Estrogen and progesterone play pivotal roles in endothelial progenitor cell proliferation

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Abstract

Background: It has been previously suggested that angiogenesis occurs during the menstrual cycle. Moreover, a rise in uterine blood flow is largely maintained by vasodilatation and substantial increases in angiogenesis. It is known that estradiol (E2) and progesterone (P4) are involved in angiogenesis. Recently, endothelial progenitor cells (EPCs) were found to be involved in neovascularization; however, their roles in uterine neovascularization have not been well characterized. We hypothesized that E2- or P4-mediated EPC proliferation plays important roles in uterine neovascularization during the menstrual cycle.

Methods: The number of EPCs in peripheral blood from subjects in the menstrual phase (n = 12), follicular phase (n = 8), and luteal phase (n = 16), was measured using flow cytometry. Peripheral blood mononuclear cells (PBMCs) were cultured for seven days with or without 17beta-estradiol (E2beta) or P4, followed by assessment of EPC proliferation based upon the uptake of acetylated low density lipoprotein (LDL) and lectin. The expression of estrogen receptor (ER) or progesterone receptor (PR) in EPCs was also evaluated using real-time PCR.

Results: E2beta and P4 significantly increased the proliferation of EPCs derived from the peripheral blood of subjects in menstrual phase, but not subjects in the luteal phase. In addition, the expression level of ERalpha was markedly higher than ERbeta in EPCs derived from women in menstrual phase.

Conclusions: EPC proliferation is induced during the menstrual phase and proliferation can be affected by estrogen through ERalpha activation.

Keywords: menstrual cycle, neovascularization, ovarian hormones

Background

Angiogenesis in female reproductive organs, including the uterus, corpus luteum, and placenta, is essential for implantation and is critical for the dramatic (30-50 fold) elevation of uterine blood flow during pregnancy [1,2]. Disturbances in uterine vascular development are associated with pregnancy loss, preeclampsia, and intrauterine growth restriction [3]. Periodic uterine endometrial neovascularization begins after menstruation and continues into the luteal phase [4]. In general, it is thought that neovascularization is mainly caused by angiogenesis, which is the sprouting of capillaries from pre-existing vessels, such as in tumors and embryos. However, vasculogenesis, which is mediated by endothelial progenitor cells (EPCs), has recently been proposed to be involved in endometrial neovascularization [5,6]. The presence of EPCs in peripheral blood provides a maintenance reservoir of endothelial cells (ECs) and contributes to up to 25% of ECs in newly formed vessels [7]. It has been hypothesized that EPCs may be involved in the growth of the uterine endometrium since EPCs localize within the vasculature and stroma of the uterine endometrium and myometrium after ovulation [8].

In female reproductive organs, neovascularization may be partially regulated by cyclic changes in sex steroids, such as estradiol (E2) and progesterone (P4) [9,10] in response to the hormones produced by the hypothalamus, pituitary gland, and ovaries [11]. Serum E2 and P4 concentrations are very low during the early follicular phase [12]. During this phase, serum estrogen levels rise in parallel to the growth of follicle size and granulosa cells. Before ovulation, follicle-produced E2 is increased...
and luteinization of the granulosa cells is stimulated, which leads to the synthesis of P₄. After ovulation, the granulosa cells continue to enlarge and become lutein. The corpus luteum, which consists of luteinized granulosa cells and theca-lutein cells, secretes P₄. There is a secondary rise in E₂ levels during the mid-luteal phase following a decrease before menstruation, and this rise in E₂ levels occurs in parallel with the rise of serum P₄ levels. However, the effects of these hormones and their interactions in reproductive organs remain unclear, especially in vasculogenesis.

