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Adult Tissue Angiogenesis: Evidence for Negative Regulation by Estrogen in the Uterus

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Increased uterine vascular permeability and angiogenesis are two major events of embryo implantation and placentation during pregnancy. These latter processes require coordinated, uterine-specific interactions between progesterone (P_4) and estrogen (E) signaling. Although roles of these steroids have long been suspected, definitive functions of E and/or P_4 in uterine angiogenesis still remain elusive. We have therefore exploited the availability of reporter and mutant mice to explore the regulation of angiogenesis in response to steroid hormonal changes *in vivo*. We present here molecular, genetic, physiological, and pharmacological evidence that E and P_4 have different effects *in vivo*: E promotes uterine vascular permeability but profoundly inhibits angiogenesis, whereas P_4 stimulates angiogenesis with little effect on vascular permeability. These effects of E and P_4 are mediated by differential spatiotemporal expression of proangiogenic factors in the uterus. (*Molecular Endocrinology* 15: 1983–1992, 2001)

UNDER PHYSIOLOGICAL CONDITIONS, angiogenesis, the process by which new blood vessels originate from preexisting vessels, primarily occurs in the uterus and ovary in the adult during the reproductive cycle and pregnancy (1). Indeed, increased vascular permeability and angiogenesis are essential to successful implantation and placentation. Several studies have provided indirect and descriptive evidence for the potential roles of estrogen (E) and progesterone (P_4) in these processes in various species (reviewed in Refs. 2–4). As described below, these studies primarily examined the changes in the whole uterus and the expression of a number of gene products known to control vascular permeability and angiogenesis, including vascular endothelial growth factor (VEGF) and its receptors, without investigating its angiogenic status. Thus, *in vivo* roles for E and P_4 in uterine angiogenesis are still unclear. In this study, we have used a combination of molecular, genetic, physiological, and pharmacological approaches to address this question.

VEGF, originally discovered as a vascular permeability factor (reviewed in Ref. 5), is also a potent mitogen for endothelial cells and a key regulatory growth factor for vasculogenesis and angiogenesis (6). Targeted disruption of even one allele of the *Vegf* gene results in embryonic death *in utero* on d 10.5 with aberrant blood vessel formation (7, 8). Differential splicing of the *Vegf* gene generates several VEGF isoforms in both humans and mice; $VEGF_{121}$ and $VEGF_{165}$ are the predominant isoforms in humans, whereas isoforms $VEGF_{120}$ and $VEGF_{164}$, which are shorter by one amino acid, are most abundant in mice (4, 9).

Abbreviations: E, Estrogen; E2, estradiol-17β; NRP1, neurophilin 1; P_4, progesterone; PECAM, platelet endothelial cell adhesion molecule; *rpL7*, ribosomal protein L7; VEGF, vascular endothelial growth factor.

VEGF effects are primarily mediated by two tyrosine kinase receptors: FLT1 (VEGF receptor 1) and FLK1/KDR (VEGF receptor 2) (10–13). Although FLT1 activation does not stimulate endothelial cell mitosis, targeted disruption of the *Flt1* gene produces impaired endothelial cell assembly into blood vessels and embryonic lethality (14). FLK1 is the major transducer of VEGF signals that induce chemotaxis, actin reorganization, and proliferation of endothelial cells (6, 15, 16). Targeted deletion of the *Flk1* gene in mice produces defects in hematopoietic and endothelial cell development leading to embryonic death by d 9.5 (17).

Recently, another multifunctional VEGF receptor has been identified as neuropilin-1 (NRP1). NRP1 was originally described as a neuronal transmembrane receptor that participates in axonal guidance in the developing nervous system (18, 19) and is a receptor for the collapsin/semaphorin family of proteins (20, 21). It is now known that NRP1 functions as a receptor for at least five different ligands, collapsin-1/semaphorin-IIII/D, semaphorin-E, semaphorin-IV, $VEGF_{165}$, and placental growth factor, which are involved in different biological processes such as nervous system development, vasculogenesis, and angiogenesis (21, 22). NRP1 is expressed in human endothelial cells as a $VEGF_{165}$-specific receptor. When coexpressed in endothelial cells with FLK1, NRP1 enhances the binding of $VEGF_{165}$ to FLK1 and $VEGF_{165}$-mediated chemotaxis severalfolds higher than that of FLK1 alone (23). Conversely, inhibition of $VEGF_{165}$ binding to NRP1 inhibits its binding to FLK1 and its mitogenic activity for endothelial cells. *Nrp1*-deficient mice show peripheral nervous system abnormalities and die in midgestation due to yolk sac vascular insufficiency and developmental anomalies of the cardiovascular system (24). Mice overexpressing NRP1 also show cardiovascu-

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lar abnormalities including increased number of blood vessels and abnormal hearts (25).

