; while the expressions of PKR1 and PKR2 were barely detectable. H-scoring confirmed that the stroma of endometriotic samples had a significantly higher ($p<0.05$) EG-VEGF protein expression than that in the paired eutopic endometrium. **Conclusion(s):** High levels of EG-VEGF expression may play an important role in angiogenesis in endometriotic tissues.

Key Words: Endometriosis, Endometrium, Laser capture micro-dissection, EG-VEGF, PK1, PKR1, PKR2

INTRODUCTION

Endometriosis refers to the presence of endometrial tissues outside the uterine cavity. It occurs spontaneously in humans and non-human primates including baboons and macaques but is rare in other animal species (1). Endometriosis is a common gynaecological disease affecting 6-10% women of reproductive age. Affected women have higher risk than the general female population in developing cancers, autoimmune and atopic disorders (2). Its etiology and pathophysiology are still unclear. Endometrial tissues in the uterus (eutopic) and outside the uterine cavity (ectopic) respond to the cyclical changes of steroid hormones. However, the proportions of estrogen and progesterone receptors, as well as the histology are different between paired eutopic and ectopic endometrial samples (3-5).

Angiogenesis is important in the development of endometriosis (6-8). Angiostatic treatment reduces the number of endometriotic lesions at implantation in the chick chorioallantoic membrane model (9) and after implantation in the nude mice model (10). Various growth factors including vascular endothelial growth factor (VEGF) have been found to be associated with angiogenesis in endometriosis (6, 10, 11).

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) belongs to the prokineticin family. It is also known as prokineticin 1 (PK1), and shares about 44% amino acid homology with prokineticin 2 (PK2) or Bv8 (12). EG-VEGF was previously thought to be selectively expressed in steroidogenic glands and stimulate the growth of endocrine gland endothelium (13). It promotes proliferation, survival, and chemotaxis of endothelial cells from steriodogenic tissue, such as adrenal cortex capillary endothelial cells (14), but not those from other tissues, such as human umbilical vein endothelial cells and other non-endothelial cell types

(12, 13). Although EG-VEGF is structurally distinct from VEGF, they induce similar angiogenic response in the ovary (15, 16).

EG-VEGF acts via G-protein coupled receptors, PKR1 and PKR2 (17, 18), and induces mitogen-activated protein kinase (MAPK) and Akt serine/threonine kinase phosphorylation in bovine adrenal capillary endothelial cells (17), HEK293 (18), adrenal cortex-derived endothelial (ACE) cells (19), neuroblastoma cell line (SK-N-SH, 20) and human uterine microvascular endothelial cell (Ut-HVEC-Myo, 21). Human PKR1 and PKR2 shared 85% amino acid identity and are most divergent in their N-terminal sequences (18).

Prokineticins and their receptors are likely to be involved in reproduction as they are expressed in male reproductive tissues including testis and prostate (22, 23), and in female reproductive tissues such as ovary, uterus and various tissues during pregnancy (16, 24, 25). EG-VEGF has also been implicated in the pathology of the reproductive tract (26), including Leydig cell neoplasms (27), prostate cancer (23), ovarian carcinoma (15) and polycystic ovarian syndrome (16). Whether EG-VEGF is involved in endometriosis is unclear. During early pregnancy and in hypoxic conditions, hypoxia-inducible factor-1α (HIF-1α) binds to the promoter of EG-VEGF and PKR1 leading to up-regulation of these genes in the placenta (13, 25, 26).

Interestingly, transient hypoxia also up-regulates HIF-1α and induces the expression of VEGF in endometrium (28) and transplanted endometriosis-like lesions (29). In the mice model, suppression of HIF-1α decreases VEGF production and inhibits growth of the transplanted endometriosis-like lesions (29).

Recently, EG-VEGF was found to be expressed in non-endocrine tissues including endometrium (26, 30, 31). In human endometrium, EG-VEGF is highly expressed in the secretory phase of the menstrual cycle when active angiogenesis occurs (30, 31). The facts that EG-VEGF is detected only in steroid (estradiol and progesterone) treated endometrial epithelial and stromal cells, and is up-regulated in

the peri-implantation endometrium after gonadotropin-stimulation and hormone replacement therapy suggests that the production of EG-VEGF is hormone dependent (30, 31). Despite all these observations, the function of EG-VEGF in the uterus remains unknown.

In view of the importance of angiogenesis in the development and progression of endometriosis, the involvement of multiple factors in the regulation of angiogenesis and HIF-1α target genes in endometriosis, and the potential angiogenic role of EG-VEGF in endometrium, we hypothesized that aberrant expression of EG-VEGF could be associated with the pathogenesis of endometriosis. In this study, we used laser micro-dissection technique to isolate paired human eutopic and ectopic endometriotic biopsies for real-time PCR analysis on VEGF, EG-VEGF and PKR1 and PKR2 mRNA expression. The increased expression of EG-VEGF mRNA and protein in ectopic endometriotic tissues suggests this molecule is associated with the progression of the disease.

