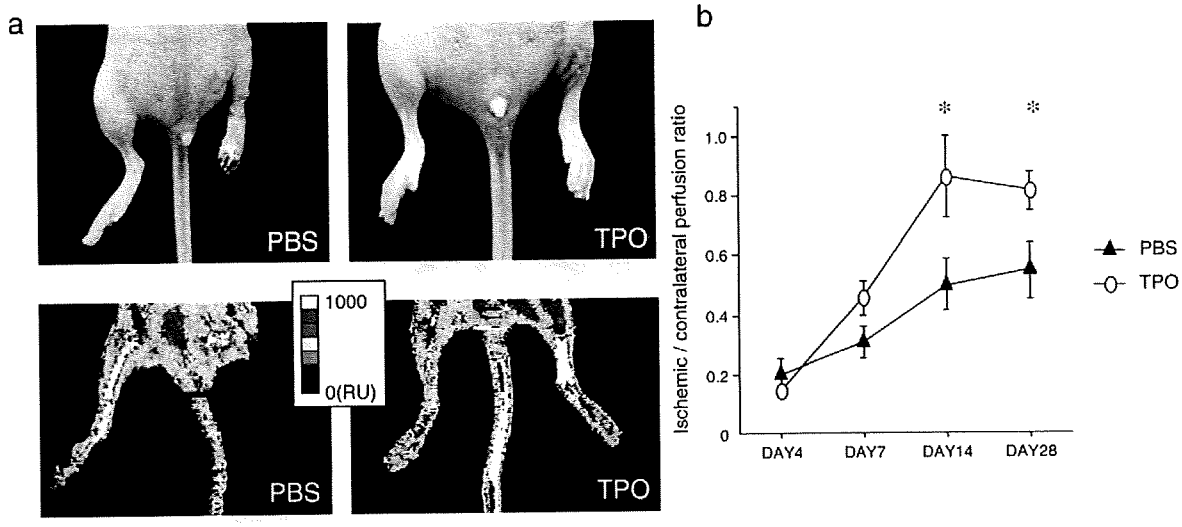

Fig. 4. Induced blood flow recovery of ischemic limb by TPO co-administration. (a) Representative photographs of hindlimbs in treated animals assessed by LDPI at 28 days after EPC transplantation. Color scale illustrates variation in blood flow from maximum perfusion (white) to minimal perfusion (dark blue). (b) Quantitative analysis of perfusion recovery measured by LDPI during the observation period. Open circle and closed triangle show TPO-treated and PBS groups, respectively (*$p< 0.05$, $N=8$).

of cultured EPCs induced by TPO. As shown in Fig. 6a, the inhibition of phosphorylation in the PI3K/Akt signaling pathway by Ly as well as another PI3K inhibitor, Wort, negated the upregulated migration potential effect of cultured EPCs induced by TPO. Furthermore, to investigate the essential role of the mTOR/p70S6K signaling pathway for the enhanced migration potential of cultured EPCs induced by TPO, we examined the effect of Rapa. Interestingly, Rapa also recovered the enhanced migration effect of cultured EPCs induced by TPO (Ratio of migrated cell No. vs cont = 1.00 ± 0.45 for control, $p< 0.01$; 1.04±0.13 for Ly, $p< 0.01$; 1.10±0.09 for Rapa, $p< 0.01$; 1.35±0.15 for Wort, $p< 0.05$ vs. 1.82±0.10 for TPO alone. 2.20±0.23 at SDF-1 as positive control) (Fig. 6a).

Alternatively, the anti-apoptotic effect of TPO on cultured EPCs was negated by Rapa in two kinds of experiments. (Ratio of pyknotic nuclei percentage vs. cont = 1.008±0.078 for control, $p< 0.01$; 0.868±0.089 for Rapa, $p< 0.05$ vs. 0.650±0.054 for TPO alone) (Fig. 6b), (O.D. 405 nm in Cell death detection assay = 0.143±0.006 for control, $p< 0.05$; 0.143±0.007 for Rapa, $p< 0.05$ vs. 0.118±0.010 for TPO alone. 0.150±0.018 at Rapa alone as negative control. 0.102±0.004 at SDF-1α 50 ng/ml as positive control.) (Fig. 6c).

Western blotting analysis showed the complete inhibition of phosphorylation of mTOR and p70S6kinase (located downstream of the PI3K/Akt pathway) by Rapa, which is an inhibitor of mTOR *per se*, as well as Ly or Wort (Fig. 6d).

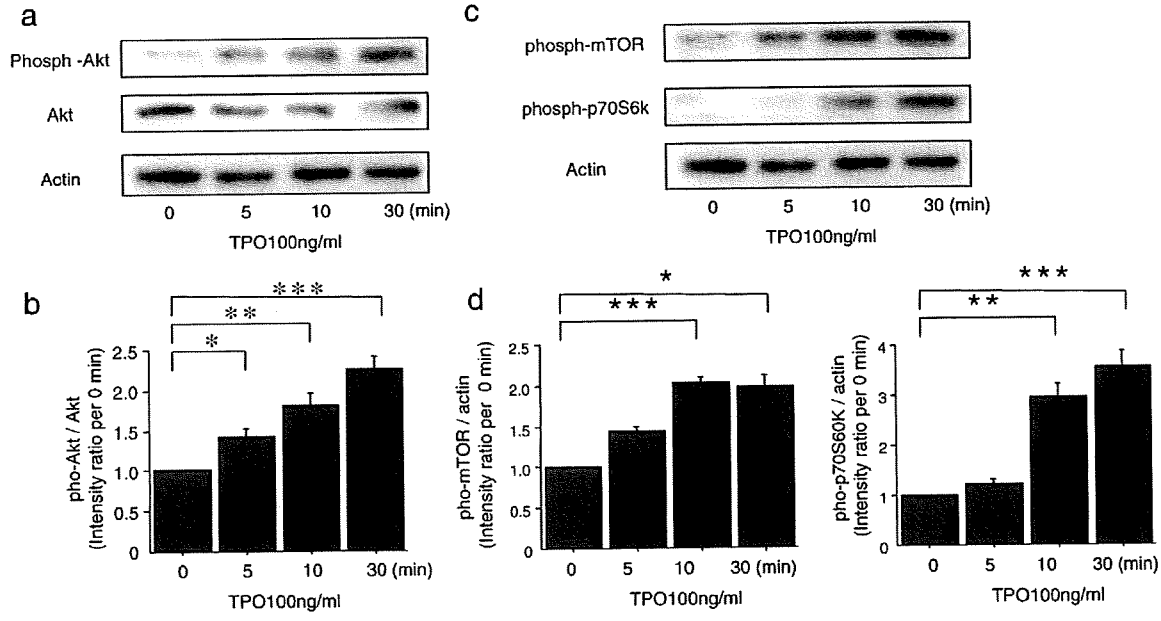

Fig. 5. TPO stimulated Akt/mTOR/p70S6kinase pathway in cultured EPCs. (a) Akt phosphorylation in TPO-stimulated EPCs by Western blotting. (b) Increment of Akt phosphorylation in TPO-stimulated EPCs at serial time points (*$p< 0.05$, **$p< 0.005$, ***$p< 0.0001$, $N=3$). (c) Phosphorylation of mTOR/p70S6kinase pathway in TPO-stimulated EPCs by Western blotting. (d) Increment of mTOR and p70S6kinase phosphorylation in TPO-stimulated EPCs at serial time points (*$p< 0.05$, **$p< 0.005$, ***$p< 0.0001$, $N=3$).