We have recently shown that the genes encoding murine VEGF isoforms and their receptors, FLT1, FLK1, and NRP1, are differentially expressed in the mouse uterus in a spatiotemporal manner during implantation, and that the predominant $VEGF_{164}$ isoform interacts with FLK1 and NRP1 (2, 4). These results provide evidence that the VEGF system is important for uterine vascular permeability and angiogenesis during implantation. Others have also shown the expression of VEGF and its receptors in the uterus as a whole during pregnancy and in response to steroid hormones (reviewed in Ref. 3). For example, E rapidly induces uterine vascular permeability and *Vegf* expression transcriptionally via nuclear ER (reviewed in Refs. 3 and 26). In addition, the *Vegf* gene contains E response elements (26). P_4 also up-regulates uterine *Vegf* expression, but at a slower rate, via activation of its nuclear receptor, PR (26). Because E rapidly stimulates uterine vascular permeability and *Vegf* expression, and because vascular permeability is considered a prerequisite for angiogenesis, it is widely believed that E is a potent stimulator of uterine angiogenesis during normal reproductive processes *in vivo*. However, there is no experimental evidence to support this conclusion.

In this paper, we have used a combination of approaches to address in detail the roles of ovarian steroids on uterine angiogenesis in mice. Unexpectedly, we found that although E promotes uterine vascular permeability, it profoundly attenuates angiogenesis. In contrast, P_4 stimulates uterine angiogenesis with little effect on vascular permeability.

RESULTS

E and P_4 Differentially Regulate the Spatiotemporal Expression of Vegf and Flk1 in the Uterus

In the mouse uterus, E elicits an early response (phase I) and a late response (phase II) (27, 28). The phase I response is characterized by increased uterine vascular permeability and edema that reach a maximum after 6 h of E administration. The phase II uterine response occurs between 12 and 24 h of E treatment and represents the "true growth" phase, *i.e.* cellular proliferation and hypertrophy (28, 29). Furthermore, P_4 attenuates the phase I estrogenic responses (reviewed in Ref. 30). Because VEGF signaling is a strong stimulator of vascular permeability, we therefore examined the temporal and cell-specific expression of *Vegf* and *Flk1* in the mouse uterus in response to estradiol-17β (E2) and/or P_4 by Northern and *in situ* hybridization. Ovariectomized mice were given an injection of oil (control), E2, P_4, or E2 plus P_4, and uterine RNA was extracted at various times. As previously reported (2), we detected multiple transcripts of *Vegf* in uterine poly(A)$^+$ RNA samples with 4.2 kb as a major transcript (Fig. 1). When normalized to a gene encoding ribosomal protein L7 (*rpL7*), the levels of *Vegf* mRNA showed an early, but transient, increase after E2 injection, with peak levels between 2 and 4 h (260% increase over the oil-treated controls) followed by a decline reaching a nadir by 24 h (Fig. 1). In contrast, *Vegf* mRNA levels after a P_4 injection were modest and remained more or less steady, except an increase (125% increase compared with controls) at 12 h. The pattern of uterine levels of *Vegf* mRNA after a combined injection of E2 and P_4 was similar to those of E2 treatment alone, except the levels were a little lower (170% increase at 4 h). These results corroborate those of a recently published report (26).

Because signaling of VEGF via FLK1 is an essential component of angiogenesis, we also examined the uterine expression of the gene encoding FLK1 in response to steroid hormones (Fig. 1). An E2 injection resulted in an early increase in uterine *Flk1* mRNA (~7.0 kb) levels that peaked at 6 h (205% over the controls). This was followed by a decline reaching a level similar to that of the control (oil) at 24 h. After an injection of P_4, however, an increase in the level of *Flk1* mRNA was observed at 6 h, reaching a peak at 12 h (165% increase over the controls), and sustained