MATERIALS AND METHODS

Human Subjects

Infertile women with endometriosis shown on diagnostic laparoscopy for assessment of tubal patency were recruited. The eutopic endometrial (DE) samples from these diseased women (n=15) were obtained by pipelle. The American Society for Reproductive Medicine (ASRM) rAFS scoring was used to classify the stages of the disease. Eutopic endometrial samples were also taken from 33 normal women (NE) who had no endometriosis as determined by laparoscopic examination and had regular menstrual cycles. There were no difference between the mean age (normal: 33, range 24-39; endometriosis: 34, range 29-38, $p>0.05$) and mean cycle length (normal: 30, range 26-35 days; endometriosis: 30, range 25-35 days, $p>0.05$) of the normal and the diseased groups. For the paired eutopic and ectopic endometriotic samples (n=16), tissues at proliferative phase (n=4) and secretory phase (n=12) were obtained from women with endometriosis (age: 31-49). All recruited subjects had not received any anti-inflammatory or hormonal medication for at least three months prior to the sample collection. The classification of the endometrial samples into proliferative or secretory phases was based on the day of the last menstrual period and was confirmed by endometrial histology (32). The Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster had approved the protocol of the study and informed consent was received from each subject.

Laser Captured Micro-dissection (LCM) and Real-time PCR

The endometrial and endometriotic tissues were snap-frozen, embedded in OCT medium and cut into 6 μm thick sections at -20^{o}C. Ectopic endometrial samples containing glandular and stromal cells were isolated by laser captured micro-dissection (LCM) to avoid contamination by ovarian stromal tissue. Eutopic

endometrial samples were processed similarly as sham procedure before RNA extraction. In brief, frozen tissue sections were mounted on a membrane slide with a 2 μm thick membrane framed by aluminum and stained with hematoxylin and eosin for histological examination. After drying in air for 10 min, the membrane slide was placed on a regular glass slide, which was put on the microscopic stage with the membrane slide facing up (Leica DM IRB, Leica Microsystems Ltd.). Glandular and stomal tissues were micro-dissected using a laser beam (VSL-337ND-S nitrogen laser, Laser Science Inc.) and picked up by the capturing caps. The attached tissues were then put directly into microcentrifuge tubes. RNA from three captured tissues were extracted with the Absolute RNA Miniprep Kit (Stratagene, CA, USA) as described previously (33) and eluted with 30 μl of distilled water. One-third of the total RNAs from each sample were reverse-transcribed into cDNA using the First-strand cDNA Synthesis Kit (Amersham Pharmacia, Buckinghamshire, UK). Real-time PCR analysis of human VEGF, EG-VEGF, PKR1 and PKR2 transcripts were performed using an ABI 7500 Sequence Detector (PE Applied Biosystems, Foster City, CA). Multiplex PCR reactions were carried out in a 20 μl reaction mixture containing 2 μl of sample cDNA; 10 μl 2X TaqMan Universal PCR Master Mix; 1 μl of 18S internal control and 1 μl of 20X Assays-on-Demand Gene Expression Assays (PE Applied Biosystems) in triplicates. The qPCR experiment was repeated thrice for the 16 pairs of RNA samples. Quantitative analysis was done by the sequence detection software supplied with the ABI 7500 Sequence Detector. The threshold cycles (Ct) for each reaction were calculated and used for quantifying the amount of starting template in the reaction. A difference of Ct values (ΔCt) was obtained by subtracting the Ct value of 18S from Ct value of the genes of interest. The relative gene expression values were calculated by the $2^{-\Delta\Delta Ct}$ method (34). The relative amount of the transcript of interest in the normal sample (X) and the diseased sample (Y) after normalization to

an endogenous reference (18S) was calculated as $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in Ct between the gene of interest and 18S, and ΔΔCt is the difference for sample X and Y i.e. $\Delta Ct_X - \Delta Ct_Y$. The expression levels in normal endometrium or in proliferative phases were used as calibrators for different PCR experiments.

Immunohistochemical Staining

Antibody against human EG-VEGF was obtained from R&D Systems (MAB1209, Minneapolis, MN). Frozen endometrial and testicular sections were acetone and then methanol fixed for 5 min and the tissues were blocked by 10% rabbit serum in PBS for 30 minutes. A 1:100 dilution of the antibody was used and the tissues sections were incubated at 4°C overnight. The tissues were washed 5 times with PBS, incubated with 1:300 diluted rabbit anti-mouse biotinylated secondary antibody for 60 minutes, and were incubated with the ABC complex (Vectastain ABC kit, Vector, Burlingame, CA) for 30 min. Specific signal were visualized by DAB staining and the tissues were counterstained with hematoxylin. Semi-quantitative assessment of staining (n=12) was done on 5 fields of epithelial and stromal tissues by H-score (35), which was calculated as: H score = ΣPi(i+1), where i = staining intensity graded as 0, 1, 2 and 3 with 3 being the highest intensity, and Pi = percentage of tissue staining at each intensity.