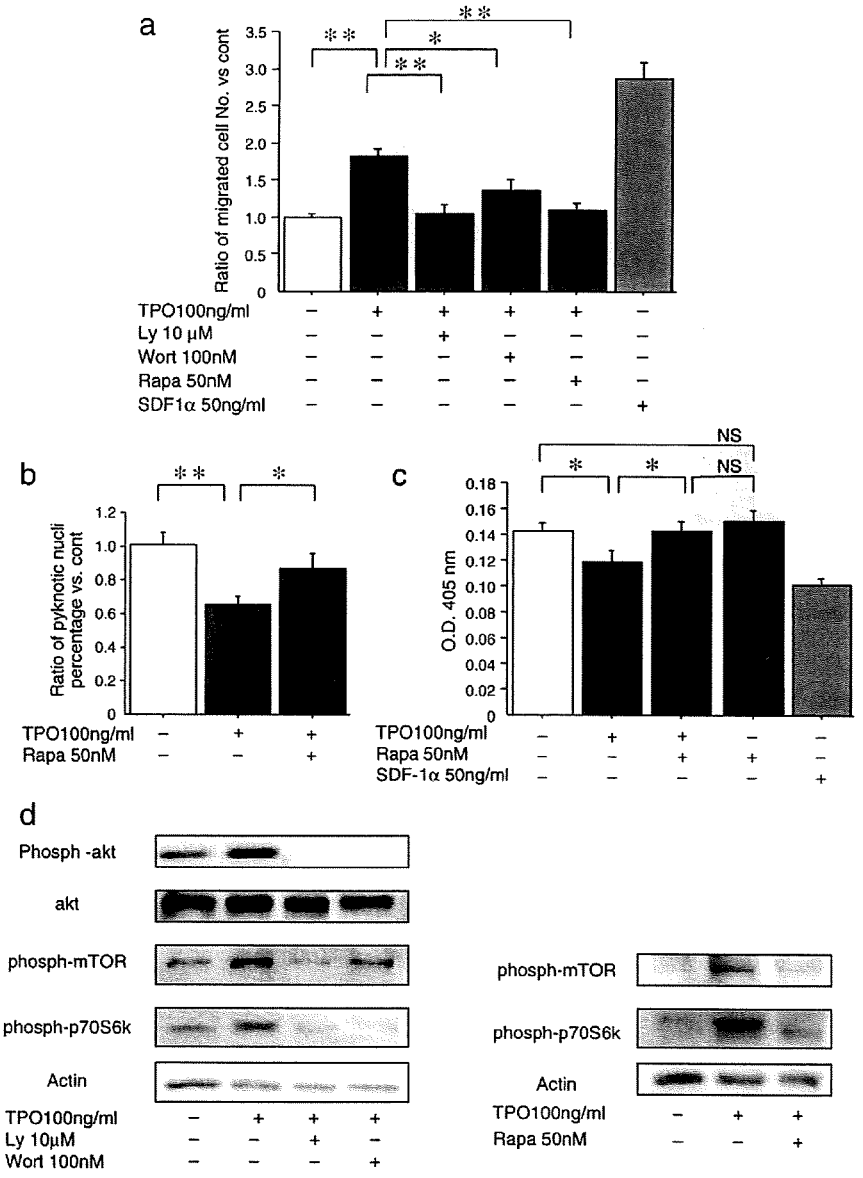

Fig. 6. The mTOR/p70S6kinase signaling pathway is required for migration and survival of cultured EPC stimulated by TPO. (a) Increment of migration of cultured EPCs treated with TPO is negated by inhibitors of the Akt/mTOR/p70S6kinase signaling pathway (*$p< 0.05$, **$p< 0.01$, $N=10$). (b) Anti-apoptotic activity of TPO in cultured EPCs, negated by inhibition of the mTOR/p70S6kinase signaling pathway. The ratio of pyknotic nuclei percentage vs. cont (*$p< 0.05$, $N=4$). (c) Similar results were obtained by cell death detection assay (*$p< 0.05$, $N=4$). (d) Left: Phosphorylation of the mTOR/p70S6kinase signaling pathway in TPO-treated cultured EPCs negated by inhibitors of the PI3K/Akt signaling pathway. Right: Inhibition of phosphorylation of the mTOR/p70S6kinase pathway in TPO-stimulated EPCs. Similar results were obtained in 3 independent experiments.

These results indicated that the PI3K/Akt/mTOR/p70S6kinase signaling pathway plays an essential role in the migration and anti-apoptotic effect on cultured EPCs augmented by TPO.

3.7. Significance of the TPO-induced mTOR/p70S6K signaling pathway for in vitro vasculogenesis in cultured EPCs

Matrigel tube formation assay for HUVECs cocultured with TPO-stimulated EPCs was performed to assess the involvement of the mTOR/p70S6K signaling pathway in the TPO-augmented vasculogenic potential of cultured EPCs. Of note, TPO-stimulated EPCs significantly enhanced the tube formation of HUVECs in Matrigel, as compared with unstimulated EPCs, which was inhibited by Rapa (tube number/×40 HPF=42.8±3.0 for TPO unstimulated EPCs, $p< 0.0001$; 46.8±3.6 for TPO-stimulated EPCs treated with Rap, $p< 0.001$; 44.500±1.916 for HUVEC alone, $p< 0.001$ vs. 62.1±3.1 for TPO-stimulated EPCs) although unstimulated EPCs possessed no effect on tube formation (Fig. 7). These findings also indicated that the mTOR/p70S6Kkinase signaling pathway plays a pivotal role in the TPO-stimulated vasculogenetic potential of cultured EPCs.

4. Discussion

In the present study, we investigated whether TPO, as a positive mediator of postnatal vasculogenesis, potentiates the vasculogenic capability of EPCs transplanted in acute ischemia. In *in vitro* experiments, TPO demonstrated the potential to enhance motility and survival of cultured EPCs, as well as tubulogenesis of HUVECs through TPO-stimulated EPCs. The functional activities of EPCs are considered to be conducted via the c-Mpl receptor of TPO expressed in EPCs (Figs. 1b,c). *In vivo*, the systemic transplantation of EPCs via tail

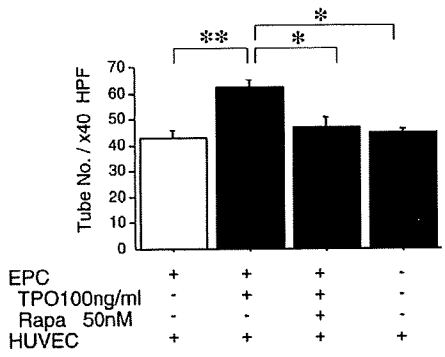

Fig. 7. The mTOR/p70S6kinase signaling pathway plays a pivotal role in vasculogenesis of cultured EPC stimulated by TPO *in vitro*. Serum starved cultured EPCs were pre-treated with TPO (100 ng/ml) and Rapa (50 nM), followed by co-culturing with HUVECs and in Matrigel-coated plates (*$p< 0.001$, **$p< 0.0001$, $N=4$).

veins, along with intramuscular local administration of TPO, showed an augmentation of EPC homing concurrent with an increase of capillary densities in ischemic hindlimbs, compared with EPC transplantation alone. Some groups reported that the maximal concentration of rhTPO (1 μg/mouse) was reached at 2 h after subcutaneous injection. The terminal half-life was 10.8 h and rhTPO was excreted within 72 h after dosing [20]. Considering this report, injection ways were different from our experiments, TPO works specially in the early period of ischemic muscle and homed transplanted EPCs into the ischemic site (Figs. 3a,b). The effect of TPO on EPC homing into ischemic tissues is figured to be mediated via the motility promoted by the concentration gradient of locally delivered TPO in the ischemic tissue. This is indicated by the preferential effect of TPO on the migratory activity of EPCs shown in Figs. 2a, 3a,b, and in similar findings from a previous report regarding local delivery of SDF-1 in ischemic tissues [6]. However, the enhanced neovascularization is not only due to the accumulation of EPCs in the foci of ischemia, but also because of the elongation of lifespan as revealed by an *in vitro* apoptosis assay (Figs. 2c and 6c).

Interestingly, the *in vitro* migration and survival of cultured EPCs boosted by TPO is mediated through the activation of the PI3K/Akt/mTOR/p70S6K signaling pathway, which is abrogated by pathway inhibitors LY294002, Wortmannin, or Rapamycin (Figs. 5, 6). As previously reported, Akt is an essential intracellular signal mediator of angiogenesis, regulating migration, survival, proliferation, and tubulogenesis in vascular ECs [21,22]. Akt signaling also plays a crucial role in the activities of immature endothelial lineage cells and EPCs undergoing vasculogenesis. Previous reports demonstrate that statins (HMG-CoA reductase inhibitors) promote vasculogenesis in terms of enhanced mobilization, migration, survival, differentiation, or proliferation of EPCs via the activation of the Akt pathway [14]. Erythropoietin directly promotes EPC proliferation and differentiation through Akt phosphorylation [5,23]. Estrogen limits EPC senescence via the PI3K/Akt signaling pathway [24]. The importance *per se* of the downstream signal pathway, mTOR/p70S6K, has been already suggested for the proliferation and differentiation of immature EPCs given the inhibitory effect of Rapamycin on the activities of CD133+ cells in peripheral blood [17]. While the universal significance of PI3K/Akt/mTOR pathway in endothelial lineage cells is unclear, its effects are not equivalent between differentiated ECs, therapeutical EPCs, and immature EPCs, thereby suggesting different roles in each cell type.

Of note, in contrast to immature EPCs [17], the pathway is unlikely to be related to the proliferative activity of adherent EPCs, as reduced by TPO, despite mTOR/p70S6K pathway phosphorylation mediating cell growth and proliferation in diverse cell types [25–27] (Fig. 2b). Considering the data, the proliferative activity of EPCs might not be influenced by TPO, because of the possible suppression of the PI3K/Akt pathway following mTOR/p70S6K activation as a negative feedback mechanism [28,29]. Consequently, we have shown for the first time that TPO enhances the migration and survival of adherent EPCs for vasculogenesis through the activation of the PI3K/Akt/mTOR/p70S6kinase pathway via c-Mpl *in vitro*, and promotes the accumulation and incorporation of EPCs in ischemic tissues *in vivo*.