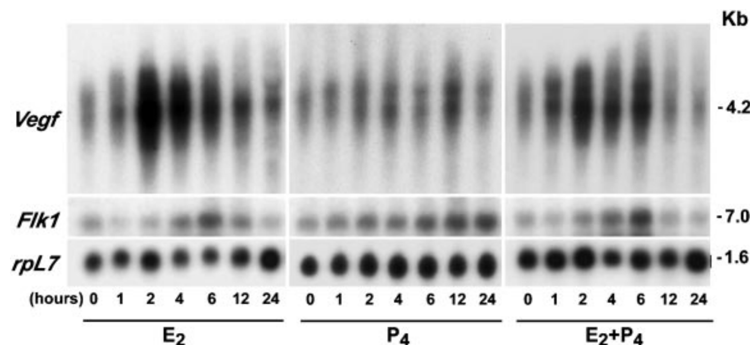

Fig. 1. Northern Blot Detection of *Vegf* and *Flk1* mRNAs in the Mouse Uterus

Poly(A)$^+$ RNA samples (2 μg) of uteri after steroid hormone treatments were separated by formaldehyde-agarose gel electrophoresis, transferred to nylon membranes, UV cross-linked, and hybridized to specific ^{32}P-cRNA probes. The same blots were stripped and rehybridized to a *rpL7* (a house-keeping gene) probe to confirm integrity of RNA samples.

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through 24 h. The pattern of levels in E2 plus P_4-treated uteri was similar to those of E2 treatment alone. These results suggest that whereas E2 regulates the uterine expression of *Vegf* and its receptor *Flk1* in an early and transient manner, P_4 has more sustained effects on the expression of these genes.

The early but transient rise in uterine levels of *Vegf* and *Flk1* mRNAs seen within 6 h of an E2 injection (Fig. 1) is consistent with a role for VEGF in uterine vascular permeability. However, Northern analysis gives no indication of the uterine cell types that are responding by increased gene expression, and levels of whole uterine mRNAs by Northern hybridization may have limited meaning because of the dilution effects resulting from heterogeneous uterine cell types in which myometrial cells constitute the major cell population. We therefore examined the cell-specific expression of *Vegf* and *Flk1* mRNAs by *in situ* hybridization in the ovariectomized uterus at different hours after an E2 injection. Indeed, differential cell-specific expression of *Vegf* and *Flk1* mRNAs were observed with changing time. As shown in Fig. 2A, *Vegf* mRNA accumulation was markedly up-regulated, primarily in stromal cells, within 2 h of an E2 injection and persisted through 6 h. In contrast, whereas the levels of *Flk1* mRNA were very low at 2 h, a marked increase was noted at 6 h of E2 treatment. The prompt increase in *Vegf* expression at 2 and 6 h was accompanied by an up-regulation of *Flk1* expression in the stromal bed at 6 h after an injection of E2 (Fig. 2A). This result suggests that VEGF is important for uterine vascular permeability changes that are induced by E during the phase I response.

Because vascular permeability is normally followed by angiogenesis, we next examined the expression of *Vegf* at 24 h after an injection of oil (control), E2, P_4, or E2 plus P_4 (Fig. 2B). Low to modest levels of *Vegf* mRNA accumulation were noted in both the epithelial and stromal cells of ovariectomized mice receiving an injection of oil (control). Surprisingly, *Vegf* expression was primarily limited to the luminal epithelium at 24 h of E2 treatment; the stromal expression was very low. In contrast, *Vegf* mRNA accumulation was prominent and primarily stromal in response to P_4 alone. The expression pattern in response to P_4 plus E2 was similar to that of P_4 alone, but at lower levels (Fig. 2B). The differential cell-specific expression of *Vegf* in response to E2 and/or P_4 prompted us to examine the cell-specific expression of *Flk1* in steroid-exposed ovariectomized uteri. As shown in Fig. 2B, the accumulation of *Flk1* mRNA in stromal endothelial cells was very low in oil-treated control uteri and was not altered 24 h after an E2 injection. In contrast, the accumulation of *Flk1* mRNA was remarkably up-regulated in endothelial cells of the stromal bed 24 h after an injection of P_4. The combined injection of P_4 with E2 resulted in an accumulation pattern similar to that of P_4 alone, albeit at lower levels. The late induction of stromal *Vegf* and *Flk1* by P_4 alone suggests that P_4 positively regulates uterine angiogenesis. E, on the other

