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Endocrine Gland-Derived Vascular Endothelial Growth Factor Is Expressed in Human Peri-implantation Endometrium, But Not in Endometrial Carcinoma

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Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) is a newly identified angiogenic and permeability-enhancing factor, predominantly expressed in steroidogenic tissues. Recently, we found that EG-VEGF is also expressed in the normal peri-implantation endometrial samples from patients of reproductive ages (80%). Immunohistochemistry analysis showed that EG-VEGF is predominantly expressed in the glandular epithelial cells and its expression is dynamic during the menstrual cycle with a peak expression at the mid-luteal phase. We also found that EG-VEGF transcripts are up-regulated in all the peri-implantation endometrial samples from the patients after the ovulating dose of human chorionic gonadotropin in gonadotropin-stimulated cycles and patients receiving hormone replacement therapy. In *in vitro* endometrial cell culture, EG-VEGF mRNA was detected in endometrial cells only in the presence of steroids, suggesting that EG-VEGF expression is highly dependent on the steroid hormones. Subsequent expression analyses on the EG-VEGF receptors showed that hPK-R1 and hPK-R2 are differentially expressed in human endometrium, but show no significant correlation with the hormonal treatments. On the other hand, EG-VEGF transcript was rarely detected in the endometrial samples from the postmenopausal patients and patients with endometrial carcinoma. It may imply that EG-VEGF may only play a role in vascular function of peri-implantation endometrium, but is unlikely to be associated with the etiology of endometrial cancer development. (*Endocrinology* 147: 88–95, 2006)

ENDOCRINE GLAND-DERIVED vascular endothelial growth factor (EG-VEGF) is a newly identified angiogenic and permeability-enhancing factor with a unique target cell selectivity (1). Although EG-VEGF functionally resembles and complements another angiogenic factor, vascular endothelial growth factor (VEGF), to regulate angiogenesis and permeability as well as to induce the formation of endothelial fenestration, the two molecules are structurally dissimilar and work through different receptors. It has been suggested that EG-VEGF may mediate a specific local mechanism, possibly to "fine tune" and complement the action of the ubiquitous VEGF/VEGF receptor system (1).

It is currently known that EG-VEGF, identical to prokineticin 1 (PK-1), is highly expressed in steroidogenic tissues, including the ovary, testis, adrenal gland, and placenta (1–6). It was also detected, but at a moderate level, in other nonsteroidogenic tissues such as human brain, colon, skeletal muscle, small intestine, spleen, thymus, liver, and uterus (7, 8). EG-VEGF/PK-1 was found to bind two closely related G protein-coupled receptors, PK-R1 and PK-R2, and lead to mobilization of calcium, stimulation of phosphoinositide turnover, and activation of p44/p42 MAPK signaling pathways (9). As demonstrated in various animal systems, EG-VEGF possesses different functions in various tissues. It promotes angiogenesis in ovary, adrenal gland (1), and testis (3); causes hyperalgesia of skin; promotes contraction of gastrointestinal smooth muscle; and control behavioral circadian rhythms (10). More recently, these two receptors were also found in human and mouse hematopoietic stem cells and specific mature blood cells, including lymphocytes. It was believed that EG-VEGF modulates growth, survival, and function of cells of the innate and adaptive immune systems, possibly through autocrine or paracrine signaling mechanisms (11).

A recent study from Battersby *et al.* (7) demonstrated that both EG-VEGF and its receptors are expressed in human endometrium. EG-VEGF expression was elevated in the secretory phase of menstrual cycle (7). Here, we have provided the additional information on the expression profile of EG-VEGF and its receptors in human endometrium. We have examined the expression of EG-VEGF and its receptors in the human peri-implantation endometrial samples from 1) the patients of reproductive age with a natural cycle, after the ovulating dose of human chorionic gonadotropin (hCG) in gonadotropin-stimulated cycle, receiving hormone replacement therapy (HRT); 2) patients with endometrial carcinoma; and 3) the endometrial samples from postmenopausal patients. It was shown that EG-VEGF likely possesses a role in the vascular functions of the peri-implantation endometrium, but is unlikely to be associated with the etiology of endometrial cancer development. Subsequent *in vitro* analysis using endometrial cell culture also demonstrated that the steroids are crucial for the EG-VEGF expression. And it

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Abbreviations: EG-VEGF, Endocrine gland-derived VEGF; hCG, human chorionic gonadotropin; hEG-VEGF, human EG-VEGF; HRT, hormone replacement therapy; PK-1, prokineticin 1; VEGF, vascular endothelial growth factor.

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may account for the absence of EG-VEGF in the endometrium of postmenopausal patients.

Materials and Methods

Patients and specimens

Women were recruited from those attending the Department of Obstetrics and Gynecology at the University of Hong Kong for treatment of infertility, endometrial cancer, and total abdominal hysterectomy for benign gynecological conditions. Ethical approval for this study was obtained from Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster, and informed consent was received from each subject.

Endometrial biopsies were taken from 17 infertile women undergoing diagnostic laparoscopy to assess tubal patency. These women had regular menstrual cycles and had not received any antiinflammatory or hormonal medication for at least 3 months before diagnostic laparoscopy. The follicular or luteal phase was based on the day of the last menstrual period and confirmed by the endometrial histology.

Endometrial biopsies were collected from 29 infertile women aged 28–38 yr. Fifteen endometrial biopsies were collected on d 7 after the LH surge in natural cycles and another 15 endometrial samples were taken d 7 (±1) after the ovulating dose of hCG in gonadotropins-stimulated cycles for *in vitro* fertilization treatment, as described previously (12).