Our former study indicated that local transfection of adenoviral vector encoding TPO in ischemic muscles induces an increase in circulating platelets by HuMK stimulation earlier than systemic transfection [30]. Though there is a difference between bolus and continuous administration of TPO, we can readily predict that locally administered TPO will trigger an increase in platelet accumulation in ischemic tissues [31,32], thereafter activating angiogenic mediators such as VEGF [33,34], or angiogenic platelet-derived lipids such as sphingosine 1-phosphate, lysophosphatidic acid, and phosphatidate [16,35,36]. Especially, sphingosine 1-phosphate (S1P) is capable of inducing angiogenetic factors *in vitro*; for example EC chemotaxis, survival, proliferation, capillary morphogenesis, etc [35]. Moreover, S1P has recently been reported to induce phosphorylation of CXCR-4, a chemokine receptor of SDF-1 through S1P receptor 3, and is thereby involved in the SDF-1/CXCR-4 signaling pathway which is important for EPC recruitment in ischemic tissues [6,37].

Alternatively, locally administered TPO is thought to encourage *in situ* ECs via c-Mpl to secrete platelet activating factor (PAF) [16,38], a candidate potent angiogenic phospholipid, mediating the secretion of angiogenic factors including VEGF [39]. Considering the descriptions above, we conclude that TPO-augmented therapeutic vasculogenesis is exerted by orchestrating the direct and indirect functions of TPO on EPC biological activities *in vitro* and *in vivo* in ischemic hindlimb.

Despite the information concerning the role of TPO exerting biological actions on cancer cells via activation of c-Mpl and its downstream signaling [40], in terms of clinical applications, rhTPO intravenous administration for delayed platelet recovery after HSC transplantation has shown no serious adverse effects including cancer disease [41]. rhTPO administration has been reported to safely induce mobilization of BM derived CD34+ cells without thrombogenic events in patients with cancer undergoing high-dose chemotherapy and autologous PB CD34+ cells transplantation [42]. Therefore, TPO may be a favorable mediator to safely augment the efficacy of EPC transplantation, especially through local administration as medicated in the present study.

In conclusion, the present study demonstrates that TPO enhances the efficacy of EPC transplantation by upregulating migration and survival of EPCs via activation of the Akt/mTOR/p70S6kinase signaling pathway, making it a promising candidate to exert a booster effect in therapeutic vasculogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2008.08.002.

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Estrogen-Mediated Endothelial Progenitor Cell Biology and Kinetics For Physiological Postnatal Vasculogenesis

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Abstract—Estrogen has been demonstrated to promote therapeutic reendothelialization after vascular injury by bone marrow (BM)–derived endothelial progenitor cell (EPC) mobilization and phenotypic modulation. We investigated the primary hypothesis that estrogen regulates physiological postnatal vasculogenesis by modulating bioactivity of BM-derived EPCs through the estrogen receptor (ER), in cyclic hormonally regulated endometrial neovascularization. Cultured human EPCs from peripheral blood mononuclear cells (PB-MNCs) disclosed consistent gene expression of ER α as well as downregulated gene expressions of ER β. Under the physiological concentrations of estrogen (17β-estradiol, E2), proliferation and migration were stimulated, whereas apoptosis was inhibited on day 7 cultured EPCs. These estrogen-induced activities were blocked by the receptor antagonist, ICI182,780 (ICI). In BM transplanted (BMT) mice with ovariectomy (OVX) from transgenic mice overexpressing β-galactosidase (lacZ) regulated by an endothelial specific Tie-2 promoter (Tie-2/lacZ/BM), the uterus demonstrated a significant increase in BM-derived EPCs (lacZ expressing cells) incorporated into neovasculatures detected by CD31 immunohistochemistry after E2 administration. The BM-derived EPCs that were incorporated into the uterus dominantly expressed ER α, rather than ER β in BMT mice from BM of transgenic mice overexpressing EGFP regulated by Tie-2 promoter with OVX (Tie-2/EGFP/BMT/OVX) by ERs fluorescence immunohistochemistry. An in vitro assay for colony forming activity as well as flow cytometry for CD133, CD34, KDR, and VE-cadherin, using human PB-MNCs at 5 stages of the female menstrual-cycle (early-proliferative, pre-ovulatory, post-ovulatory, mid-luteal, late-luteal), revealed cycle-specific regulation of EPC kinetics. These findings demonstrate that physiological postnatal vasculogenesis involves cyclic, E2-regulated bioactivity of BM-derived EPCs, predominantly through the ERα. (***Circ Res.* 2007;101:598-606.**)

Key Words: estrogen ■ endothelial progenitor cell ■ estrogen receptor ■ physiological postnatal vasculogenesis

In the female reproductive system, neovascularization is a recurring phenomemnon controlled by cyclic development of transient structure and cyclical repair of damaged tissue.[1] The ovarian sex steroid hormones, estrogen and progesterone, are primarily uterotropic and control the cyclical patterns of uterine cell proliferation and vascular growth that occur throughout the nonpregnant menstrual cycle. Given the synchronized nature of neovascularization in this cyclical mannter, it is assumed that angiogenic growth factor expression is induced by steroid hormones and regulates blood vessel formation in reproductive organogenesis.[2–5]

Despite clinical evidence for the significant role of steroid hormones in endometrial neovascularization, further investigation using in vitro and in vivo experiments have yielded inconclusive results regarding pathophysiological mechanisms in angiogenesis.[6–10] Moreover, estrogen has been shown to exhibit an inhibitory effect on certain hematopoietic kinetics, including lymphocytes and monocytes, both in terms of number and function.[11–14] Endometrial vascularization has formerly been considered to develop via "angiogenesis", ie, proliferation and migration of fully differentiated endothelial cells (ECs) from preexisting "parent" vessels.[15] However, circulating EPCs have been shown to incorporate into foci of neovascularization in adult species,[16] consistent with the notion of postnatal "vasculogenesis".[17] EPCs comprise of undifferentiated blood MNCs which are mobilized from BM by ischemic stimuli and angiogenic/hematopoietic factors,[17] and subsequently home to, differentiate, and proliferate in foci of neovascularization.[18]

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Given this new understanding of adult neovascularization, it is also possible that vasculogenesis could also be responsible for ovarian hormonal regulation of endometrial neovascularization. Recently, 2 groups have demonstrated promotive effect of estrogen on reendothelialization after vascular injury via EPC incorporation. Iwakura et al disclosed an NO-dependent estrogen effect on EPCs using eNOS KO mice,[19,20] and Strehlow et al indicated an estrogen dependent antiapoptotic effect on EPC biology. Hamada et al demonstrated the functional importance of ER expression by EPCs.[21] Together, these reports suggest a therapeutic application of altering estrogen levels directly, or its receptor agonists, for vascular repair.

Therefore, in the current study, we investigated the hypothesis that E2 regulates physiological neovascularization of the endometrium by modulating the biology and kinetics of BM-derived EPCs.

Methods

EPC Culture of Human and Mouse EPCs

Human and mouse EPCs were cultured by using a modified protocol that has previously been reported.[18,22,23] Phenol-red free (PRF) endothelial basal media (EBM, Clonetics) was used to delete estrogenic effect, as described in Supplementary Method (SM)-I, available online at http://circres.ahajournals.org.[24,25]

Effects of Estrogen on EPCs: Differentiation, Proliferation, Migration, and Antiapoptosis In Vitro

The assays for EPC bioactivity effected by E2 were performed, according to the detailed description in SM-II, as previously reported.[19,26]

RT-PCR for Endothelial Gene Expression in Cultured Human EPCs

The protocol for RT-PCR assay was described in SM-III.

Real Time PCR Assay for Gene Expression of Estrogen Receptors in Cultured Human EPCs

The protocol of real time PCR assay was described in SM-IV.

Mouse Cultured EPC Assay

The protocol of mouse cultured EPC assay was described in SM-V.[18,22,23]

Mouse Cornea Neovascularization Assay

The effect of E2-induced EPC kinetics on neovascularization was studied by E2 pretreated OVX mice for 4 days as described in SM-6.[18,22,27]

Study Design of BMT Animal Experiments

BMT animal models with endogenous sex hormone depletion were developed as follows: female nude SCID mice (NIHS-bg-nu-xid, Taconic, Albany, NY; 4 weeks) were lethally irradiated and received BM cells from age-matched female Tie-2 transgenic mice overexpressing β-galactosidase by Tie-2 promoter (FVB/N-TgN[TIE2LacZ]182Sato, Jackson Laboratory, Bar Harbor, Me).[17,22,28] The protocol of BMT animal experiments was depicted in SM-VII.