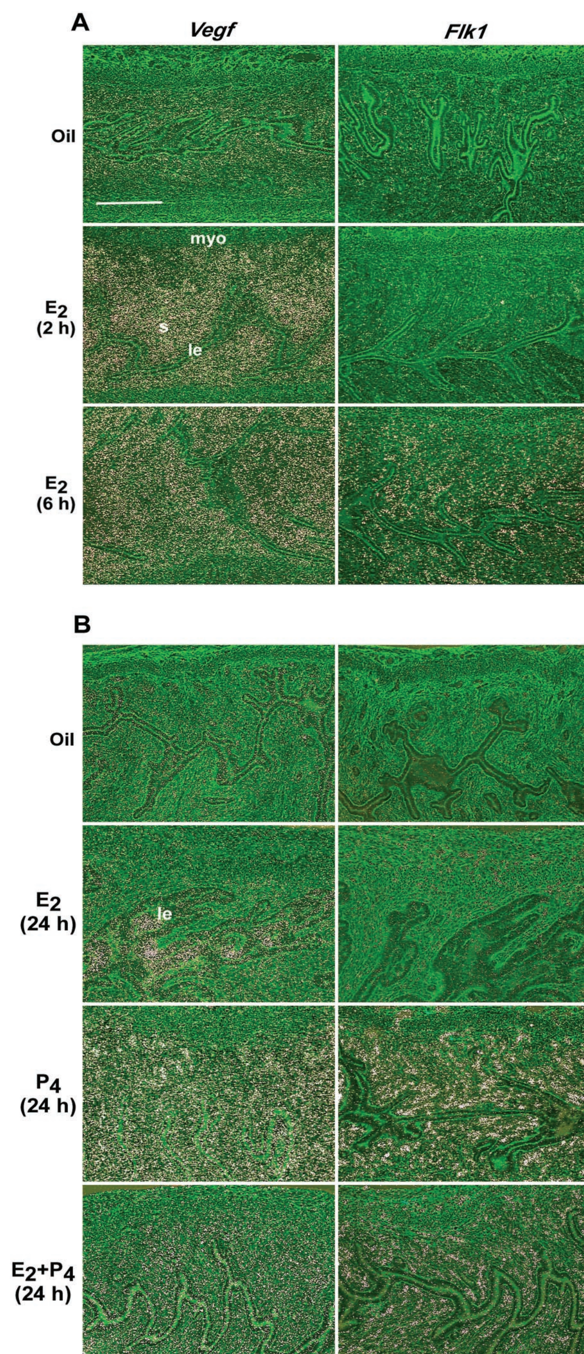

Fig. 2. *In Situ* Hybridization of *Vegf* and *Flk1* mRNA in Mouse Uteri After Steroid Hormone Treatments

A, Two and 6 h after oil (control) or E2 treatment. B, Twenty-four hours after oil, E2, P_4, or E2 plus P_4 treatment. Representative dark-field photomicrographs of longitudinal sections of uteri are shown (*bar*, 60 μm). le, luminal epithelium; s, stroma; myo, myometrium.

hand, appears to be a negative regulator of uterine angiogenesis, because *Vegf* and *Flk1* expression in the stromal bed is very low in response to this steroid during the late phase, and P_4-induced changes are attenuated by E.

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Effects of E and P_4 on Uterine Expression of Angiogenic Factors are Mediated by Their Nuclear Receptors

E and P_4 effects in the uterus are primarily mediated via activation of nuclear ERα and PR, respectively. Because these steroids differentially regulate uterine expression of *Vegf* and *Flk1* in a spatiotemporal manner, we sought to examine whether the effects of E or P_4 on the expression of these genes are direct and mediated via their nuclear receptors. Thus, we examined the expression of *Vegf* and *Flk1* in uteri of mice with a null mutation for the *ERα* or *PR* gene by *in situ* hybridization. Our results show distinct expression of stromal *Vegf* and endothelial *Flk1* in the endometrial bed in *ERα(−/−)* mice (Fig. 3A). In contrast, uterine expression of *Vegf* and *Flk1* in *PR(−/−)* mice was very scanty (Fig. 3B), and the response was not altered in ovariectomized *PR(−/−)* mice by treatment with E2 or P_4 (data not shown). The level and pattern of expression of *Flk1* mRNA in *ERα(−/−)* and *PR(−/−)* uteri correlate well with *lacZ*-stained endometrial blood vessels under a *Flk1* promoter in *ERα(−/−)* × *Flk1(+/−)^{lacZ}* and *PR(−/−)* × *Flk1(+/−)^{lacZ}* double mutant female mice, respectively (compare Fig. 3 *vs.* Fig. 6A). Uteri of *ERα(−/−)* mice contain PR and respond to P_4 with respect to gene expression and decidualization (31–33). The results provide genetic evidence that higher *Flk1* expression in *ERα(−/−)* uteri is primarily due to P_4 effects, whereas the attenuated expression in *PR(−/−)* uteri is the result of predominant E action. We next sought to determine the effects of E and/or P_4 on uterine angiogenesis.