Thirteen infertile patients with regular menstrual cycles received either one or three monthly injections of depot GnRH agonist (Decapeptyl, 3.75 mg im, Triptorelin; Ferring Pharmaceuticals Ltd., Berkshire, UK). Standard regimen of HRT (13) was started on the second of the subsequent periods or after the last dose of Decapeptyl. Endometrial biopsy was then taken on the 7th d after progesterone was given.

Endometrial cancer patients were collected from 23 patients before the operation. The average age of patients was 59 yr (ranged from 43–80 yr). The stage of carcinoma was diagnosed according to the criteria of the International Federation of Gynaecology and Obstetrics classification. Histological cell types of tumors were classified according to World Health Organization classification.

Endometrial tissue was obtained from 15 postmenopausal women undergoing total abdominal hysterectomy for benign gynecological disease. Their age ranged from 46–73 yr, with a mean age of 57 yr.

RT-PCR

mRNA was isolated from 100–500 mg of frozen endometrial tissues by TRIzol reagent (Life Technologies, Inc., Rockville, MD) and mRNA purification kit (Amersham, Piscataway, NJ). mRNA (1 μg) was reverse transcribed in a 33-μl reaction system using first-strand cDNA synthesis kit (Amersham) under conditions described by the supplier. Reversed-transcribed cDNA (1 μl) was amplified with specific primers: EG-VEGF (GenBank accession no. AF333024) (forward, 5′-ATGAGAGGTGCCACGCGAGTCTC-3′; reverse, 5′-CCTAAAAATTGATGTTCTTCAAG-3′) for 30 cycles; PK-R1 (GenBank accession no. AY089976) (forward, 5′-GCGGCATTGGAAACTTCA-3′; reverse, 5′-GGCCCACGAATTCTATGCC-3′) for 40 cycles; PK-R2 (GenBank accession no. AF506288) (forward, 5′-CGGCAGCTCTCCTGGGAGCATGGC-3′; reverse, 5′-CGTCTGGAACCCAGGGACTGCC-3′) for 45 cycles; VEGF (GenBank accession no. AB021221) (forward, 5′-GCGAGCCGCGCGCCCCGGTCGGG-3′; reverse, 5′-CAGGAATCCCAGAAATAAAAC-3′) for 35 cycles; and β-actin (forward, 5′-GAATTCATTTTTGAGACCTTCAA-3′; reverse, 5′-CCGGATCCATCTCTGCCTCGAAGTCCA-3′) for 25 cycles. The estimated sizes of RT-PCR products for EG-VEGF, PK-R1, PK-R2, and β-actin were 317, 506, 432, and 306 bp, respectively. VEGF primers were able to detect the two major isoforms of VEGF including VEGF165 and VEGF121 of the expected sizes 944 and 812 bp, respectively. The specificity of these primers was confirmed by sequencing. All the PCR were performed separately and in the linear range of amplification (30–42 cycles). The product bands were then scanned for relative expression by comparing with that of the standard, β-actin.

Immunohistochemistry

Immunohistochemistry study was performed on at least five different tissue samples from each of five stages of the menstrual cycle: early proliferative phase (d 4–7); mid- to late proliferative phase (d 8–14); early secretory (d 15–18), mid-secretory phase (d 19–23), and late secretory phase (d 24–28). Rabbit polyclonal antiserum to human EG-VEGF (hEG-VEGF) was generated against a synthetic peptide of the C terminus of hEG-VEGF (residues 87–105) (Zymed Laboratories, San Francisco, CA). For histological analysis, paraffin sections of endometrial tissues were rehydrated using standard protocols and microwaved for 20 min in 10 mM sodium citrate. Sections were then incubated with anti-hEG-VEGF antibodies (1:100) followed by incubation with secondary antibodies (Calbiochem, San Diego, CA) and mounted with aqueous mounting media (Vector Laboratories, Burlingame, CA).

In situ hybridization

Human endometrial samples were fixed and embedded in paraffin, subsequently sectioned, and mounted on glass slides. The hEG-VEGF containing plasmid was linearized with the appropriate restriction enzymes (1). The [^{35}S]uridine triphosphate-labeled antisense and sense transcripts were generated using T3 and T7 RNA polymerase separately (Promega, Madison, WI). Slides were dewaxed, rehydrated, and hybridized overnight in a humid box (containing 50% formamide, 5× SSC) with a sense or antisense ^{35}S-labeled probe in hybridization solution (50% deionized formamide, 0.3 M NaCl, 5 mM EDTA, 20 mM Tris-HCl, 10% dextran sulfate, 2× Denhardt's buffer, 0.5 mg/ml yeast tRNA, 10 mM dithiothreitol) at 55 C. After a series of washings under stringent conditions, the slides were dehydrated and subsequently dipped into a photographic emulsion (Amersham). The slides covered with emulsion were dried and stored in the dark at 4 C. After 3 d of exposure, the sections were developed.