Statistical Analysis

Data were analyzed using the SigmaPlot software package (Jandel Scientific, San Rafael, CA). Statistical analysis was performed by Kruskal–Wallis and Mann–Whitney *U* test or paired *t-test* as appropriate. Linear associations of American Society for Reproductive Medicine's classification rAFS scores on the endometriosis

patients with EG-VEGF, PKR1 and PKR2 mRNA expression were tested using Pearson's correlation coefficient. A p-value <0.05 was considered statistically significant.

RESULTS

Expression of EG-VEGF, PKR1 and PKR2 in Normal and Eutopic Endometrial Tissues

To determine if there were differences in the mRNA expression levels of EG-VEGF, PKR1 and PKR2 between eutopic endometrium from normal women (NE, n=33) and eutopic endometrium of diseased women with endometriosis (DE, n=15), tissue samples were divided into 4 groups: normal proliferative endometrium (n = 14), normal secretory endometrium (n = 19); eutopic proliferative endometrium with endometriosis (n = 9) and eutopic secretory endometrium with endometriosis (n = 6). The relative expression of the three transcripts was normalized to 18S and calculated by the equation: $2^{-\Delta\Delta Ct}$ using the proliferative phase normal endometrium (NE) as calibrator. It was found that normal endometrium (NE) at secretory phase expressed significantly ($p<0.05$) higher EG-VEGF (50-fold) but lower PKR1 (6-fold) mRNA when compared with that of the normal proliferative phase samples (Table 1, Figure 1A and B). PKR2 transcript was detected in the normal proliferative endometrium but not in the normal secretory endometrium. (Table 1 and Figure 1C). The expression of PKR2 in the eutopic proliferative endometrium from patients with endometriosis (DE; Table 1 and Figure 1C) was significantly higher (4-fold, $p<0.05$) than that in the eutopic secretory endometrium. However, no difference was found in EG-VEGF and PKR1 expression between the proliferative and secretory eutopic endometria from patients with endometriosis (Figure 1A and B). When the expression of the three genes in endometrial biopsies of the same menstrual phase was compared, no difference was found between the normal and the endometriosis groups. The rAFS scores of the endometriosis patients ranges from 1 to 85 with a median value of 4. Analysis of the relationship between rAFS scores of the endometriosis patients and the mRNA expression of EG-VEGF, PKR1 and PKR2 had a coefficient of correlation

(*r*) of -0.136, 0.0122 and -0.0152, respectively (p>0.05), indicating that the gene expressions were not correlated with the rAFS scores (data not shown).

Expression of EG-VEGF, PKR1 and PKR2 in the Paired Eutopic and Ectopic Endometriotic Samples

We further analyzed the expression of VEGF and EG-VEGF mRNA in another sixteen paired human endometriotic samples (Figure 2A) in which 4 were in proliferative and 12 were in secretory phases by real-time PCR. The LCM isolated tissues from eutopic and ectopic samples contained a mixture of glandular and stromal tissues. Multiplex real-time PCR were carried out and the data were normalized with 18S and calculated by the equation: $2^{-\Delta\Delta Ct}$ using the ectopic endometrium as calibrator.. No significant difference was found in VEGF expression (Figure 2B) between the eutopic and ectopic endometiotic samples (1.922 vs 1.151, p>0.05), but a significant increase in EG-VEGF expression (Figure 2B) between the eutopic and ectopic endometriotic samples (0.140 vs 2.131, p=0.00879) was observed. Subgroup analysis of the secretory phase samples demonstrated the expression of EG-VEGF in ectopic endometrium is significantly higher than that of the eutopic endometrium (0.202 vs 1.970, p=0.0159); while no significant change in VEGF expression was found. Very low or undetectable expression of PKR1 and PKR2 were observed, i.e. Ct value close to 40 cycles, in both the eutopic and ectopic endometriotic samples (data not shown).

EG-VEGF Protein Expression in Ectopic Endometriotic Tissues

Immunohistochemical staining was performed using EG-VEGF antibody on frozen paired endometrial tissues. EG-VEGF immunoreactivity was strongly localized to the glandular and luminal epithelium of the eutopic endometrial samples (Figure

3A) and the expression of EG-VEGF in the stromal cells were weak. In ectopic endometriotic samples, intense EG-VEGF immunostaining was found in the stromal cells (Figure 3B). No immunostaining was found when the primary antibody was omitted (Figure 3C). The specificity of the antibody was confirmed when frozen human testicular section was used (Figure 3D) and specific signal was observed in the Leydig cells of the testis. The expression of EG-VEGF protein in twelve pairs of endometriotic samples was determined by H-scoring (Figure 3E and F). Significant difference was found in the stromal cells (Median and range: 1.5 (0.5-2.4) vs 0.5 (0-2.2), $p=0.011$), but not in the glandular epithelium (Median and range: 1.4 (0.2-2.0) vs 1.5 (1.0-2.4), $p>0.05$) of ectopic and eutopic sample, respectively.