Estrogen receptors (ERs) are expressed in uterine arterial ECs [13,14], as well as in other types of ECs [15], which suggests that 17β-estradiol (Eβ) can act directly on the cells and alter uterine vascular function. It has been reported that Eβ can enhance angiogenesis [16,17] and vasculogenesis by increasing the number of EPCs [18]. These data support the theory that Eβ is an important factor in promoting neovascularization in female reproductive organs. Similar to Eβ, P₄ has also been shown to influence uterine angiogenesis [19]. P₄ may enhance Eβ-induced angiogenesis by increasing endothelial nitric oxide synthase (eNOS) expression [20]. In contrast, it has been reported that P₄ inhibits Eβ-reduced neointimal proliferation [21] and decreases ER and P₄ receptor (PR) expression in human uterine vascular endothelium [22], which consequently attenuates Eβ-induced angiogenic responses [23,24].

The aim of this study was to evaluate the influence of sex steroids on the proliferation of EPCs during the menstrual cycle. The findings of this study could help elucidate the role of vasculogenesis in cyclic endometrial neovascularization.

**Methods**

**EPC isolation**

This study was approved by the Ehime University Institutional Review Board. All participants gave informed consent for participation in this study. Peripheral venous blood (20 ml) from healthy young volunteers in the menstrual phase (n = 12; 30 ± 4 years-old), follicular phase (n = 8; 30 ± 5 years-old), luteal phase (n = 16; 28 ± 4 years-old) with regular menstrual cycles (24-35 d) were analyzed according to a previously described technique [25]. Briefly, PBMCs (8 × 10⁵ cells) were seeded into each well of 96-well culture plates coated with human fibronectin (Sigma-Aldrich, St. Louis, MO) and cultured in endothelial basal medium-2 (EBM-2, Clonetics; San Diego, CA) supplemented with EGM-2MV (Clonetics) consisting of 5% FBS, VEGF, fibroblast growth factor-2 (FGF2), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF1), and ascorbic acid. After four days in culture, non-adherent cells were removed and adherent cells were subsequently cultured for an additional three days.

**Low density lipoprotein (LDL)/lectin assay**

After seven days in culture, adherent cells were assessed as EPCs based on the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated LDL (DiL-Ac-LDL; Biogenesis; Poole, UK) and FITC-labeled Ulex europaeus agglutinin I (FITC-lectin, Sigma-Aldrich). Cells were incubated with DiL-Ac-LDL (2.4 μg/ml) for one hour at 37°C and fixed with 2% paraformaldehyde for 10 min. Cells were washed twice with DPBS and then incubated with FITC-lectin (10 μg/ml) for one hour at 37°C. Cells were washed twice with DPBS and detached with 0.25% trypsin/EDTA.

**FACS analysis**

Before culturing the cells, the cEPCs were confirmed by FACS with a PE-labeled monoclonal antibody against human KDR (Genzyme Technne; Cambridge, MA), a FITC-labeled monoclonal antibody against human AC133 (Genzyme Technne), and a PerCP-labeled monoclonal antibody against human CD34 (Becton Dickinson). Then, 1.0 × 10⁵ PBMCs were incubated with the three antibodies for 30 min at 4°C in the dark. Isotype antibodies were used as controls. After incubation, cells were analyzed with a FACScan flow cytometer (Becton Dickinson) and Cell Quest software (Becton Dickinson) (Figure 1). On the 7th day of culture, adherent cells were incubated with DiL-AcLDL and FITC-lectin, and double-stained cells were analyzed by FACS (Figure 2).

**EPC culture with Eβ or P₄**

To assess the effects of Eβ or P₄ on EPC proliferation of EPCs, 8 × 10⁵ PBMCs, derived from the peripheral
blood of women in the menstrual or luteal phases, were seeded into each well of 96-well culture plates coated with human fibronectin (Sigma-Aldrich), and cultured in endothelial basal medium (phenol red free EBM-2, Clonetics; San Diego, CA) supplemented with EGM-2MV (Clonetics) consisting of 5% charcoal stripped serum (Invitrogen), VEGF, FGF2, EGF, IGF1, and ascorbic acid with or without 10^-9-10^-7 Mo fE or P4. ICI 182,780 (10^-5 M, Wako Pure Chemical Industries, Ltd., Osaka, Japan) or RU486 (10^-5 M, Sigma-Aldrich) was used as an inhibitor of ER or PR, respectively [26]. After four days in culture, nonadherent cells were removed and adherent cells were subsequently cultured for an additional three days.