Cell culture

Primary endometrial cell culture was established using endometrial tissues obtained from the Assisted Reproduction Unit at the University of Hong Kong. In brief, endometrial fragments was digested with 0.25% type I collagenase for 30 min in a shaking water bath at 37 C. The digestate was then filtered through a 40-mm stainless steel sieve. The glands were retained by the sieve and collected after backwashing. The stromal cells passed through the sieve along with some epithelial cells and blood elements. The stromal cell-enriched fraction was further purified using a Percoll gradient together with incubation for 30 min at 37 C in a standard humidified 95% air/5% CO_2 incubator. During this period, the stromal cells attached to polystyrene tissue culture plastic. Conversely, incubation of the glandular epithelial fraction for 30 min at 37 C enables contaminating stromal cells to adhere to the tissue culture plastic surface, whereas purified glands and glandular epithelial cells remain floating in the supernatant. Finally, the isolated glandular epithelial and stromal cells was maintained in DMEM/F12/penicillin/streptomycin medium.

Epithelial and stromal cells were seeded 0.5×10^6 cells per well in the six-well plates and treated with various concentrations of estrogen and progesterone (10^{-10}–10^{-7} M) alone or in combination for 1–3 d. The expression of EG-VEGF mRNA was examined using RT-PCR analysis.

Statistical analysis

Statistical analysis was performed using the SPSS statistics software package (SPSS, Chicago, IL). Statistical comparisons for the EG-VEGF expression in patients were performed with Kruskall-Wallis signed rank test as appropriate. The effects of steroids on EG-VEGF mRNA expression were compared using Student's t test. A P value less than 0.05 was representing statistical significance. Survival distributions were depicted with Kaplan-Meier curves, and Log-rank statistics were used to compare curves.

Results

Differential expression of EG-VEGF and VEGF in human peri-implantation endometrium during natural cycles

The existence of EG-VEGF in the human uterus suggested that it may play a role in the cyclical and/or peri-implantation angiogenesis of the endometrium (7, 8). To elucidate

the potential physiological function(s) of this molecule, we examined the expression of EG-VEGF in 15 normal peri-implantation endometrial samples on d 7 after the LH surge in natural cycles from women aged between 28 and 38 yr. RT-PCR with a specific pair of primers detected EG-VEGF mRNA in 80% of these samples. On the other hand, VEGF-specific primers were able to detect two major forms of VEGF, VEGF121 and VEGF165. Either one or two forms of VEGF transcripts were detected in the samples examined with different intensities (Fig. 1). Subsequent comparative studies on the expression of EG-VEGF and VEGFs among this group of patients showed no significant correlation between these two factors (data not shown).

Expression of EG-VEGF in human endometrium during natural cycle

To examine the temporal expression pattern of EG-VEGF in human endometrium during the menstrual cycle, we used an antibody specific to EG-VEGF to perform the immunohistochemistry (Fig. 2A). EG-VEGF expression in human endometrium was found to be dynamic. It was expressed in both stromal and glandular epithelial cell, but the expression level was minimal at the early follicular phase. Until the late follicular phase, a higher EG-VEGF fluorescence signal was detected, predominantly, at the epithelial cells, but not in the stromal cells. Its expression persisted throughout the early to mid-luteal phase, whereas peak EG-VEGF expression was found at the mid-luteal phase. Finally, the EG-VEGF was much reduced at the late luteal phase.

To further elucidate the hormonal effects on the EG-VEGF mRNA, the expression level of EG-VEGF transcript in the human endometrium was also examined. Seventeen paraffin sections of human endometrial samples taken from patients undergoing laparoscopy, nine during follicular phase; eight during the luteal phase, were subjected to *in situ* hybridization analysis with specific ^{35}S-labeled sense and antisense probes. Unlike protein, EG-VEGF transcript was expressed at a comparable level in the stromal and the glandular epithelial cells and showed no obvious difference in the mRNA expression level between these two cell types. Consistently, a stronger hybridization signal was detected in the endometrial samples during the luteal phase than those from the follicular phase of menstrual cycle (Fig. 2B).

Up-regulated EG-VEGF expression in human peri-implantation endometrium during the stimulated cycle

Ovarian steroids are thought to restore the endometrial vasculature shed during menstruation by elevating the an-