Cellular Identification of LacZ Expressing Cells in Uterus or Cornea of Tie-2/LacZ/BMT/OVX Mice

Uterus Experiments

The uterus of mice euthanized at day 2, day 4, and day 7 after subcutaneous E2 pellet implantation was processed for CD31 immunohistochemistry as well as LacZ staining,[17] as described in SM-VIII. LacZ positive cells in whole area, or localized in vascular wall or stroma per uterus tissue section were counted. The percentage of LacZ positive cells localized in each part versus whole area was assayed.

Cornea Experiments

Six days after making the cornea model, the cornea was observed, after staining eye balls with LacZ solution. LacZ stained tissues embedded in paraffin were processed to CD31 immunohistochemistry.

Investigation of ER α and ER β Expression by BM-Derived EPCs Incorporated Into the Uterus of Tie-2/EGFP/BMT/OVX Mice

The protocol was described in SM-IX.

EPC Culture Assay and Flow Cytometry in Menstrual Cycle of Premenopausal Women

EPC culture assay and flow cytometrical analysis were performed, using PB-MNCs of 6 healthy premenopausal females (aged 20 to 40) at 5 separate stages of the menstrual cycle: T1=early proliferative, T2=preovulatory, T3=postovulatory, T4=mid luteal, and T5=late luteal, as previously described.[29] Flow cytometrical analysis (FACS) was performed on a FACStar flow cytometer (Becton Dickinson) and a Cell Quest software (Becton Dickinson), as described in SM-X.

EPC Colony Forming Activity of PB in Menstrual Cycle of Premenopausal Women

EPC colony forming activity was also assessed by SM-XI and XII.

Notice on Experiments in Animal and Human Subjects

Notice on experiments in animal and human subjects was described in SM-XIII.

Statistical Analysis

All results are expressed as mean±SE. Statistical significance was evaluated using unpaired Student *t* test for the comparison between 2 groups and ANOVA followed by Fisher post hoc test for the comparison among multiple groups. A probability value less than 0.05 was interpreted to denote statistical significance.

Results

Quantitative Real Time PCR Assay of ER α and ER β Gene Expressions in Cultured Human EPCs

In day 7 cultured human EPCs, endothelial gene expressions of von Willbrand Factor (vWF) and CD31 were detected (Figure 1A). To establish the potential for a direct effect of E2 on EPCs, mRNA expression of ER α and ER β was assessed by quantitative real time PCR assay. The gene expressions of ER α and ER β in cultured EPCs varied during the culture period. The expression of ER α did not change between day 4 and day 7, whereas the expression of ER β was remarkably downregulated at day 7 to the level of 0.12 fold relative to day 4 (Figure 1B).

Receptor Mediated E2 Effects on EPC Activity

EPC bioactivities upregulated by E2 were deleted in the presence of ICI, suggesting the ER mediated bioactivities, as described in supplemental Figure (SF)-I.

Upregulation of EPC Kinetics After E2 Administration

To explore the systemic effects of E2 on EPC kinetics, we administered E2 to OVX female and CST male mice. The

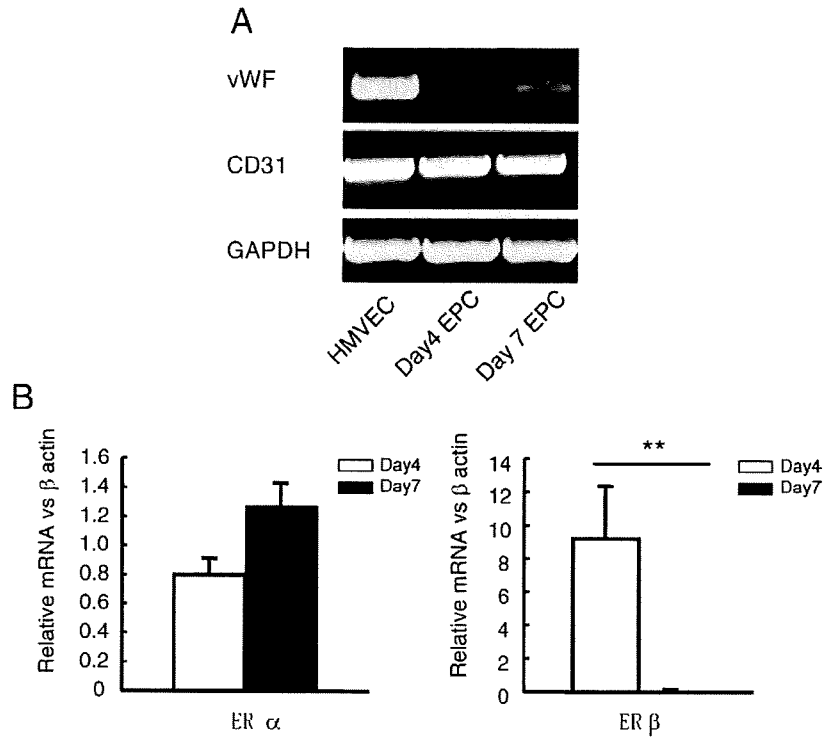

Figure 1. Quantitative real-time PCR assay of ERs gene expressions in cultured human EPCs. A, Endothelial gene expressions of vWF and CD31 in cultured human EPCs supplied for in vitro experiments. HMVECs, human microvascular ECs served as a positive control. B, Quantitative real-time PCR assay of ERs gene expressions in cultured human EPCs. The assay was performed on 3 human subjects. The analysis was performed using ddCt method. dCt values of ERs vs β-actin from each Ct value were acquired at each time point. ddCt values at each time point on each ER were calculated from each dCt value at day 4 or day 7. Relative mRNA vs β-actin presents $2^{(-ddCt)}$, n=3, $^{**}p<0.01$.

EPC culture assay[22] revealed a significant increase in endothelial lineage cells in cultures of PB-MNCs isolated at 2 to 4 days after subcutaneous implantation of E2 pellet in both OVX and CST mice. EPCs, identified by acLDL-DiI uptake and BS-1 lectin- FITC reactivity, consisted principally of spindle-shaped cells, often forming colonies. The number of cultured EPCs decreased to or below the level of pre-implantation by day 7 (Figure 2A and 2B). In controls, pellet implant did not increase EPC numbers significantly. These results thus provide quantitative evidence that E2 mobilizes EPCs from BM into the peripheral circulation.

Enhanced Cornea Neovascularization After E2 Administration

Examination of the cornea in E2- or P-treated mice established the extent of vascular development induced by implantation of a VEGF-containing pellet in the mouse cornea, presented in SF-II.

Identification of BM-Derived EPCs Within the Endometrium of Tie-2/LacZ/BMT/OVX Mice After E2 Administration

Before pellet implantation, macroscopic examination of the uterus of Tie-2/LacZ/BMT/OVX mice from both groups

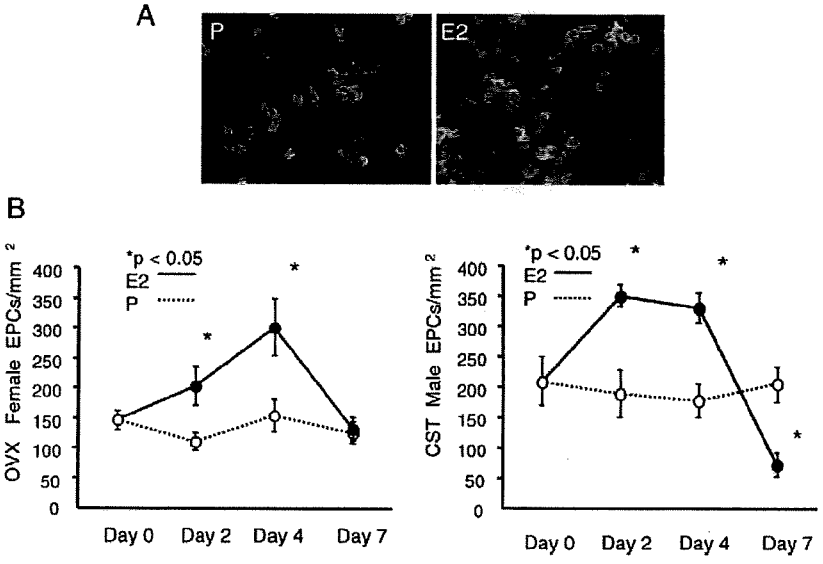

Figure 2. EPC culture assay in mice after E2 administration. A, Representative fluorescent photomicrographs of cultured EPCs merged acLDL- DiI with BS-1 lectin-FITC at day 4 in P and E2 treated OVX female mouse. ×10 magnification. B, Time course of EPC frequency in culture of PB-MNCs from E2- or P- treated mice. The results are shown for both OVX and CST mice, n=6 mice /each group.