**Isolation of RNA and semi-quantitative RT-PCR**

Total RNA was collected from cells grown in 3.5 cm cell culture dishes. Cells were washed with cold DPBS and subsequently frozen at -80°C until isolation of total RNA. The cells were disrupted and homogenized with an ultrasonic homogenizer. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN; Valencia, CA) according to the manufacturer’s instructions. Total RNA (2 μg) was reverse transcribed to cDNA by incubating the samples with a random primer (100 pmol/μl) at 70°C for 10 min, followed by 4°C for 10 min. The samples were then incubated with Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 2.5 mM dNTPs, ribonuclease inhibitor (40 U/μl, Promega Corp. Madison, WI), AMV reverse transcriptase XL (37 U/μl, Takara Biochemicals Tokyo, Japan), and a dNTP mixture (2.5 mM) and then incubated at 42°C for one hour. The samples were subsequently heated at 99°C for 5 min to terminate the reaction, and stored at 4°C.

ERα, ERβ, progesterone receptor AB (PR AB), progesterone receptor B (PR B), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide primers were constructed from published nucleotide sequence databases (Table 1). The level of GAPDH mRNA served as an internal standard for normalization of ER and PR mRNA levels. RT-PCR conditions were optimized to ensure that amplification proceeded within the linear portion of the reaction. The semi-quantitative RT-PCR amplification profile consisted of denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 10 s and 65°C for 1 min.

The iQ SYBR Green Supermix (QIAGEN), 0.2 μM of sense and antisense oligonucleotides (ER, PR, or GAPDH), and 0.1 μg cDNA were used in a final volume of 30 μl for each RT-PCR reaction. The PCR products were digitally analyzed using a luminescent image analyzer (FluoImager, Beckton Dickinson) and quantified using Image Quant software (Beckton Dickinson).

**Statistical analysis**

The results were expressed as mean ± standard error (SE). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by a post-hoc test (Bonferroni). Statistical significance was determined as p < 0.05.

**Results**

**Detection of cEPCs in peripheral blood during the menstrual cycle**

The number of cEPCs in the peripheral blood obtained from subjects in the menstrual cycle was significantly
increased during the luteal (124.0 ± 29.1 cells/ml) and menstrual (165.3 ± 38.5 cells/ml) phases compared to the follicular phase (44.1 ± 11.1 cells/ml; \( p < 0.05 \); Figure 3A).

**LDL/Lectin assay of cells cultured for seven days**

The number of EPCs that were LDL⁺/lectin⁺ derived from the peripheral blood of subjects in the luteal phase was significantly increased on the seventh day of culture (481.2 ± 91.2 cells/well) compared to the menstrual phase (227.1 ± 26.5 cells/well; \( p < 0.05 \); Figure 3B).

**Effects of E₂β and P₄ on the proliferation of EPCs in peripheral blood**

E₂β or P₄ significantly increased the proliferation of EPCs derived from the peripheral blood of subjects in the menstrual phase by activating the respective receptors in a dose-dependent manner (Figure 4). Receptor antagonists of ER and PR, ICI 182,780 and RU 486, respectively, reduced the proliferation of EPCs from menstrual phase. In addition, P₄ did not influence the effect of E₂β on EPC proliferation. In contrast, the proliferation of EPCs from luteal phase was not influenced by E₂β or P₄ (Figure 5). Moreover, these receptor antagonists did not affect the proliferation of EPCs from luteal phase.