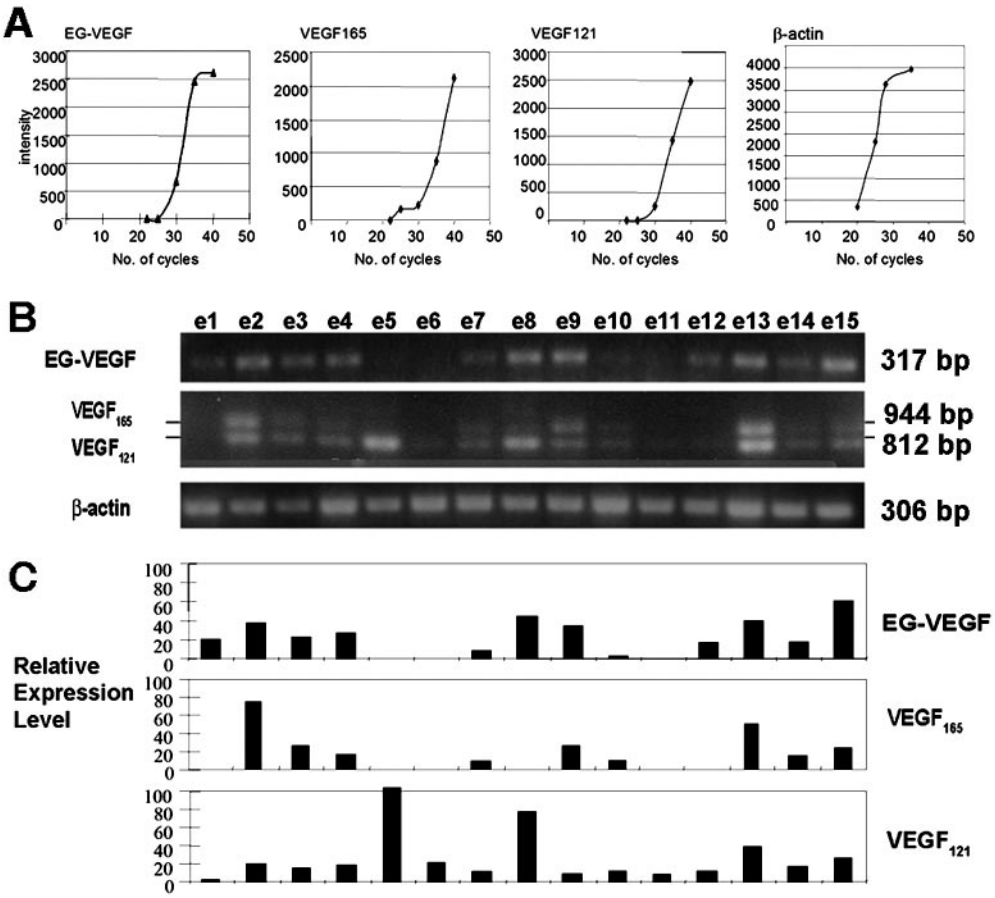

FIG. 1. Expression of EG-VEGF and VEGF mRNA in the human peri-implantation endometrium. RT-PCR analysis: A, graphs show that the 30, 35, and 25 cycles used for EG-VEGF/VEGFs and β-actin are in the linear range of amplification. B, PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide, and photographed. C, The *bar charts* represent the data from the densitometric analysis of the EG-VEGF and VEGF relative to β-actin expression. (the *bars higher than the bottom line* were considered as positive).

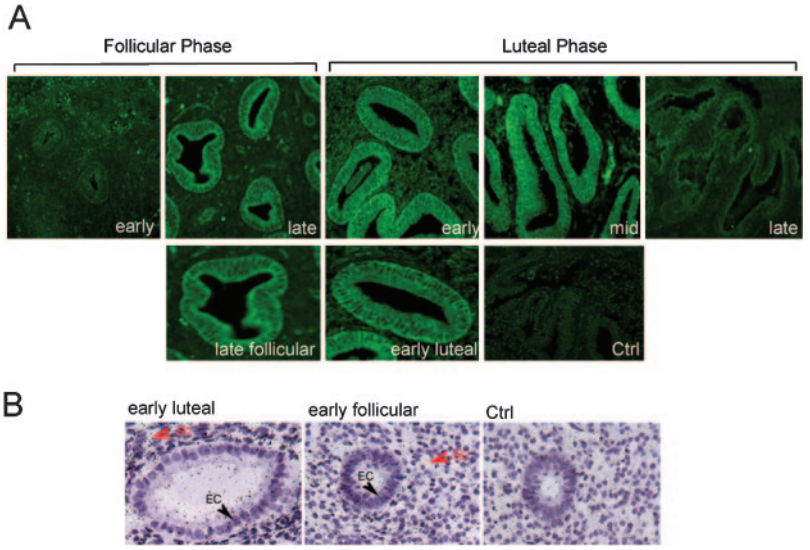

FIG. 2. A, Immunohistochemistry analysis of EG-VEGF expression in human endometrium during menstrual cycle. Sections of human endometrium from five different phases of menstrual cycle, including early and late follicular phase and early, mid-, and late luteal phase, were stained with anti-EG-VEGF antibody. EG-VEGF-positive signals visualized with FITC (*green*) (*upper panel*). With higher magnification, immunosignals are predominantly detected in glandular epithelial (*lower panel*). The immunosignals are specific because no signal was detected when the section was incubated with the peptide preabsorbed anti-EG-VEGF antibody (Ctrl). *B*, *In situ* hybridization analysis of EG-VEGF expression in human endometrium. Representative figures from the *in situ* hybridization analysis with human endometrium samples at luteal phase and follicular phases using ^{35}S-labeled antisense and sense (Ctrl) probes. Consistently, higher expression levels of EG-VEGF RNA were detected as *black* precipitates in both the stromal cells (SC) and epithelial cells (EC) of the samples at luteal phase than those at the follicular phase of menstrual cycle.

giogenic factor such as VEGF. To initially demonstrate the hormone responsiveness of EG-VEGF expression, we have examined 15 peri-implantation endometrial samples taken on d 7 (±1) after the ovulating dose of hCG in gonadotropin-stimulated cycles for *in vitro* fertilization treatment. RT-PCR analysis showed that EG-VEGF mRNA was detected in all these samples (100%). In addition, an up-regulation of the expression level of EG-VEGF mRNA was observed in this group of samples when compared with those from natural cycles (Fig. 3, A and C; $P < 0.05$, Kruskall-Wallis test).

Using the RT-PCR, we have also examined another 13 endometrial samples from the patients receiving HRT taken on d 21 of the artificial cycle, *i.e.* the 7th d after progesterone was started. Similarly, all the samples examined showed the high level of EG-VEGF expression when compared with those from natural cycle (Fig. 3, B and C; $P < 0.05$, Kruskall-Wallis test). Taken together, the patients receiving hormonal treatments always exhibited high level expression of EG-VEGF in their endometrium when compared with untreated group.