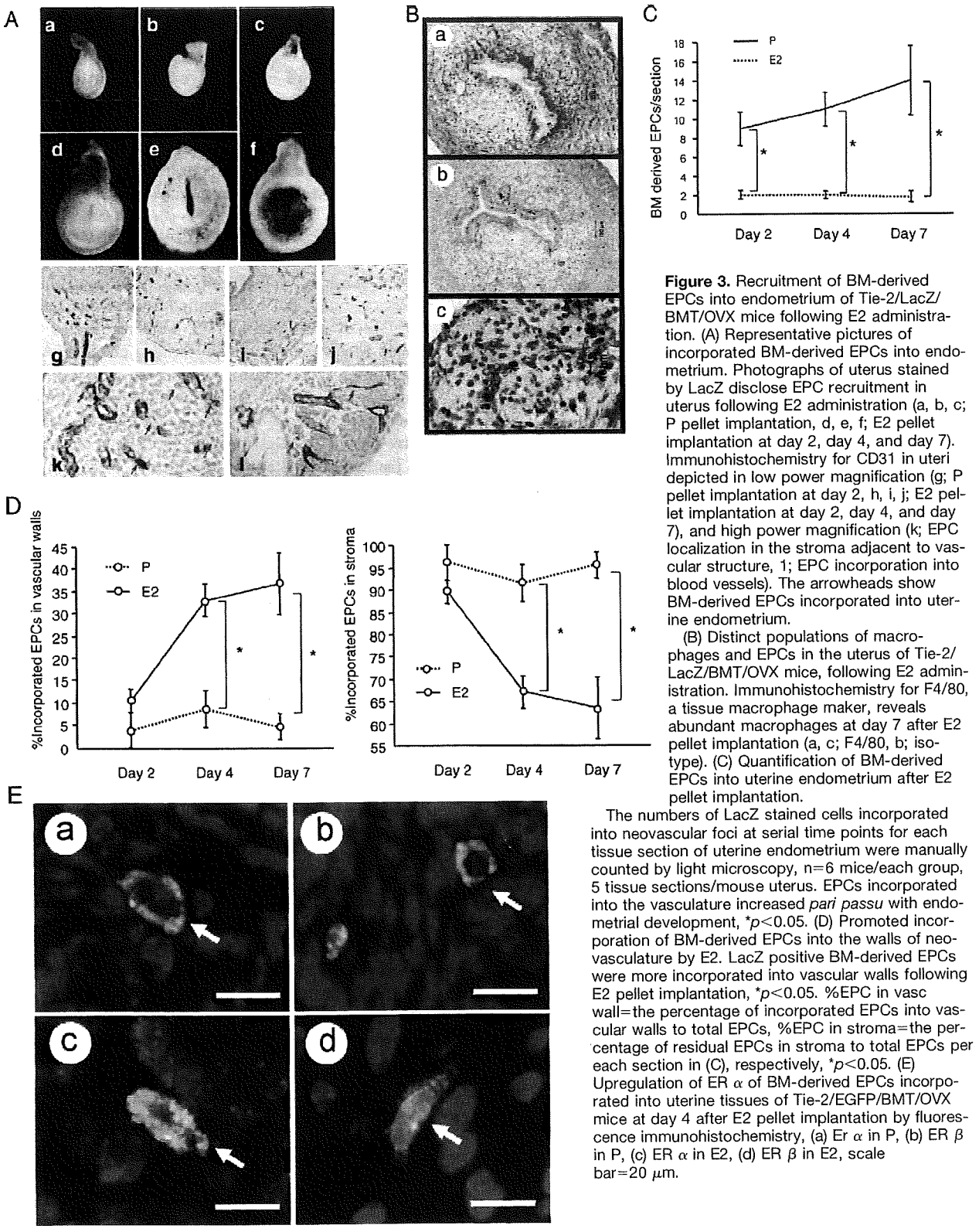

Figure 3. Recruitment of BM-derived EPCs into endometrium of Tie-2/LacZ/BMT/OVX mice following E2 administration. (A) Representative pictures of incorporated BM-derived EPCs into endometrium. Photographs of uterus stained by LacZ disclose EPC recruitment in uterus following E2 administration (a, b, c; P pellet implantation, d, e, f; E2 pellet implantation at day 2, day 4, and day 7). Immunohistochemistry for CD31 in uteri depicted in low power magnification (g; P pellet implantation at day 2, h, i, j; E2 pellet implantation at day 2, day 4, and day 7), and high power magnification (k; EPC localization in the stroma adjacent to vascular structure, 1; EPC incorporation into blood vessels). The arrowheads show BM-derived EPCs incorporated into uterine endometrium.

(B) Distinct populations of macrophages and EPCs in the uterus of Tie-2/LacZ/BMT/OVX mice, following E2 administration. Immunohistochemistry for F4/80, a tissue macrophage maker, reveals abundant macrophages at day 7 after E2 pellet implantation (a, c; F4/80, b; isotype). (C) Quantification of BM-derived EPCs into uterine endometrium after E2 pellet implantation.

The numbers of LacZ stained cells incorporated into neovascular foci at serial time points for each tissue section of uterine endometrium were manually counted by light microscopy, n=6 mice/each group, 5 tissue sections/mouse uterus. EPCs incorporated into the vasculature increased *pari passu* with endometrial development, $^*p<0.05$. (D) Promoted incorporation of BM-derived EPCs into the walls of neovasculature by E2. LacZ positive BM-derived EPCs were more incorporated into vascular walls following E2 pellet implantation, $^*p<0.05$. %EPC in vasc wall=the percentage of incorporated EPCs into vascular walls to total EPCs, %EPC in stroma=the percentage of residual EPCs in stroma to total EPCs per each section in (C), respectively, $^*p<0.05$. (E) Upregulation of ER α of BM-derived EPCs incorporated into uterine tissues of Tie-2/EGFP/BMT/OVX mice at day 4 after E2 pellet implantation by fluorescence immunohistochemistry, (a) Er α in P, (b) ER β in P, (c) ER α in E2, (d) ER β in E2, scale bar=20 μm.

disclosed occasional blue LacZ-stained cells located mainly in the mesometrium. In mice with P pellet implants, the uterus remained atrophic, and the location and frequency of LacZ-stained BM-derived EPCs did not change during observation. In contrast, Tie-2/LacZ/BMT/OVX mice with implanted E2 pellet revealed an evolving pattern of BM-derived, LacZ-positive cells within the uterus. Two days after E2 pellet implant, the frequency of LacZ-positive cells increased

throughout the uterus but remained concentrated in the outer layer of myometrium. By day 4 after implantation, EPCs continued to increase in number and were now identified in the outer and inner layers of myometrium and endometrium. EPCs finally accumulated in large numbers within the endometrium 7 days after E2 implantation. Immunohistochemical staining for CD31 in the P group demonstrated LacZ-stained CD31-positive EPCs appearing as round cells localized in the stroma adjacent to established vessels and incorporated as spindle-shaped cells into vascular walls. After E2 pellet implantation, EPCs were more frequently found to be incorporated within the vascular walls of the endometrium (Figure 3A). Counterstaining with antibody F4/80, as a tissue macrophage marker, and LacZ staining, revealed that the macrophages did not express β-gal; thereby indicating that they do not express Tie-2 and are therefore a completely separate population from the EPCs identified by Tie-2 promoter driven LacZ expression (Figure 3B). EPCs incorporation into foci of uterine neovascularization increased significantly by approximately 4.5-, 5.5-, or 7.8-fold at day 2, day 4, or day 7 after E2 pellet implantation versus endometrial tissues harvested from P pellet implants examined at identical time points. The number of incorporated EPCs per uterine section were as follows: for E2 group, day 2=9.0±1.8, day 4=11.0±1.7, day 7=14.0±3.6; in contrast, for P group, day 2=2.1±0.4, day 4=2.0±0.3, and day 7=1.8±0.5 (Figure 3C). Also, after E2 pellet implantation, the percentage of BM-derived EPCs incorporated into the neovasculature (of the total BM-derived EPCs per uterine section) increased significantly (day 2=10.44±2.7%, day 4=33.01±3.7%, day 7=36.73±6.9%); in contrast, the percentage for P group remained low (day 2=3.85±3.8%, day 4=8.56±4.1%, and day 7=4.44±2.9%; Figure 3D). On the other hand, the percentage of BM-derived EPCs in the stroma of the E2 group decreased inversely (day 2=89.57±2.7%, day 4=66.99±3.7%, day 7=63.27±6.9%), whereas the percentage for P group remained high (day 2=96.15±3.8%, day 4=91.44±4.1%, and day 7=95.56±2.9%; Figure 3D).

These findings indicate that BM-derived EPCs incorporate into foci of neovascularization during E2-induced endometrial maturation. This effect was restricted to E2-responsive organs: incorporated EPCs in other organs such as lung, liver, or skin,[17] could not be enhanced by E2 (data not shown). The sequence of histologic patterns observed suggests that E2 mobilizes BM-derived EPCs via the circulation (vide infra) into the myometrium from mesometrium, which precedes accumulation and incorporation into the neovasculature of the endometrium. The representative feature of BM-derived EPC incorporation into endothelial layer of vessel wall in uterine endometrium was recognized at day 7 after E2 pellet implantation by fluorescence immunohistochemistry of EGFP cellular positivity in CD31 positive endothelial layer (SF-III).