DISCUSSION

The expression of EG-VEGF in human endometrium was up-regulated in the secretory phase of the menstrual cycle (Figure 1A), consistent with a steroid regulatory mechanism of EG-VEGF production. Higher expression of EG-VEGF was also found in the gonadotropin-stimulated cycle when compared with the natural cycle of patients undergoing assisted reproduction treatment (30). The hormonal regulation of EG-VEGF expression by progesterone has also been demonstrated using cultured endometrial tissues (31). Furthermore, our recent study demonstrated that estrogen and/or progesterone stimulate EG-VEGF expression in the human primary endometrial stromal and glandular epithelial cells (30).

We detected a higher PKR1 and PKR2 mRNA expression in eutopic endometrial samples from normal women (NE) in the proliferative phase (Figure 1B and C) than in the secretory phase suggesting that the receptors for EG-VEGF (PKR1 and PKR2) may be mainly regulated by estrogen. The differential expression pattern of PKR2, but not PKR1, was also found in the proliferative and secretory eutopic endometria from patients with endometriosis (DE). However, no significant temporal variation of PKR1 and PKR2 mRNA expression was found between the human proliferative and secretory endometria in another study (31). The reasons for this discrepancy remain unknown. It could be due to the inclusion of the full-thickness of the endometrium with basal endometrial-myometrial region expressing low level of PKR2 in the previous study, whereas only the superficial endometrial layer was analyzed in the present study.

It was interesting to note that the level of EG-VEGF was low whilst that of the transcripts of EG-VEGF receptors (PKR1 and PKR2) was up-regulated in the proliferative endometrium when compared to the secretory endometrium in normal samples. The reason for the dissonance in the expression between EG-VEGF and its

receptors was not clear. It was possible that EG-VEGF/PK1 could act on another receptor yet to be defined in the secretory endometrium. In line with this, endometrium expresses another ligand of PKR1 and PKR2, PK2 with level that does not vary significantly in the menstrual cycle (31). PK2 has a long form spliced variant, PK2L, which is co-expressed with PK2 in many tissues (36). PK2L produces a peptide, PK2β that can selectively activate PKR1 (36). The abundance of PK2L in endometrium at different phases of the menstrual cycle is not known. Its determination would be useful to verify the existence of the second possibility.

PKR1 and PKR2 share 87% homology in amino acid sequences and have almost identical transmembrane domain sequence (37), suggesting that they may have similar activation mechanism (26). However, it was reported that EG-VEGF induces phosphorylation of p44/42 MAPK in the PKR1 transfected cells but not in the PKR2 transfected cells (18). In mouse, PKR2 is essential for the maturation of the reproductive system as its deficiency is associated with atrophy of the reproductive tract including gonads and endometrium, which has been linked to the lack of gonadotropin-releasing hormone neurons in the hypothalamus (38). Yet, no abnormality in the reproductive tract of PKR1 knockout mice was found (38, 39).

When endometrial tissues in the same menstrual phase were compared, there was no significant difference in the mRNA expression of EG-VEGF and its receptors between the normal endometrium and eutopic endometrium from women with endometriosis, indicating that eutopic EG-VEGF expression was not associated with the occurrence of endometriosis. This observation was supported by the present results showing no correlation between the severity of endometriosis (ASRM endometriosis scores) and the mRNA expression of EG-VEGF, PKR1 and PKR2 in the eutopic endometrium.

Human ovary abundantly expresses EG-VEGF and PKR2 but not PKR1 (14, 18). In order to avoid contamination of endometriotic tissues by neighboring ovarian

tissue, we used laser captured micro-dissection to isolate endometriotic tissues. The purity of the laser micro-dissected samples was demonstrated by the very low or undetectable level of PKR2, which is known to be produced abundantly in the ovary. The micro-dissected ectopic endometriotic tissues used in this study contained both epithelial and stromal cells. They produced EG-VEGF but not its receptors, suggesting paracrine actions of the EG-VEGF most probably act on the neighboring ovarian cells which express PKR2. Consistent with this possibility is the observation that over-expression of EG-VEGF in the rat ovary resulted in excessive angiogenesis and cysts formation (13). Thus, the increase in EG-VEGF expression in ectopic endometrium when compared with that in the eutopic endometrium is likely to be an event secondary to the initiation of ovarian endometriosis. The expression of EG-VEGF is also associated with polycystic ovary syndrome (16). The aetiology of EG-VEGF over-expression in ovarian endometriotic cyst warrants further investigation.

Angiogenesis is important in the establishment and growth of endometriosis. In the ovary, the angiogenic response induced by EG-VEGF is indistinguishable from that by VEGF (14). However, the expression of ovarian EG-VEGF and VEGF is temporally and spatially complementary to each other (16), suggesting that the two growth factors have distinct roles and targets in ovarian functions. This may explain why up-regulated levels of EG-VEGF could induce pathophysiological changes in endometriosis even in the presence of other potent angiogenic factors like VEGF.

In the present study, a significant increase in EG-VEGF protein expression was found in the stromal cells of ectopic endometrium (Fig. 3B and E). It is possible that the stromal cells may synthesize more EG-VEGF and/or EG-VEGF is synthesized in the epithelial cells but is accumulated in the extracellular matrix of the stroma. The latter explanation was supported by the fact that VEGF binds to the extracellular matrix upon secretion (40) and releases during tissue breakdown or

hypoxia. EG-VEGF is a heparin-binding molecule (13) and the heparin-binding domain of VEGF interacts with the matrix proteins (41). Thus, EG-VEGF may also be accumulated in the extracellular matrix giving an intense immuno-positive signal. The current data cannot distinguish these two possibilities.