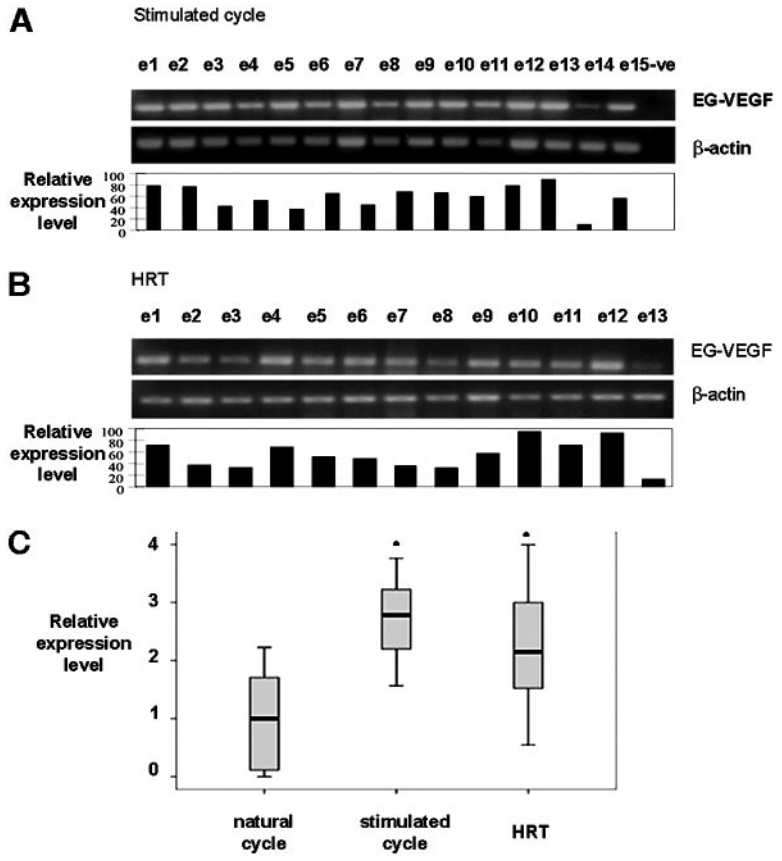

FIG. 3. Expression of EG-VEGF mRNA in the human peri-implantation endometrium from patients given hCG and receiving HRT. A, EG-VEGF expression analysis using semiquantitative RT-PCR with endometrial samples from patients taken on d 7 (±1) after the patients given ovulating dose of hCG in stimulated cycles. β-Actin was used as the standard. The *bar chart* represents the data from the densitometric analysis of the EG-VEGF relative to β-actin expression. B, RT-PCR analysis of endometrial biopsy taken on d 21 of the artificial cycle, *i.e.* the 7th d after progesterone is started, from patients receiving HRT. Again, β-actin was used as the standard. The *bar chart* represents the data from the densitometric analysis of the EG-VEGF relative to β-actin expression. C, Kruskall-Wallis signed rank test was performed and showed significant difference on the expression of EG-VEGF in natural cycle, stimulated cycle, and HRT groups (*, $P < 0.001$).

Hormone dependency of EG-VEGF expression in the in vitro endometrial cell culture

In an attempt to directly demonstrate hormone-dependency of EG-VEGF expression, a primary endometrial cell culture was established. As illustrated in Fig. 4, EG-VEGF mRNA was detected in both glandular epithelial cells (EC) and stromal cells (SC) isolated from the human endometrial biopsies. Additionally, we found that EG-VEGF mRNA was only detected in primary cells cultured in the presence of steroids (4×10^{-10} M estrogen and 5×10^{-8} M progesterone), confirming the hormone-dependent expression of EG-VEGF (Fig. 4, B and C, *left panel*). Consistently, endometrial stromal and glandular epithelial cells showed similar response to the steroid stimulation. Estrogen or progesterone alone, with concentration of 10^{-9} M or more, was sufficient to induce the EG-VEGF mRNA expression in both endometrial stromal and glandular epithelial cells (Fig. 4, B and C, *middle and right panels*).

Existence of EG-VEGF receptors (hPK-R1 and hPK-R2) in human endometrium

It was demonstrated that EG-VEGF activates p44/p42 MAPK pathway by interacting with two G protein-coupled receptors, prokineticin receptors 1 and 2 (hPK-R1 and hPK-R2), to induce angiogenesis. Thus, the expression of hPK receptors in the human endometrium was also examined using RT-PCR. As shown in Fig. 5A, hPK-R1 and hPK-R2 mRNAs are differentially expressed in 80% (12 of 15) of human peri-implantation endometrium samples from patients of natural cycle. Consistently, weaker hPK-R2 bands were detected in these endometrial samples, possibly due to the lower expression level of hPK-R2 in endometrium as shown in previous study (7). hPK-R1 and hPK-R2 transcripts were also detected in the patients after the ovulating dose of hCG in gonadotropin-stimulated cycle (hPK-R1: 26% (four of 15) and hPK-R2: 53% (eight of 15); Fig. 5B); and also the patients receiving HRT (seven of 13, 53% for hPK-R1 and 10 of 13, 76% for hPK-R2) (Fig. 5C). Subsequent statistical analyses using Kruskall-Wallis signed rank test, showed that there is no significant difference on the expressions of hPK-R1 and hPK-R2 in these three groups of patients ($P > 0.05$) (Fig. 5D).