BM-derived EPCs (Tie-2/EGFP positive cells) incorporated into uterine tissues in Tie-2/EGFP/BMT/OVX mice, expressing ER α by stimulation of E2 pellet implantation for 4 days, but not ER β by fluorescence immunohistochemistry (Figure 3E). In this context, it is intriguing to note that the pattern of EPC recruitment and incorporation is identical to the previously established pattern of in situ VEGF expression in the hormone-regulated cycle of endometrial development and regression.[3]

Recruitment of BM-Derived EPCs into Cornea Neovascularization of Tie-2/LacZ/BMT/OVX Mice Following E2 Administration

Macroscopically, BM-derived EPCs stained with LacZ were observed more frequently in cornea of E2 pellet implanted mice, as compared with P pellet implanted, as presented in SF-4.

EPC Kinetics Through Human Menstrual Cycle

The morphology of cultured EPCs varied at different phases of the menstrual cycle (Figure 4A). At the preovulatory phase (T2), cultured EPCs, identified by double staining with acLDL-DiI and UEA-1-FITC, were recognized as isolated round adhesive cells, seldom forming colonies. After ovulation through the luteal phase (T3 to T5), EPCs appeared spindle-shaped, frequently exhibiting colony formation. Frequent colony formation was noted during the early proliferative phase (T1). The frequency of EPCs in culture decreased to the lowest level at the preovulatory phase (T2), increased gradually through ovulation, and remained high even during the early proliferative phase (Figure 4B).

As shown in Figure 4C, ovulation was identified between T2 and T3 by a surge of luteinizing hormone; the associated expression patterns of E2 and progesterone conform to the typical pattern of the menstrual cycle. VEGF levels were lowest at T1, then increased rapidly, reaching a peak at the T2 before slowly decreasing through the luteal phase. The pattern of VEGF expression was thus synchronized with E2. The numeric values of hormones and EPC numbers during menstrual cycle are shown in supplemental Table I.

Flow cytometrical analysis of PB-MNCs was used to determine the frequency of endothelial-specific antigen expressing cells as well as circulating immature EPCs according to the phase of the menstrual cycle. KDR was more frequently expressed by circulating cells from the preovulatory through luteal phases (Figure 4D-b). VE-cadherin antigen positive cells also increased, but the peak expression was until the later luteal phase. Differentiated ECs, positive for P1H12 antigen,[30] were identified at T1, immediately after menstruation, and did not augment, whereas KDR or VE-cadherin–positive endothelial lineage cells increased, after steroid hormone peaks (Figure 4D-b). Of note, the cell populations of CD133-positive or CD34-positive cells involving circulating immature EPCs in PB during the menstrual cycle disclosed a fluctuating pattern with significant amelioration at T3 following E2 peak (Figure 4D-c and 4D-d).

Discussion

The female reproductive system constitutes a unique exception to the quiescent vasculature of the normal healthy adult as the requirement for neovascularization recurring on a cyclic basis. Specifically, in every estrous cycle, the sequential maturation of the endometrium, as well as ovarian follicles and corpora lutea, is accompanied by concomitant development of elaborate capillary networks. Given the extent of newly forming vascular volume in endometrial

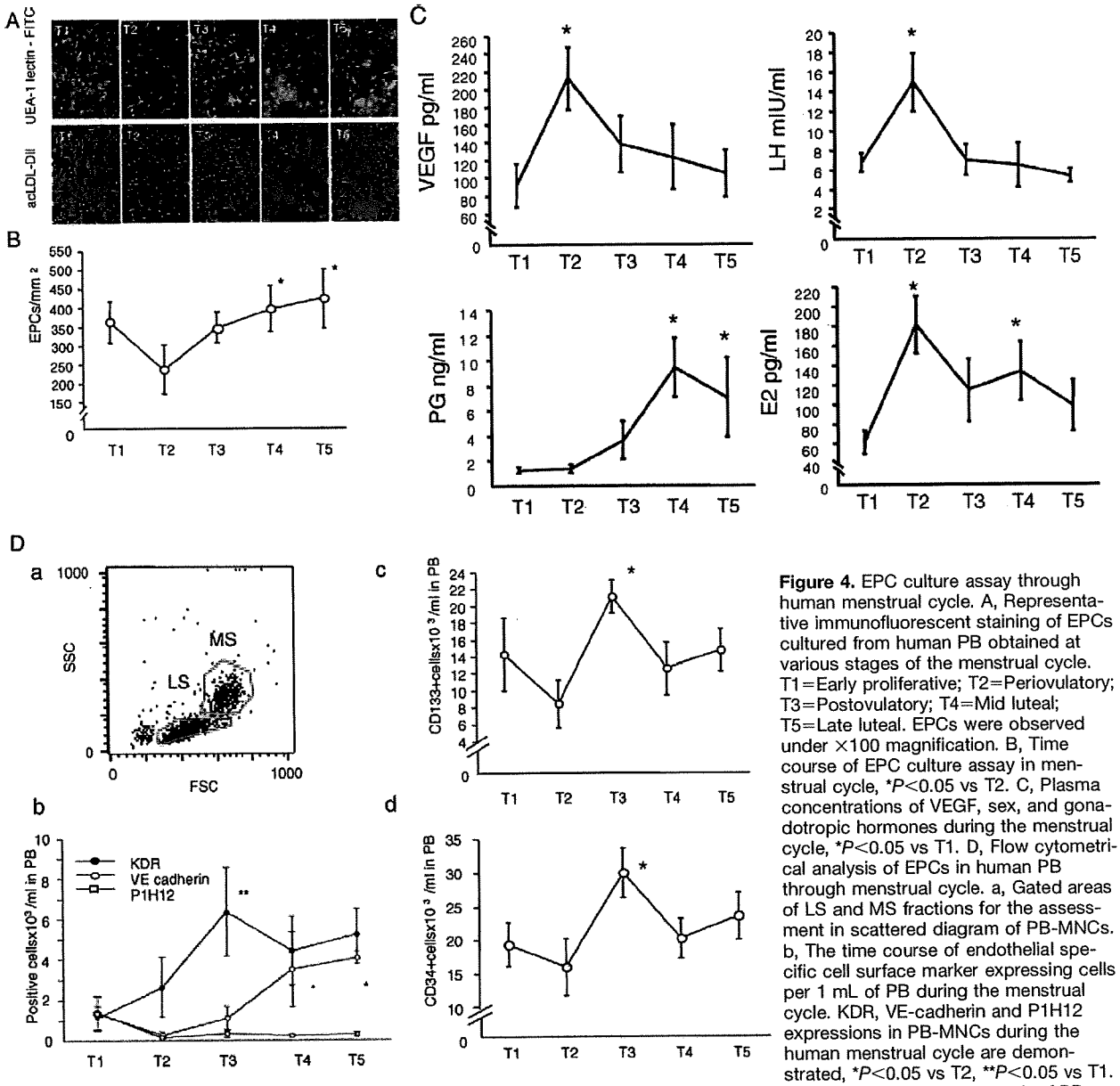

Figure 4. EPC culture assay through human menstrual cycle. A, Representative immunofluorescent staining of EPCs cultured from human PB obtained at various stages of the menstrual cycle. T1=Early proliferative; T2=Periovulatory; T3=Postovulatory; T4=Mid luteal; T5=Late luteal. EPCs were observed under ×100 magnification. B, Time course of EPC culture assay in menstrual cycle, *$P<0.05$ vs T2. C, Plasma concentrations of VEGF, sex, and gonadotropic hormones during the menstrual cycle, *$P<0.05$ vs T1. D, Flow cytometrical analysis of EPCs in human PB through menstrual cycle. a, Gated areas of LS and MS fractions for the assessment in scattered diagram of PB-MNCs. b, The time course of endothelial specific cell surface marker expressing cells per 1 mL of PB during the menstrual cycle. KDR, VE-cadherin and P1H12 expressions in PB-MNCs during the human menstrual cycle are demonstrated, *$P<0.05$ vs T2, **$P<0.05$ vs T1. c and d, The time course of immature EPC surface marker expressing cells (CD133-positive or CD34-positive cells) per 1 mL of PB, *$P<0.05$ vs T2.

development, it is possible that vasculogenic mechanisms may play a significant role in this cyclic organization. In this regard, we hypothesized that one of the main gender hormones, estrogen, controls EPC biology for cyclic neovascularization. The present findings provide evidence that the physiologic cycle of estrogen regulates EPC kinetics, ie, differentiation, proliferation, migration, apoptosis, mobilization, and ultimately incorporation into foci of neovascularization in the developing endometrium. Although the therapeutic potential of E2 for enhancing the contributions of EPCs for reendothelialization after vascular injury has been suggested, the physiological role of estrogen for EPC mediated vascular development has not been well established.[19,20,31]