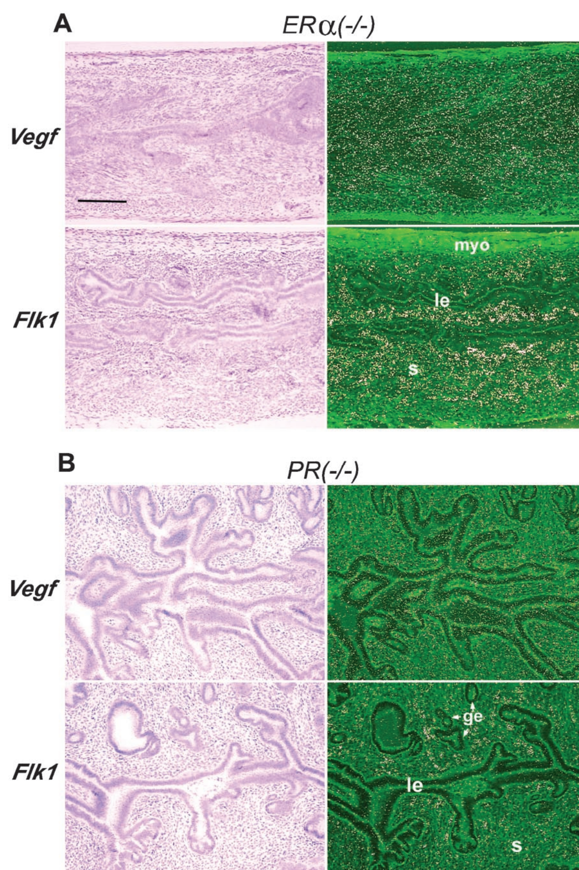

Fig. 3. *In Situ* Hybridization of *Vegf* and *Flk1* mRNAs in Uteri of Intact *ERα(−/−)* (A) and *PR(−/−)* (B) Mice

Representative bright- and dark-field photomicrographs of longitudinal uterine sections are shown (*bar*, 60 μm). le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium.

E and P_4 Differentially Regulate Uterine Angiogenesis

FLK1 (VEGF receptor 2) is a well established marker of endothelial cell development and angiogenesis (34, 35). Mice in which the *Flk1* gene has been disrupted with an insertion of the *Escherichia coli β-galactosidase* (*lacZ*) gene (17) were used to study uterine angiogenesis. Although the *Flk1(−/−)^{lacZ}* embryos die *in utero*, *Flk1(+/−)^{lacZ}* embryos are viable, and *Flk1(+/−)^{lacZ}* females have normal fertility. Thus, *Flk1(+/−)^{lacZ}* mice serve as a powerful genetic model, with β-galactosidase expression as a read-out for *Flk1* promoter activity and as an endothelial cell marker to examine uterine angiogenesis under different physiological and experimental conditions.

To examine the status of uterine angiogenesis in response to E2 and/or P_4, ovariectomized *Flk1(+/−)^{lacZ}* mice received injections of oil, P_4, and/or E2 once daily for 2 d and were killed 24 h after the last injection. When compared with oil-treated (control) mice, endometria of mice treated with P_4 showed an increased density of *lacZ*-stained blood vessels (Fig. 4A). In contrast, mice treated with E2 exhibited a remarkably reduced number of such endometrial blood vessels, even lower than those of the oil-treated mice. Mice that received both P_4 and E2 injections displayed a number of *lacZ*-stained endometrial blood vessels intermediate between P_4- and E2-treated mice (Fig. 4A). To quantify the extent of endometrial angiogenesis, we measured the area of uterine sections occupied by *lacZ*-stained blood vessels. The mean percentage of endometrial area occupied by *lacZ*-stained blood vessels is shown in Fig. 4B. Furthermore, the inhibitory response of E2 or stimulatory effects of P_4 on uterine angiogenesis were reversed by IC1–182,780, an ER antagonist, or RU-486, a PR antagonist (Fig. 4C). These results suggest that whereas E2 is profoundly inhibitory, P_4 is stimulatory to uterine angiogenesis. Furthermore, E2 is capable of counteracting the stimulatory effects of P_4 on uterine angiogenesis. This observation prompted us to examine the long-term effects of sustained stimulation of the uterus with P_4 or E2 using SILASTIC brand (Dow Corning Corp, Midland, MI) implants (36). In ovariectomized *Flk1(+/−)^{lacZ}* mice with SILASTIC brand implants containing P_4 or E2 for 4 d, a similar pattern of *lacZ* staining was noted as observed for steroid treatments of shorter duration (data not shown). We also examined by *in situ* hybrid-