**Change in ER and PR expression in cEPCs**

ERα mRNA expression levels in EPCs from menstrual phase were higher than ERβ mRNA as well as ERα mRNA in EPCs from luteal phase (\( p < 0.05 \); Figure 6). In contrast, no significant difference was observed between the expression level of total PR mRNA and isoform B mRNA in EPCs; however, the expression levels of total PR mRNA and isoform B mRNA were limited in EPCs (Figure 6).

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**Table 1 List of oligonucleotide primers used.**

| GeneBank accession No. | mRNA    | Sequence                                                                 | Length of DNA product (bp) |
|------------------------|---------|--------------------------------------------------------------------------|-----------------------------|
| NM000125               | ERα     | 5′ AAG AGC TGC CAG GCC TGC C 3′ (sense)                                  | 168                         |
|                        |         | 5′ TTG GCA GCT TCA ATG TCT CC 3′ (antisense)                             |                             |
| AB006590.1             | ERβ     | 5′ TAA AAG CAT TCA AGG ACA TAA T 3′ (sense)                              | 160                         |
|                        |         | 5′ GCA GTT CTC TCT ATT C 3′ (antisense)                                  |                             |
| AY382151               | PR AB   | 5′ TGG AAG AAA TGA CGT CAT CG 3′ (sense)                                 | 196                         |
|                        |         | 5′ TAG GGC TTG GCT TCT ATT TG 3′ (antisense)                             |                             |
| AB085583               | PR B    | 5′ ACA CTT TGC CGT AAC TTT CG 3′ (sense)                                 | 196                         |
|                        |         | 5′ CTG TCC TTT GGG GGA CT 3′ (antisense)                                 |                             |
| NM0002046              | GAPDH   | 5′ CCA CCC ATG GCA AAT TCC ATG GCA 3′ (sense)                            | 622                         |
|                        |         | 5′ TCT AGG CAG GTC AGG ACC 3′ (antisense)                                |                             |

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**Figure 3** Changes in circulatory EPC proliferation during the menstrual cycle.

A) The number of EPCs in peripheral blood. \( *: p < 0.05 \) vs. the follicular period.

B) The proliferation of EPCs on the 7th day in culture. \( **: p < 0.05 \) vs. the luteal period.

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**Matsubara and Matsubara Reproductive Biology and Endocrinology 2012, 10:2**

http://www.rbej.com/content/10/1/2

Page 4 of 8
Discussion
cEPCs in the peripheral blood serve as a reserve for EC maintenance, and contribute up to 25% of ECs in newly formed vessels of ischemic lesions [25,27-32]. In addition, estrogen preserves ischemic heart function during myocardial infarction by augmenting the mobilization and incorporation of bone marrow-derived EPCs into sites of neovascularization [31]. In female reproductive organs, EPCs appear to play a crucial role in vascularization of the uterine endometrium at the site of embryo attachment.

![Figure 4](image1.png)

**Figure 4** Effects of E$_2$ or P$_4$ on the proliferation of EPCs from menstrual phase. A) The effect of E$_2$ on the proliferation of EPCs. B) The effect of P$_4$ on the proliferation of EPCs. *: p<0.05 vs. control. #: p<0.05 vs. cells treated with RU486.

![Figure 5](image2.png)

**Figure 5** The effect of E$_2$ or P$_4$ on the proliferation of EPCs from luteal phase. A) The effect of E$_2$ on the proliferation of EPCs. B) The effect of P$_4$ on the proliferation of EPCs.
implantation and placentation [33-35]. During the menstrual cycle, the number of cEPCs in peripheral blood exhibits a cyclic change, whereby it is markedly increased during the luteal and menstrual phase, and decreased during the follicular phase. The number of cEPCs has been shown to decrease over the course of pregnancy [36]. Since EPCs were also found in the corpus luteum and uterine endometrium, the estrogen-mediated increase in EPCs is thought to be involved in periodic neovascularization during the menstrual cycle [28,37,38].