Our EPC culture assay experiments demonstrated variations in EPC number and morphology throughout the phases of the menstrual cycle. These morphological changes are indicative of enhanced differentiation potential of circulating EPCs corresponding to cyclic hormonal changes. The peak increase in EPC number followed the peak serum concentrations of estrogen, as well as VEGF. This interval may potentially reflect a combination of estrogen effect on EPC proliferation, differentiation, and estrogen-induced mobilization from BM that has been suggested previously.[19,20,31]

The increase in the number of circulating EPCs expressing KDR or VE-cadherin antigen after peak estrogen levels and the decrease after downregulation of sex hormones were demonstrated by flow cytometrical analysis. Given their

essential function in embryonic vasculogenesis,[32–35] KDR and VE-cadherin were used to detect EPCs in PB-MNC population. Similarly, Strehlow et al have shown that estrogen mobilizes BM-derived EPCs (CD34 positive/KDR positive cells) into circulation of human subjects.[31] A temporal discrepancy in expression between KDR and VE-cadherin antigen in preovulatory and postovulatory phases was observed, which may suggest a differential effect of ovulatory estrogen on EPC biology. The basis for this differential expression may be related to the former findings that the expression of Flk-1 (homologue of mouse KDR) has been considered to represent a very early endothelial lineage marker during embryogenesis, whereas VE-cadherin–positive EPCs are considered differentiated from Flk-1–positive/VE-cadherin–negative cells.[35] The initial increase in KDR-positive cells seen during pre- to post-ovulatory phases may result from mobilization of immature EPCs into circulation, followed by an increase in committing EPCs during the luteal phase.[35] In contrast to KDR and VE-cadherin, P1H12 was used as a marker for differentiated ECs and was present at lesser frequency during preovulatory to luteal phases than other markers. Thus, differentiated ECs may circulate in PB only when dislodged by physiological blood vessel regression during menstruation.

The animal experiments provide potential insights into the significance of cyclic changes in EPC kinetics observed in human subjects. Systemic E2 pretreatment enhanced EPC mobilization detected in EPC culture assay and promoted enhanced neovascularization in the mouse cornea micropocket model. These results indicate that systemic estrogen stimulates EPC kinetics in the circulation, subsequently contributing to neovascularization via vasculogenesis. BMT experiments have demonstrated recruitment and incorporation of BM-derived Tie-2 receptor expressing cells, putative EPCs, during estrogen-induced endometrial development. It is intriguing to note that this pattern of EPC recruitment and incorporation is identical to the previously established pattern of in situ VEGF expression in the hormone-regulated cycle of endometrial development and regression.[3] In addition to EPCs found have incorporated into vascular structures, other round and at times even spindle-shaped cells were frequently identified in the uterine stroma. Regarding the finding, the cells could represent tissue macrophages derived from BM incorporating into uterine stroma during estrous cycle. The existence of BM-derived cells defined as macrophages (stained by F4/80) is also important to discuss because this may identify a significant role of blood bone cells in endometrial formation, especially regarding neovascularization. The similar findings were pointed out by several publications.[36,37] Tie-2 expressing BM-derived EPCs need vasculogenic environments introduced by angiogenic cytokines secreted from BM-derived macrophages. Therefore, this balance of EPCs and macrophages might play a pivotal role in neovascularization in endometrial formation.

Although the fate of the EPCs is currently uncertain, such cells may comprise EC reservoirs for the next round of endometrial development. The concept of BM-derived progenitor cell reservoirs in normal tissues is consistent with the notion of BM-derived satellite myoblasts and mesenchymal stem cells in muscle or other normal organs.[5,38] Using the same BMT models, we have previously demonstrated similar stroma-localized EPCs in growing neoplasms, wound healing, severe ischemia, and even though more sparsely—in normal organs.[17] Flk-1–positive cells previously demonstrated in the uterine myometrium[39] may represent similar cells. We have considered that the effect of estrogen may be direct or indirect (eg, mediated via VEGF). Evidence for a direct effect was given by the fact that EPC kinetics in CST male mice, lacking reproductive organs to respond to estrogen, responded equivalently in the case of OVX female mice, leaving a potentially estrogen-responsive (ie, VEGF-producing) uterus. This finding suggests that estrogen enhances EPC kinetics by direct interaction with EPCs or associated cells, such as in the BM microenvironment.

Our in vitro assay presented E2 promotion on EPC differentiation, migration, proliferation, and apoptosis inhibition, as partly indicated previously.[19,31] The findings that enhanced biological activities, such as proliferation, migration, antiapoptosis, and differentiation stimulated by E2 were blocked by an nonselective ERs antagonist (ICI), supported the fact that these effects of estrogen on EPCs were via functional ERs which were detected by mRNA in EPCs.

The importance of ERs on EPC bioactivity, using a myocardial infarcted model of ER α and ER β knockout mice has been recently documented by Hamada et al.[21] The authors described that ER α expressed in EPCs plays a more potent role in pathological vasculogenesis, rather than ER β. The present study disclosed the higher significance of ER α versus ER β in physiological vasculogenesis as well. During the culture period of human EPCs for 7 days through 4 days, the ER α expression was remained at the high level with the downregulation of ER β expression by real time PCR assay, as shown in Figure 1B. Accordingly, the EPC bioactivities disclosed in vitro study of day 7 cultured EPCs are considered to be brought through ER α. Furthermore, in vivo study of Tie-2/EGFP/BMT/OVX mice, the incorporated EPCs in uterus via E2 stimulation disclosed the expression of ER α, but not ER β, as shown in Figure 3E.

These findings may suggest that each ER shares the roles on EPC differentiation cascade, ie, the predominant function of ER β for EPC immature stage at provasculogenic state or ER α for EPC maturing stage at vasculogenic state, although the ER β function especially has yet to be elucidated.

Given the consideration, even in pathological vasculogenesis, ie, coronary vessel formation in infracted hearts, as shown by Hamada et al[21] as well as physiological vasculogenesis, each ER may have a unique role on EPC differentiation.

The basis for organogenesis has become a seminal issue for organ transplantation or therapeutic regeneration of damaged organs. Embryogenesis as well as physiological organogenesis in adult species reveal essential elements of organogenesis, devoid of pathological stimuli, including inflammation. The physiological regenerative processes constitute natural models that indicate how organs are established and survive. Blood vessel development is clearly one of the essential processes for organogenesis. The present study demonstrates that the unique system of cyclical blood vessel development and regression during the menstrual cycle, which occurs more

than 300 times in a female life span, involves hormone-mediated in situ proliferation, incorporation, differentiation, and survival of BM-derived progenitor cells. Above all, the unique EPC kinetics during menstrual cycle provides "dogma" for EPC biology as shown in supplemental Table I. Also, these findings have important implications for the impact of estrogen on vessel formation in disease states. Further insights regarding the precise mechanisms responsible for such physiological vasculogenesis will likely contribute to advanced methods and concepts for organ development in vivo as well as ex vivo.

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Disclosures

None.

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Supplementary Methods (SM)-I;

EPC Culture of Human and Mouse EPCs

Phenol-red free (PRF) endothelial basal medium (EBM, Clonetics) including 5% heat-inactivated defined FBS (Hyclone), endothelial growth supplement kit (EGM-2 MV single quots, Clonetics) without hydrocortisone was used for in vitro assays to investigate the effect of ERs on cultured human EPCs as well as for human or mouse EPC culture assay, using PB-MNCs. In brief, human or mouse PB-MNCs were isolated by density gradient centrifugation method, using Histopaque-1077 or -1083 (Sigma), respectively. Human EPCs from PB-MNCs ($1x10^7$/well) were cultured on 6 well tissue culture plates (Primaria, BD Labware) coated with human fibronectin. The adherent cells were reseeded at day 4 (2×10^5 cells/well), cultured for 3 more days, and then harvested with PBS containing 2 mM EDTA, as day 7 harvested EPCs, applied for in vitro experiments except differentiation assay, as described below. For human or mouse EPC culture assay, isolated PB-MNCs per 500 μl of blood were seeded per well of 4 well chamber glass slides coated with fibronectin or vitronectin containing gelatin, and cultured for 4 days. Cultured EPCs were applied for uptaking of acetylated LDL-DiI (acLDL-DiI)(Biomedical Technologies) and binding of Ulex europaeus agglutinin-1-FITC (UEA- 1 lectin-FITC)(20 μg/ml in PBS)(FL-1061, Vector Lab) for human and Griffonia Simplicifolia Lectin-1-FITC (BS-1 lectin-FITC)(20 μg/ml in PBS)(FL-1101, Vector Lab) for mouse subjects. The slides were embedded with VECTASHIELD Mounting Medium with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (H-1200, Vecter Lab), and cultured EPCs were counted under fluorescence microscopy (IX70, Olympus).