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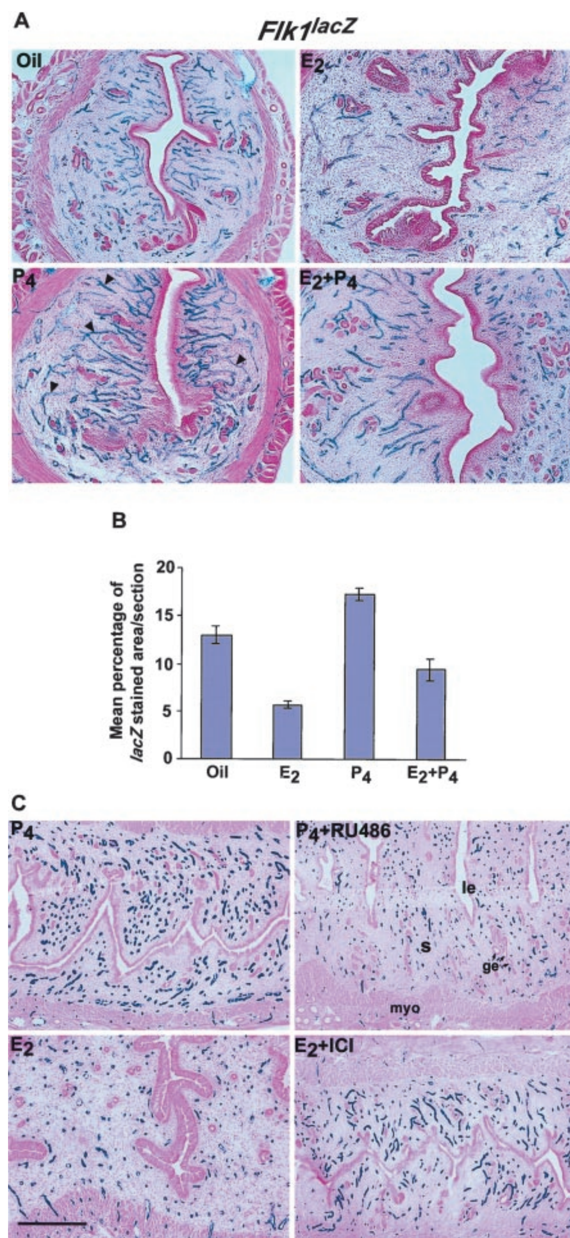

Fig. 4. *lacZ*-Stained Endometrial Blood Vessels in *Flk1(+/−)^{lacZ}* Mice After Steroid Hormone Treatments

Ovariectomized mice were treated with oil, E2, P_4, or E2 plus P_4 once daily for 2 d and killed 24 h after the last injection. A, Photomicrographs of *lacZ* staining of representative uterine cross-sections are shown. B, Quantification of *lacZ* staining is shown as mean percentage of stained endometrial area. Four to six sections from two to three mice in each group were evaluated for quantification. Results are presented as mean ± SEM. Values were statistically significant from each other ($P < 0.05$, ANOVA). C, Ovariectomized mice were treated for 2 d with either P_4, E2, P_4 plus RU-486, or E2 plus ICI-182,780 (ICI). RU-486 and ICI were injected 30 min prior to steroid injections. Mice were killed 24 h after the last injection. Photomicrographs of *lacZ* staining of representative uterine longitudinal sections are shown (*bar*, 60 μm). *Arrowhead*s indicate the location of *lacZ*-stained blood vessels. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium.