Loss of EG-VEGF transcript in the human endometrial carcinoma

VEGF has been implicated as a major angiogenic factor in endometrial cancer (14). To elucidate the potential role of EG-VEGF on the etiology of endometrial cancer, we have examined the expression of EG-VEGF in the endometrial cancer samples. The samples were obtained from a total of 23 women including tumors with different stages (19 at early stage and four at late stage). Surprisingly, EG-VEGF mRNA was not detected in the majority of patient samples (21 of 23; 92%) independent of the stage of carcinoma (Fig. 6, *upper panel*). In addition, 15 "normal" endometrial samples from postmenopausal patients were also examined as age-matched control (Fig. 6, *lower panel*). Again, the EG-VEGF transcript was not detected in most of these controls.

To assess whether the expression of EG-VEGF could predict clinical outcome, these 23 patient specimens with complete clinical response to chemotherapy and documented relapse were analyzed. There was no correlation between the

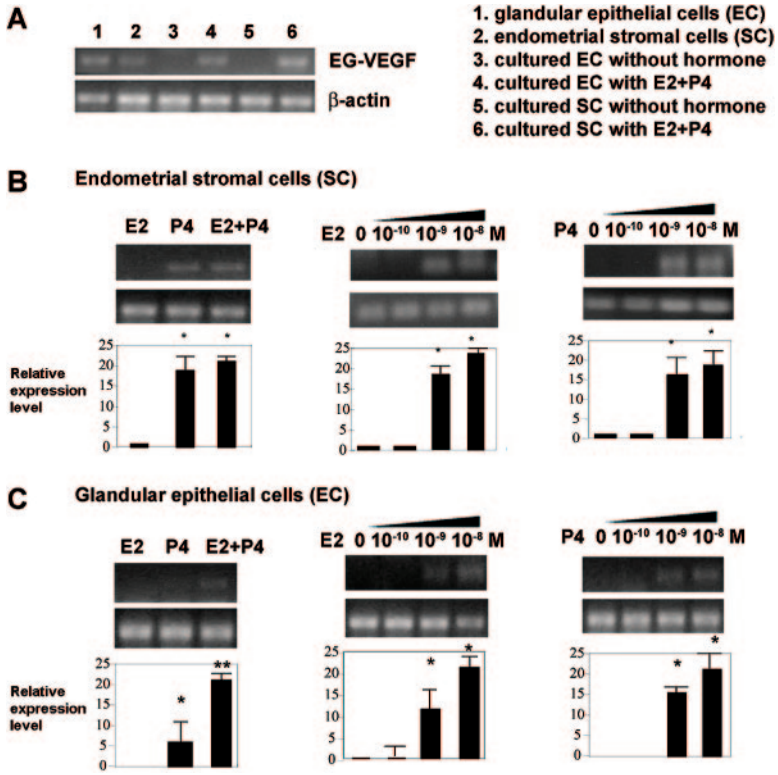

FIG. 4. The hormone-dependent expression of EG-VEGF in the human endometrial cell culture. A, RT-PCR analysis on the EG-VEGF mRNA expression in the (1) isolated endometrial glandular epithelial cell (EC) and (2) stromal cell (SC) and cultured endometrial cells in presence (lanes 4 and 6) or absence (lanes 3 and 5) of physiological dose of estrogen and progesterone (E2, 4×10^{-10} M and P4, 5×10^{-8} M). The hormone-dependent expressions of EG-VEGF mRNA in the cultured endometrial stromal (B) and glandular epithelial (C) cells were analyzed using RT-PCR. *Left*, The cells treated with physiological doses of E2 (4×10^{-10} M), P4 (5×10^{-8} M), or E2 plus P4 (E2, 4×10^{-10} M and P4, 5×10^{-8} M). Cells treated with increasing concentrations of estrogen (*middle*) (10^{-10}–10^{-8} M) and progesterone (*right*) (10^{-10}–10^{-8} M). The *bar charts* represent the data from the densitometric analysis of the EG-VEGF relative to β-actin expression. The value reported in the *bar charts* represent the mean ± SEM of four independent PCR from two patients. *Bars* bearing an *asterisk* (*) and *two asterisks* (**) are statistically different (by Student's *t* test, $P < 0.05$)