VEGF is generally accepted as a critical angiogenic factor associated with implantation and early pregnancy (42), though there are opposing opinions (43, 44). Although the expression of VEGF mRNA and protein was higher in red peritoneal endometriotic lesion than in other type of peritoneal lesions (45, 46), no difference in the mRNA expression of VEGF between eutopic endometrium and ovarian endometriotic tissues was found in the present study. This observation supports a recent report on lack of difference in VEGF immunorectivities between eutopic endometrium and ovarian endometriosis (47). There is also no difference in the mRNA and protein production of VEGF by granulose-luteal cells in women with or without ovarian endometriomas (48).

In conclusion, we have demonstrated an up-regulation of EG-VEGF but not VEGF in ectopic endometriotic tissues obtained from same patients. The absence and/or low expression of EG-VEGF receptors (PKR1 and PKR2) in the ectopic endometriotic tissues suggested that EG-VEGF may act as paracrine and endocrine factors to promote angiogenesis in adjacent tissues leading the paracrine disorder in women. Further studies on the functional role of EG-VEGF, PKR1, PKR2 and VEGF on the pathophysiological changes in ectopic endometrium *in vitro* and in animal model will provide a better understanding on the causes of endometriosis and may lead to possible medical treatments for the prevention of this disease.

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Figure Legends

Figure 1

Real-time PCR analysis of EG-VEGF, PKR1 and PKR2 mRNA expression levels between normal and eutopic endometrium. Eutopic endometrial samples from normal women (NE) in proliferative (Proli, n = 14) and secretory phase (Sec, n = 19); and from women with endometriosis (DE) in proliferative (n = 9) and secretory phase (n = 6) were studied. The relative expression of the (A) EG-VEGF, (B) PKR1 and (C) PKR2 mRNA expressions were normalized to 18S expression and calculated by the equation: $2^{-\Delta\Delta Ct}$ using the proliferative phase normal endometrium (NE) as calibrator. A difference of Ct values (ΔCt) was obtained by subtracting the Ct value of 18S from Ct value of the genes of interest. The relative gene expression values were calculated by the $2^{-\Delta\Delta Ct}$ method (see materials and methods). Data were presented as median with 10^{th}, 25^{th}, 75^{th} and 90^{th} percentiles and outliers (●). Statistical analysis was performed by Kruskal-Wallis test and p-values were shown. A p-value less than 0.05 was considered significant different from different samples.

Figure 2

Real-time PCR analysis of EG-VEGF and VEGF mRNA expression levels in paired LCM isolated eutopic and ectopic endometriotic samples. (A) Three LCM sections from ectopic or eutopic samples were used for RNA extraction. (B) The relative expressions of EG-VEGF and VEGF transcripts were normalized to 18S and calculated by the equation: $2^{-\Delta\Delta Ct}$ using the ectopic endometrium as calibrator. A p-value less than 0.05 was considered significant different from different samples. NS: Not significant.

Figure 3

Immunohistochemical staining of EG-VEGF in eutopic and ectopic endometrium. Frozen eutopic (A and C) and ectopic (B) endometrial, and archive testis (D) samples were stained with EG-VEGF (A, B and D) antibody. Specific brown signal was found in the glandular (GE) and luminal epithelium (LE) of eutopic endometrium (A), and in the stromal cells (SC) of the ectopic endometrium (B) of the same patient and no signal was found when the primary antibody was omitted (C). Leydig cells (Le) of frozen human testicular sample were positively stained with EG-VEGF antibody, but not in spermatozoa (Sp) in the seminiferous tubule (D). The expression of EG-VEGF protein in twelve pairs of endometriotic samples was determined by H-scoring. Significant difference was found in the stromal cells (E), but not glandular epithelium (F) of ectopic and eutopic endometrium ($p=0.011$).

Table 1 Messenger RNA expression of EG-VEGF, PKR1 and PKR2 in eutopic endometria of normal women and diseased women with endometriosis. The transcript expression levels were expressed as median of Ct values (ranges). * P<0.05 when compared with the proliferative phase.

mRNA	Normal women		Women with endometriosis	
	Proliferative	Secretory	Proliferative	Secretory
EG-VEGF	0.03 [a] (5.2×10^{-6}-1.0)	1.50 [a] (0.0-27.9)	0.04 (2.9×10^{-4}-0.6)	0.28 (2.0×10^{-4}-5.6)
PKR1	3.92 [b] (0.0-23.4)	0.64 [b] (0.0-6.7)	1.89 (0.1-5.5)	0.43 (0.0-2.1)
PKR2	0.75 [c] (0.0-6.6)	0.00 [c] (0.0-1.0)	0.93 [d] (0.0-10.7)	0.24 [d] (0.0-1.2)