ization the expression of *Vegf* and *Nrp1* in uteri of these mice (Fig. 5A). In ovariectomized uteri without the steroid treatment, the expression of *Vegf* and *Nrp1* was modest throughout the endometrium. E2 treatment again suppressed stromal expression of *Vegf*; the expression was primarily restricted to epithelial cells, as observed at 24 h after an E2 injection (compare Fig. 2B *vs.* Fig. 5A). In contrast, the expression was distinct and mostly stromal in P_4-treated mice. The expression of *Nrp1* in E2-treated uteri was observed in both epithelial and stromal cells at very low levels. In contrast, *Nrp1* was abundantly expressed in stromal cells of P_4-treated mice (Fig. 5A). Our observation of up-regulated expression of *Nrp1* in the stroma by P_4 is consistent with a recent report of up-regulated *Nrp1* expression in the rat uterus by P_4, but not by E2 (37). Although the observation of heightened expression of *Vegf*, *Flk1*, and *Nrp1* in the stromal bed in response to P_4 suggests its stimulatory role, their attenuated expression in the stromal bed by E2 suggests its inhibitory role in uterine angiogenesis. Immunolocalization of the platelet endothelial cell adhesion molecule (PECAM), another endothelial cell marker (35), showed similar distribution patterns as *lacZ* staining in steroid-treated uteri (Fig. 5B).

Heightened Angiogenesis with the Loss of ERα Function Is Consistent with Reduced Angiogenesis with the Loss of PR Function in the Uterus

Although uterine estrogenic effects are virtually absent in ERα-deficient mice, uteri in PR-deficient mice are highly estrogenized due to the absence of P_4 effects. These findings led us to examine the status of uterine angiogenesis in mice homozygous for *ERα* or *PR* null mutations but heterozygous for the *Flk1^{lacZ}* mutation. *ERα(+/−)* or *PR(−/−)* males were mated with *Flk1(+/−)^{lacZ}* females to generate *ERα(−/−)* × *Flk1(+/−)^{lacZ}* or *PR(−/−)* × *Flk1(+/−)^{lacZ}* double mutant female mice. Our results of *lacZ* staining show that the density of endometrial blood vessels in *ERα(−/−)* × *Flk1(+/−)^{lacZ}* mice was remarkably higher than that observed in *PR(−/−)* × *Flk1(+/−)^{lacZ}* mice (Fig. 6A). In fact, the lower density of endometrial vessels in the latter group of mice was similar to that observed in ovariectomized *Flk1(+/−)^{lacZ}* mice treated with E2 (Fig. 4A *vs.* Fig. 6A). The density of these endometrial blood vessels was even higher in ovariectomized *PR(−/−)* × *Flk1(+/−)^{lacZ}* mice, and P_4 did not alter this response. However, treatment of such ovariectomized mice with E2 reduced the number of *lacZ*-stained endometrial blood vessels, and as expected, treatment with P_4 did not alter the E2 response (Fig. 6B). However, the angiogenic status in the ovarian theca was similar in both *ERα(−/−)* × *Flk1(+/−)^{lacZ}* and *PR(−/−)* × *Flk1(+/−)^{lacZ}* mice. Likewise, there was no significant alteration in *lacZ* staining in adult brains or skeletal muscles of these double mutant mice (Fig. 6A).