SM-II;

Mouse Cultured EPC Assay

C57BL6J female or male mice (4 wks) (Clea Japan) were underwent OVX or CST

(Castration) under anesthesia by intraperitoneal pentobarbital injection. EPC culture assay was performed, using MNCs isolated from blood drawn by heart puncture at day 2, day 4 and day 7 after subcutaneous E2 pellet implantation.

SM-III;

Effects of Estrogen on EPCs: Differentiation, Proliferation and Migration Activity, Antiapoptosis In vitro

Differentiation assay: Isolated human PB-MNCs (1x 10^7 cells/well) on fibronectin coated 6 well plates (BD Labware) were cultured with E2 (Sigma) and/or ICI (Astra Zeneca) for 4 days. After the replacement of medium, the number of adherent colonies double positive for acLDL-DiI uptake and UEA-1 lectin-FITC binding was counted as colony forming unit (CFU)-EPCs under fluorescence microscopy.

Proliferation assay: Using a Cell Proliferation ELISA BrdU kit (Roche Diagnostics), colorimetric immunoassay for the quantification of cell proliferation was performed, based on the measurement of BrdU incorporation during DNA synthesis, according to the supplementary protocol. Cultured EPCs were plated at 1x 10^4 cells/100 μl serum depleted medium per a well on human fibronectin coated 96 wells (Primaria). After 24 hours, the medium was replaced to serum depleted medium including the serial doses of E2 and/or ICI. After 48 hours culture with the conditional medium, supplementary BrdU solution was added to the culture medium at the final concentration of 10 μM. After 12 hours of BrdU exposure, the medium was removed and dried up for 1 hour at room temperature (RT). Finally, the colorimetric immuno-reaction was stopped by adding 25 μl of 1N H_2SO_4 per well, and the absorbance at 450 nm was measured by a ELISA plate reader (Spectra Max 250, Molecular Devices). The experiment was performed with 10 wells per each group.

Migration assay: A modified Boyden chamber assay was performed. Briefly, using a 24 well- transwell plate with 5 μm pore size polycarbonate membranes (Corning Costar), E2 and/or ICI in PRF-EBM with 0.1% BSA were in the bottom chamber, and day 7 harvested EPCs (1x 10^5 cells/well) were seeded in the upper chamber. The cells on the bottom chamber, stained with DAPI in v Mounting Medium after fixation with Giemsa solution (Baxter Diagnostics) were counted in random fields in each well under fluorescence microscopy.

Apoptosis assay: The proportion of apoptotic cells to 100 cells in each well after serum starvation of EPCs reseeded onto 4-chamber glass slides (1x 10^5 cells/well) with PRF-EBM medium with E2 and/or ICI for 72 hours was quantified by counting pyknotic nuclei in VECTASHIELD HardSet Mounting Medium with DAPI. The function of ERs on antiapoptosis induced by E2 was also evaluated by a cell death detection ELISA^{PLUS} kit (Roche Diagnostics), photometric enzyme immunoassay for the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death. Day 7 harvested EPCs (3x 10^4 cells/well) were cultured on 96 well Primaria plate coated fibronectin with PRF-EBM alone containing E2 and/or ICI for 72 hours, according to the supplementary protocol. After removal of the medium with decantation following centrifugation (200x g, 10 minutes) of the plate, the cell pellet was suspended with 200 μl Lysis buffer per well, incubated for 30 minutes at RT and centrifugated. Twenty μl of the supernatant per well was applied to a well of streptoavidin coated Microplate Module and reacted with 80 μl of Immunomix adjusted the supplementary solutions of Anti DNA antibody-peroxidase, Anti histone antibody-biotin and Incubation buffer (1:1:18) for 2 hours at RT. After washing with Incubation buffer following decantation of the supernatant, the immunoreacted nucleosome was added 100 μl ABTS substrate buffer, incubated for 15 minutes at RT, and then the absorbance was measured at 405 nm by a ELISA plate reader. The experiment was performed with 10 wells per each group.

SM-IV;

RT-PCR for Endothelial Gene Expression in Cultured Human EPCs

Isolated total RNA from cultured EPCs for 4 or 7 days, using RNeasy total RNA isolation kit (QIAGEN) underwent RT-PCR. Two μg of isolated RNA was reverse transcribed into cDNA by Superscript III (Invitrogen). The reverse transcripts were amplified by Advantage 2 PCR kit (Clontech). The primer sequences and product sizes were as follows:

PCR primer pairs with the product sizes

vWF (383 bp);

sense 5'- ACA AGG CTG TGT ACT CAG TCA TG -3'

antisense 5'- GCA CAA TGT CCT CTC CAG ACT C -3'

CD31 (704 bp);

sense 5'- ACA AGG CTG TGT ACT CAG TCA TG -3'

antisense 5'- GCA CAA TGT CCT CTC CAG ACT C -3'

GAPDH (982 bp);

sense 5'- TGA AGG TCG GAG TCA ACG GAT TTG – 3

antisense 5'- CAT GTG GGC CAT GAG GTC CAC CAC -3'

SM-V;

Real Time PCR assay for the Assessment of ER α and ER β Expression in Cultured Human EPCs

Real time PCR assay for the same transcripts by RT reaction was also performed for the assessment of ER α or ER β gene expression of day 4 and day 7 harvested EPCs. Using the reaction mixture of Taq Man Universal PCR Master Mix (Roche), the transcripts were

amplified by real time PCR system, ABI 7700 (Applied Biosystems). The primer pairs were purchased (Hs 00174860_A1 ESR1 for ER α, Hs 00230957_A1 ESR2 for ER β, and Human ACTB 435297 for β-actin, Applied Biosystems Inc), and adjusted with Taq Man Gene Expression Assay Mix (Applied Biosystems). The reaction protocol was as follows: 45 cycles of 95 C°/15 secs for denature, and 60 C°/1 minute for annealing/extension following 95 C°/20 secs for denature in 25 μl volume per each reaction.

SM-VI;

Mouse Cornea Neovascularization Assay

Following 4 days E2 exposure, E2 pellet was removed and a cornea micropocket with VEGF pellet was created. At 6 days after cornea micropocket surgery, neovascularization was assessed in OVX mice.

SM-VII;

Study Design of BMT animal Experiments

At 3 wks post-BMT, the mice underwent OVX (Tie-2/LacZ/BMT/OVX mice). A 1.7 mg sustained (90 days)-release pellet of E2 (E2 pellet) was delivered in order to reach serum levels similar to that of ovulatory women (500-600 pg/ml). The pellet of E2 or placebo (P pellet) (Innovative Research of America) was subcutaneously implanted to each mouse under anesthesia, 3 wks after surgery.

SM-VIII;

Cellular Identification of LacZ Expressing Cells in Uterus or Cornea of Tie-2/LacZ/BMT/OVX Mice

Immunohistochemistry was performed with a rat monoclonal antibody against mouse CD31 (553370, BD Pharmingen). To differentiate between BM-derived EPCs and tissue macrophages, the tissues at day 7 were stained using a rat/anti-mouse F4/80 antibody (MCA497GA, Serotec), a specific marker for tissue macrophages and the isotype of rat /anti-mouse IgG2b.

SM-IX;

Investigation of ER α and ER β expression by BM-derived EPCs incorporated into the Uterus of Tie-2/EGFP/BMT/OVX Mice

Tie-2/EGFP/BMT/OVX mice were also constructed to investigate ER α or ER β expression by BM-derived EPCs incorporated into the uterus. Using female Tie-2 transgenic mice overexpressing EGFP by Tie-2 promoter (FVB/N-TgN(TIE2GFP)287Sato, Jackson Lab, Bar Harbor, ME) as donors and the age-matched female nude SCID mice as recipients, Tie-2/EGFP/BMT/OVX mice were developed, according to the same BMT protocol as Tie-2 LacZ/BMT/OVX mice. The uteri were harvested for fluorescence immunohistochemistry of ER α and ER β expressions, following the E2 pellet administration.

Fluorescence Immunohistochemistry to detect ERs in Uterus of Tie-2/EGFP/BMT/OVX Mice

The frozen sections of uterus embedded in OCT compound were fixed with 4% paraformaldehyde, washed with PBS for 5 minutes, three times, blocked with 10% rabbit serum in PBS containing 0.1% Triton-X (PBST) for 60 minutes at RT. The sections were incubated with goat anti mouse ER α (1:50, ERRa (V-19): sc-32971, Santa Cruz Biotec.) or ER β (1:200, ERRa (Y-19): sc-6821, Santa Cruz Biotec.) antibody diluted in 5% rabbit serum containing PBST (ER α; 1:50, ER β; 1:200) at 4 C° for overnight. After washing with PBS,