a-a, b-b, c-c, d-d are statistically difference (p<0.05) between groups

Figure 1

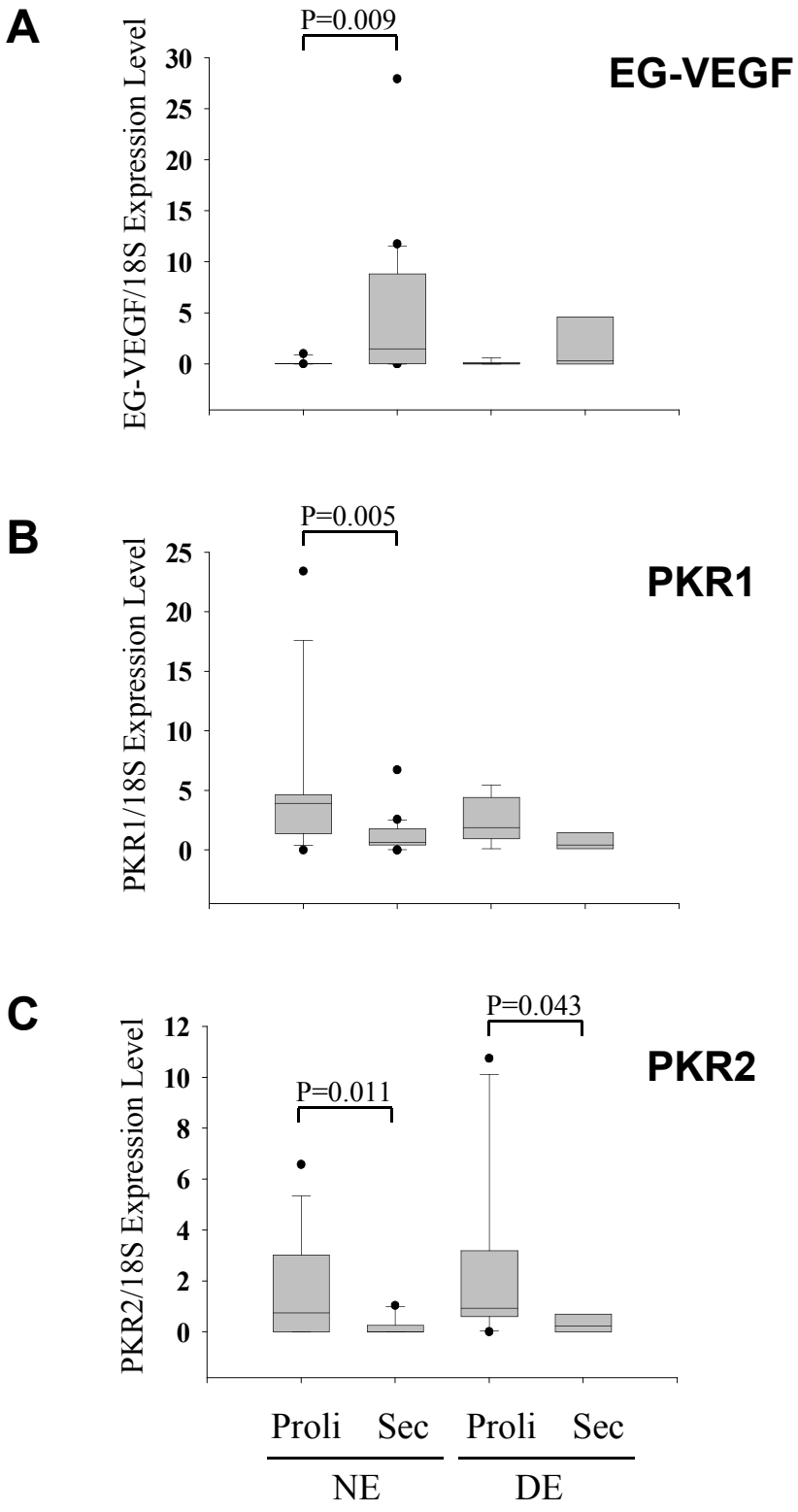
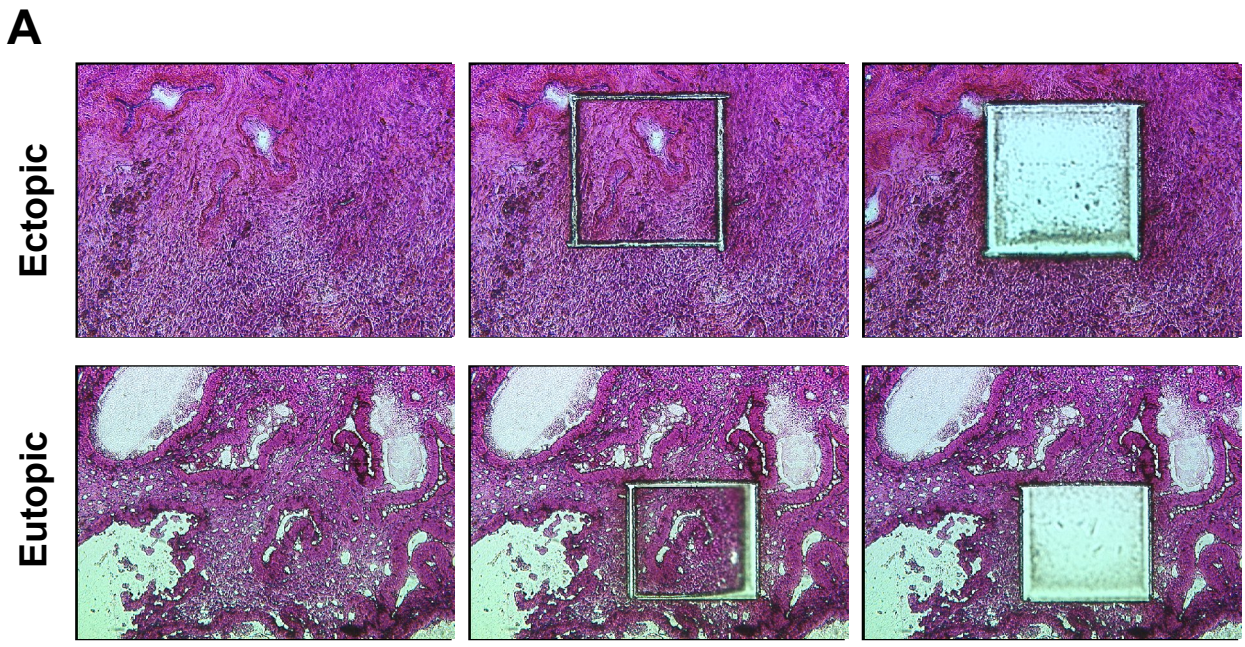