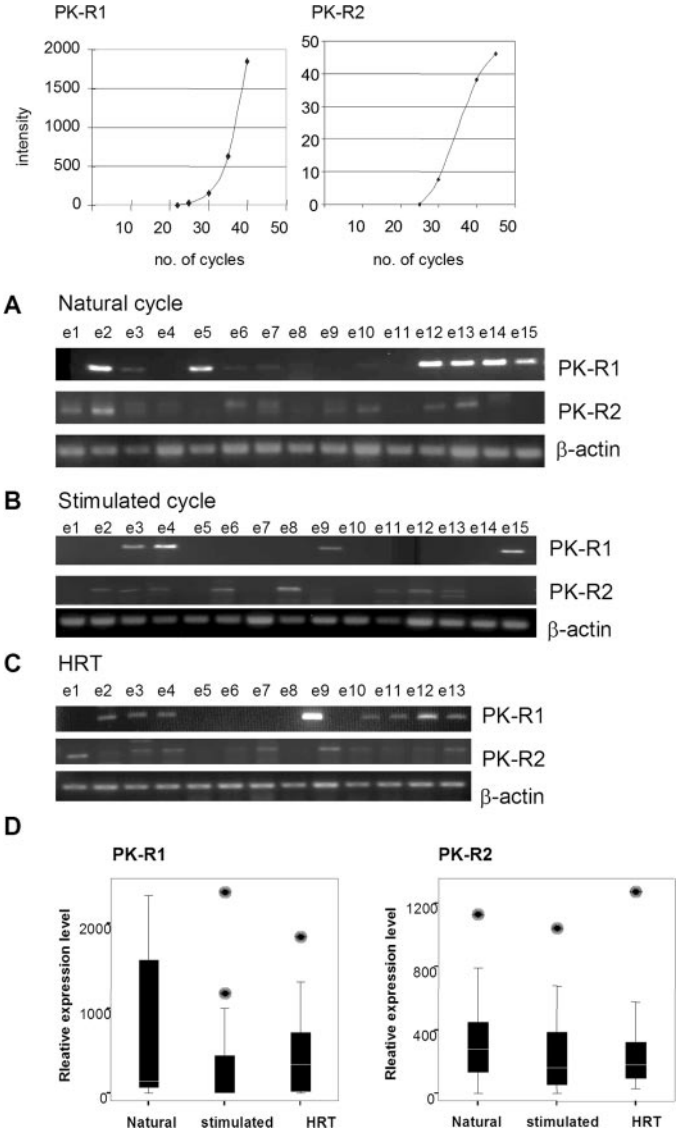

FIG. 5. Presence of PK-R1 and PK-R2 in human endometrium. mRNA was prepared from human endometrial samples and reverse transcribed and amplified with PK-R1, PK-R2, and β-actin primers, respectively. PCR products were electrophoresed, stained with ethidium bromide, and photographed. Human peri-implantation endometrium from patients of natural cycle (A), stimulated cycle (B) (given hCG), and receiving HRT (C). D, Kruskall-Wallis signed rank test was performed, and it showed that there is no significant difference on the expression of hPK-R1 and hPK-R2 in natural cycle, stimulated cycle, and HRT groups ($P > 0.05$).

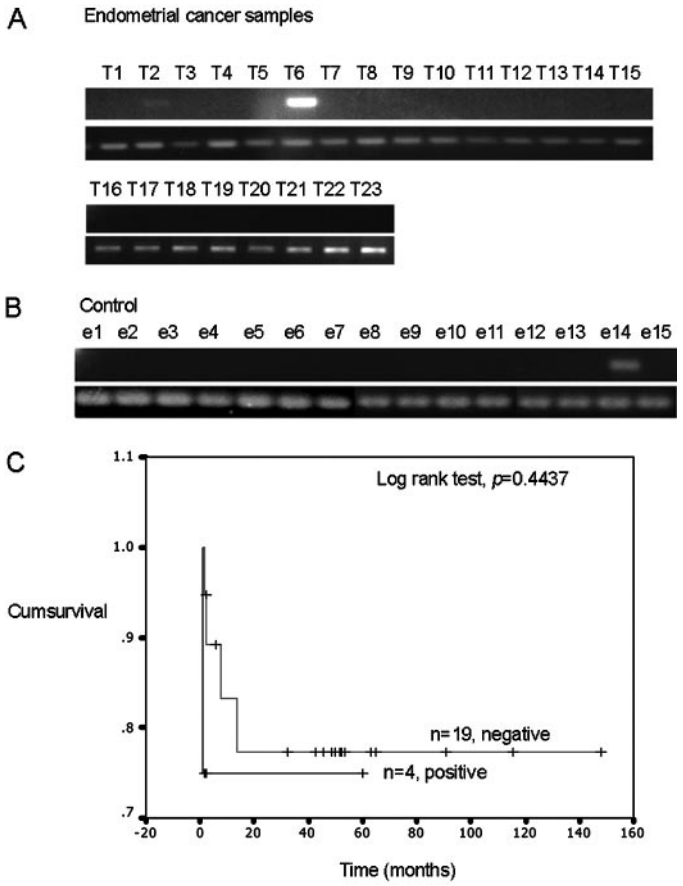

FIG. 6. EG-VEGF mRNA is down-regulated in endometrial carcinoma. EG-VEGF expression analysis was conducted using semiquantitative RT-PCR with endometrial samples from patients with endometrial carcinoma (A) and age-matched control (B). C, EG-VEGF mRNA expression does not correlate with patient outcome in endometrial cancer. A total of 23 endometrial cancer specimens with complete clinical response to chemotherapy and detailed follow-up information were used for outcome analysis (Log rank test, $P = 0.4437$).

patient outcome and the expression of EG-VEGF transcript (Fig. 6B; Log rank test, $P = 0.4437$). Taken together, EG-VEGF expression was not associated with the development of the endometrial carcinoma. Unlike VEGF, EG-VEGF was only expressed in the endometrium of the patients of the reproductive age.