The differences in EPC proliferation and cEPC concentration between the menstrual and luteal phases were the most important findings of this study. We demonstrated that EPCs from menstrual phase did not proliferate after seven days in culture; however, there was a dose-dependent increase in proliferation after cells were treated with E2β or P4. On the other hand, proliferation was increased in EPCs from luteal phase after seven days in culture; however, the proliferation was not influenced by addition of E2β or P4. Therefore, we hypothesize that luteal EPCs may have already reached maximum stimulation in vivo and could not be activated further.

Decreased proliferation of EPCs from menstrual phase suggests that this observed effect was due to decreased concentration of E2β or P4 during menstruation. Therefore, the increase in proliferation was a sex steroid-mediated dose-dependent increase. Since the expression of ERα and ERβ in EPCs was different between the menstrual and luteal phases, E2β-induced neovascularization may be mediated by both receptors, and the differential expression of ERα and ERβ indicates that they have different effects on neovascularization. ERα mRNA expression levels in EPCs from menstrual phase was higher than those from luteal phase as shown in Figure 6. On the other hand, the ERβ mRNA expression level in EPCs from luteal phase was higher than ERα expression. Since estrogen-responsive element-dependent gene transcription activities are severely impaired in EPCs obtained from ERα-knockout mice, vascular growth is down-regulated in ERα-knockout EPCs [39,40], and epithelial cell proliferation can be reduced through the activation of ERβ [30], the increased expression of ERα may at least partially explain the increase in E2β-induced proliferation of EPCs derived from subjects in the menstrual phase. However, the roles of ERα and ERβ in EPC biology during the menstrual cycle will require further elucidation.

The cEPC concentration was lowest in the follicular phase, and EPC proliferation in the cultures was not different compared to the other cultures. It has been well established that the serum concentration of E2 and VEGF is very low during the early follicular phase [12,41]. Therefore, it is possible that decreased VEGF and E2 expression during the follicular phase could reduce the recruitment of cells from bone marrow into blood, since VEGF can induce the recruitment of EPCs from bone marrow [42]. These observations suggest that the initial priming of the ovarian hormone is required for EPC proliferation during the menstrual cycle.

Estrogen also stimulates extensive placental neovascularization through the up-regulation of angiogenic factors in the uterus during pregnancy [43], and the number of cEPCs peaks from luteal phase through the 1st trimester [44]. This finding is consistent with the fact that vasculogenesis peaks during embryogenesis [35,45]. On the other hand, estrogen is reduced after menopause, which leads to the depletion of EPCs [38], and estrogen replacement therapy can delay the onset of senescence in bone marrow-derived EPCs [46]. Therefore, estrogen is critical in promoting angiogenesis and vasculogenesis in female reproductive organs.

**Conclusion**

In the female reproductive system, vasculogenesis is a recurring phenomenon controlled by the cyclical development of a transient structure and the cyclical repair of damaged tissue by E2 and P4 during the menstrual cycle. It is hypothesized that neovascularization is regulated by the crosstalk between these hormones in utero during the ovarian cycle and pregnancy in preparation for implantation and the maintenance of pregnancy. The findings described in this study provide evidence that the physiologic cycle of estrogen regulates EPC proliferation through the alternating balance of ER mRNA expression.
Regulation of angiogenic growth factors in the

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The authors declare that they have no competing interests.

Competing interests

participated in the design of the study, performed the statistical analysis,
and helped to review the manuscript. All authors read and approved the final manuscript.

Authors

Matsubara and Matsubara Reproductive Biology and Endocrinology 2012, 10:2
http://www.ncbi.nlm.nih.gov/content/10/1/2

Page 7 of 8

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Authors’ contributions

YM collected all samples, carried out the molecular genetic studies, participated in the sequence alignment, and drafted the manuscript. KM

participated in the sequence alignment, and drafted the manuscript. KM

participated in the statistical analysis,

Kalka C:

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