A
P=0.009
EG-VEGF
EG-VEGF/18S Expression Level
30
25
20
15
10
5
0
B
P=0.005
PKR1
PKR1/18S Expression Level
25
20
15
10
5
0
C
P=0.043
P=0.011
PKR2
PKR2/18S Expression Level
12
10
8
6
4
2
0
Proli
Sec
Proli
Sec
NE
DE

Figure 2


A
Ectopic
Eutopic

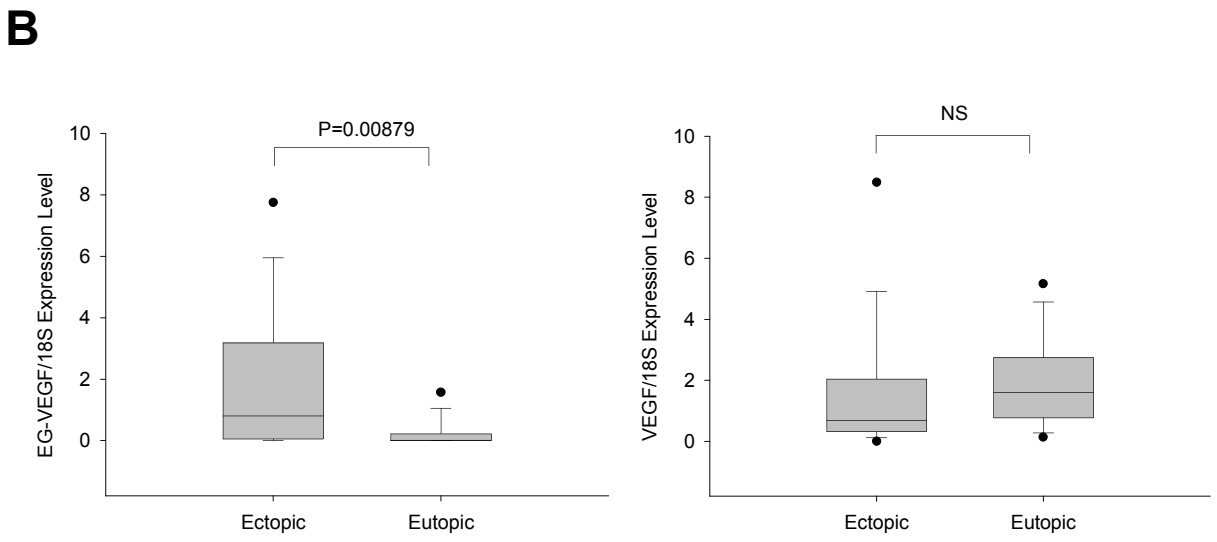
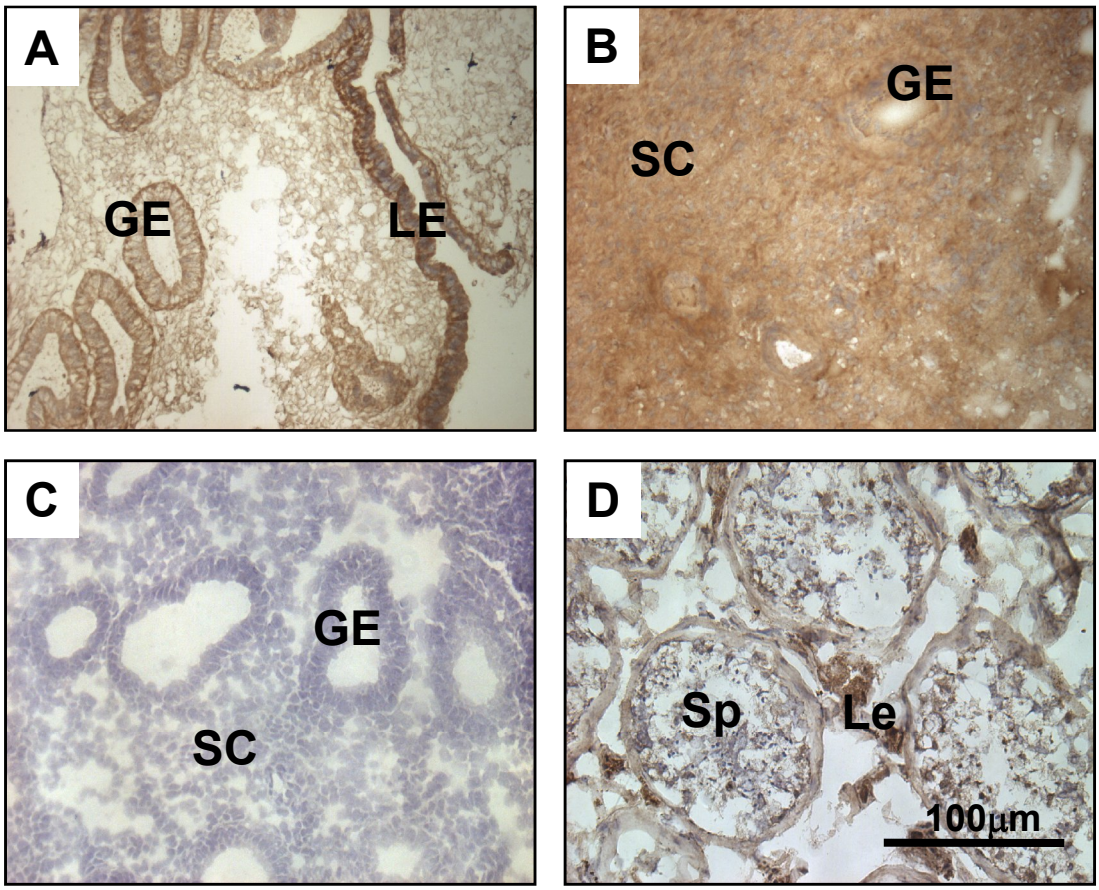
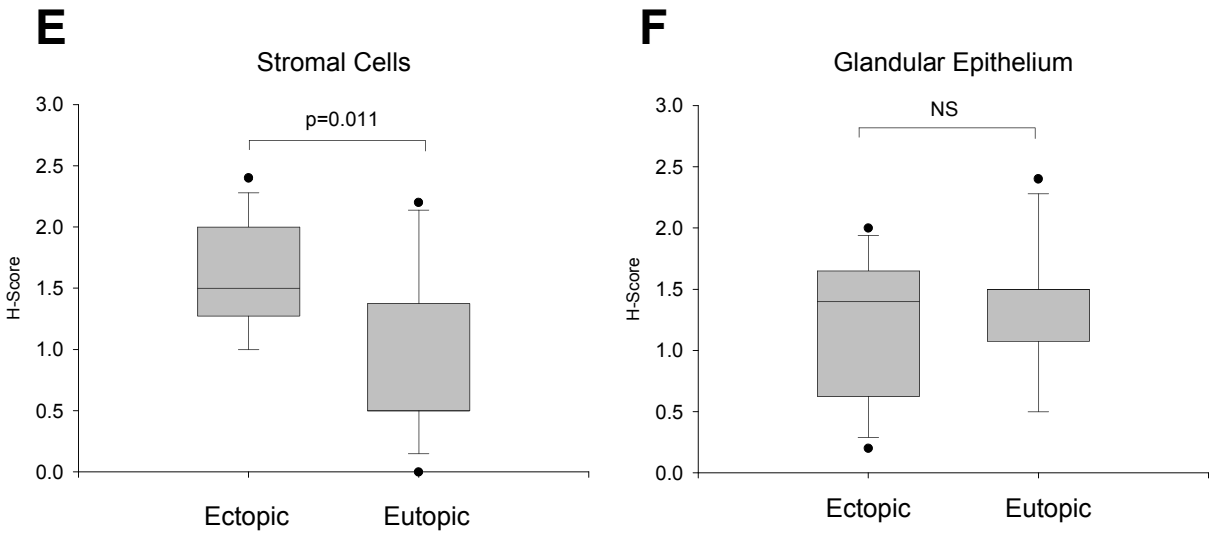

B
P=0.00879
EG-VEGF/18S Expression Level
10
8
6
4
2
0
Ectopic
Eutopic
NS
VEGF/18S Expression Level
10
8
6
4
2
0
Ectopic
Eutopic

Figure 3


A
GE
LE
B
GE
SC
C
GE
SC
D
Sp
Le
100μm


E
Stromal Cells
p=0.011
H-Score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ectopic
Eutopic
F
Glandular Epithelium
NS
H-Score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ectopic
Eutopic