Discussion

The present study is to examine the expression of EG-VEGF in the human peri-implantation endometrium. We have demonstrated that EG-VEGF is expressed in the human peri-implantation endometrium during a natural cycle and its expression shows no direct correlation with that of the VEGF (15). This finding is consistent with the previous finding in the ovary (6). VEGF and EG-VEGF were found to be differentially expressed and regulated, although both act as an angiogenic factor in the ovary. *In vitro* studies using the immortalized human granulosa-lutein cells immortalized with simian virus 40 large T antigen showed that EG-VEGF and VEGF have opposite response to two common angiogenic inducers causing chemical hypoxia: $CoCl_2$ and desferrioxamine mesylate. EG-VEGF mRNA, in contrast to VEGF, was markedly reduced by hypoxia (16). In addition, *in situ* hybridization studies by Ferrara and colleagues (6, 17) also showed that EG-VEGF expression in normal ovaries is dynamic and generally complementary to VEGF expression in both follicles and corpora lutea. Interestingly, unlike in ovary, we found that these two angiogenic factors exhibit similar spatial and temporal (mRNA and protein) expression patterns in endometrium across the menstrual cycle (15). Our *in situ* hybridization and *in vitro* data consistently showed that both stromal and glandular epithelial cells express EG-VEGF transcript and its expression in these two cell types is

highly dependent on the steroids. In addition, our immunohistochemistry data also indicated that the peak protein expressions of these two factors are at mid-luteal phase of the menstrual cycle and both are predominantly expressed in the endothelial glandular epithelium (Fig. 2). The low protein expression of EG-VEGF in stromal cells may be due to the low protein stability in stromal cells. Taken together, it is believed that EG-VEGF may not be functionally complementary to VEGF in human endometrium. However, the existence of EG-VEGF, at least, may permit an additional layer of signaling refinement to establish and maintain the differentiated endothelial structure and functions of the tissues.

The coexistence of EG-VEGF/PK-1 receptors in human endometrium further supports the notion that EG-VEGF may directly act on human endometrium as a mitogen and/or angiogenic factor. Consistent with the previous work done by Battersby *et al.* (7), our RT-PCR results also showed that these two types of receptors are expressed, but at different levels and pattern, in human endometrium. As a matter of fact, a distinct pattern of PK receptor expression was also observed in various tissues. In the adrenal gland, only hPK-R1 was detected (18). In the ovary, follicular cells predominantly express PK-R1, whereas corpus luteum-derived cells express high levels of both PK-R1 and PK-R2 (16). Therefore, it is believed EG-VEGF may have different angiogenic as well as nonangiogenic functions in these tissues via acting on either PK-R1 or PK-R2. However, the exact role(s) of PK-R1 and PK-R2 remains unclear. The coexistence of these two receptors may imply that EG-VEGF possesses various functions in the human endometrium. Regarding the effect of the steroids on receptors, there is no obvious difference on the expression of these two receptors in patients, of reproductive age, with a natural cycle; and receiving HRT. However, these two receptors seem to be slightly (but not significant) down-regulated in the patients after receiving the ovulating dose of hCG in gonadotropin-stimulated cycle. One possible explanation on this reduction may be due to the negative feedback of persistent high level of EG-VEGF expression. Nevertheless, the steroid effect on the expression of the receptors is less obvious than that of EG-VEGF.

Increasing evidence showed that EG-VEGF exhibits different physiological functions in steroidogenic and nonsteroidogenic tissues. Similarly, it seems to play different roles in the etiology of various diseases. EG-VEGF has been associated in the development of colorectal cancer. *In vivo* experiments demonstrated that EG-VEGF overexpressing human colorectal cancer cell line (SW620) significantly induces the angiogenesis and tumor proliferation/metastasis after implanted into cecum and sc (19). In contrast, our data showed that EG-VEGF mRNA is not present in most of the endometrial cancer (Fig. 6), and is unlikely to be involved in the development of endometrial carcinoma. A similar observation was found in ovarian carcinoma (8). A significantly lower amount of EG-VEGF mRNA was detected in benign, low malignant potential neoplasms or stage I ovarian cancer when compared with the normal ovarian tissues. Interestingly, a further decrease in the late-stage carcinomas when compared with early stage carcinomas (8) was observed. Therefore, EG-VEGF is likely playing diverse roles in various tissues and diseases. The underlying mechanism(s) for the absence of EG-VEGF in endometrial and ovarian cancer is still not clear.

In most assisted reproduction programs, gonadotropins are given to stimulate the development of multiple oocytes so that multiple embryos can be transferred to enhance the success rate. However, we have previously demonstrated that the supraphysiological hormonal concentrations resulting from gonadotropin stimulation could significantly reduce the implantation and pregnancy rates in patients with serum estradiol concentration of ≥20,000 pmol/liter (excessive responders) (13). The low implantation rate is likely the result of the gland-stromal dyssynchrony of the peri-implantation endometrium (12). In the natural cycle, the endometrial glands were either more tortuous or numerous at the presumed time of implantation. There appears to be a trend toward a decline in the glandular volume fraction and glandular diameter with the rise in the serum estradiol concentrations. More importantly, significant differences in the glandular and stromal components in these high responders were associated with enhanced vessel development (12). Thus, we believed that the marked increase in stromal vessels leads to in advanced stromal maturation and gland-stromal dyssynchrony. Because EG-VEGF expression is highly dependent on the ovarian steroids, it would be interesting to investigate the possible involvement of EG-VEGF in the estradiol induced gland-stromal dyssynchrony in peri-implantation endometrium. The data from this study could give useful information on the correlation of the EG-VEGF expression and the stromal vessels development.

Taken together, we have provided additional data complementary to a recent study by Battersby *et al.* (7). We demonstrated that 1) EG-VEGF is expressed in the human peri-implantation endometrium; 2) the expression of EG-VEGF, but not the receptors, is hormone dependent; and 3) its expression is not associated with the development of the endometrial carcinoma. Unlike VEGF, EG-VEGF is only expressed in the endometrium of the patients of the reproductive age. Thus, we believe this novel angiogenic factor and its receptors would play a significant role in peri-implantation endometrium through autocrine/paracrine signaling mechanisms.

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