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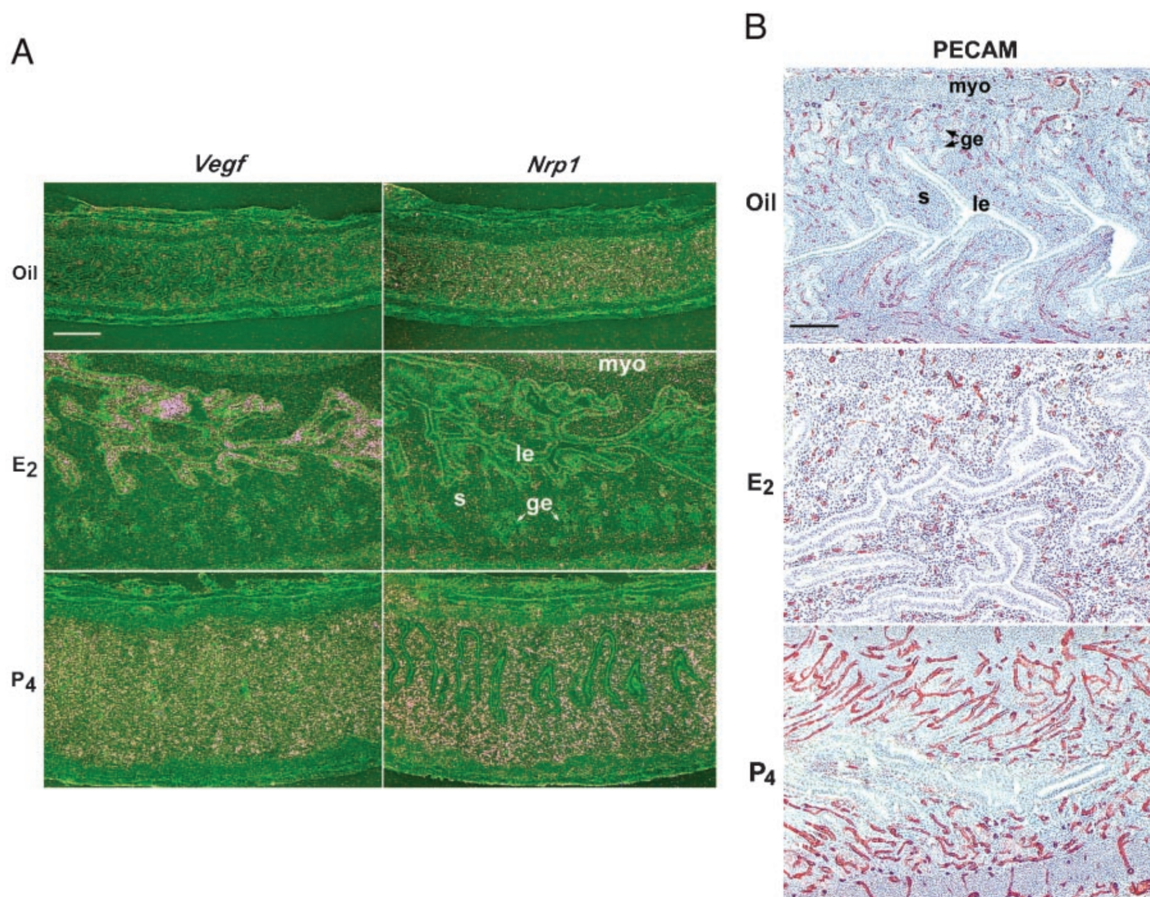

Fig. 5. *In Situ* Hybridization of *Vegf* and *Nrp1* mRNAs and Immunolocalization of PECAM in Mouse Uteri After Long-Term E2 or P_4 Treatment

Ovariectomized mice carrying SILASTIC brand implants containing oil, E2, or P_4 were killed after 4 d, and uteri were processed for *in situ* hybridization and immunohistochemistry. A, Representative dark-field autoradiographic signals of *in situ* hybridization in longitudinal uterine sections are shown (*bar*, 75 μm). B, Frozen longitudinal uterine sections (10 μm) were subjected to immunostaining using a rat-antimouse monoclonal antibody to PECAM (PECAM-1). *Red color* indicates the sites of immunoreactive PECAM (*bar*, 60 μm). le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium.

DISCUSSION

The present investigation provides molecular, genetic, physiological, and pharmacological evidence that E is a potent inhibitor of uterine angiogenesis in mice, and surprisingly, that P_4 is a stimulator of uterine angiogenesis. Furthermore, these effects of E and P_4 are mediated via activation of their cognate nuclear receptors and are specific to the uterus, because angiogenesis in the ovary, brain, and skeletal muscle were similar in mice with null mutations for the *ER*α or *PR* gene. These findings challenge the prevailing notion of E-induced stimulation of uterine angiogenesis (reviewed in Ref. 3). This notion is built on the correlative findings that vascular permeability is followed by increased angiogenesis and that E rapidly induces uterine vascular permeability coincident with increased VEGF expression. Indeed, our observation of rapid, but transient, induction of *Vegf* and *Flk1* in the uterine stromal bed by E supports the idea that E increases uterine vascular permeability during the phase I response. However, our findings of reduced uterine vascular density and decreased stromal endothelial *Flk1* expression during the late estrogenic response suggest that this steroid has an inhibitory role in uterine angiogenesis. This is further confirmed by genetic evidence in *ER*α*(−/−)* mice that display increased density of *lacZ*-stained endometrial blood vessels and increased stromal expression of *Vegf* and *Flk1* in the absence of ERα functions. The role of ERβ is questionable, because uterine expression of *ER*β is very low in *ER*α*(−/−)* mice. Moreover, an ER antagonist (ICI 182,780), which negates both ERα and ERβ functions (38), reversed the inhibitory effects of E2 on uterine angiogenesis. Thus, our findings demonstrate that uterine vascular permeability is not always followed by angiogenesis. This is consistent with the recent observation that although mice deficient in individual Src family kinases show normal angiogenesis, mice lacking in pp60c-src or pp62c-yes, but not fyn, failed to exhibit VEGF-mediated vascular permeability (39).

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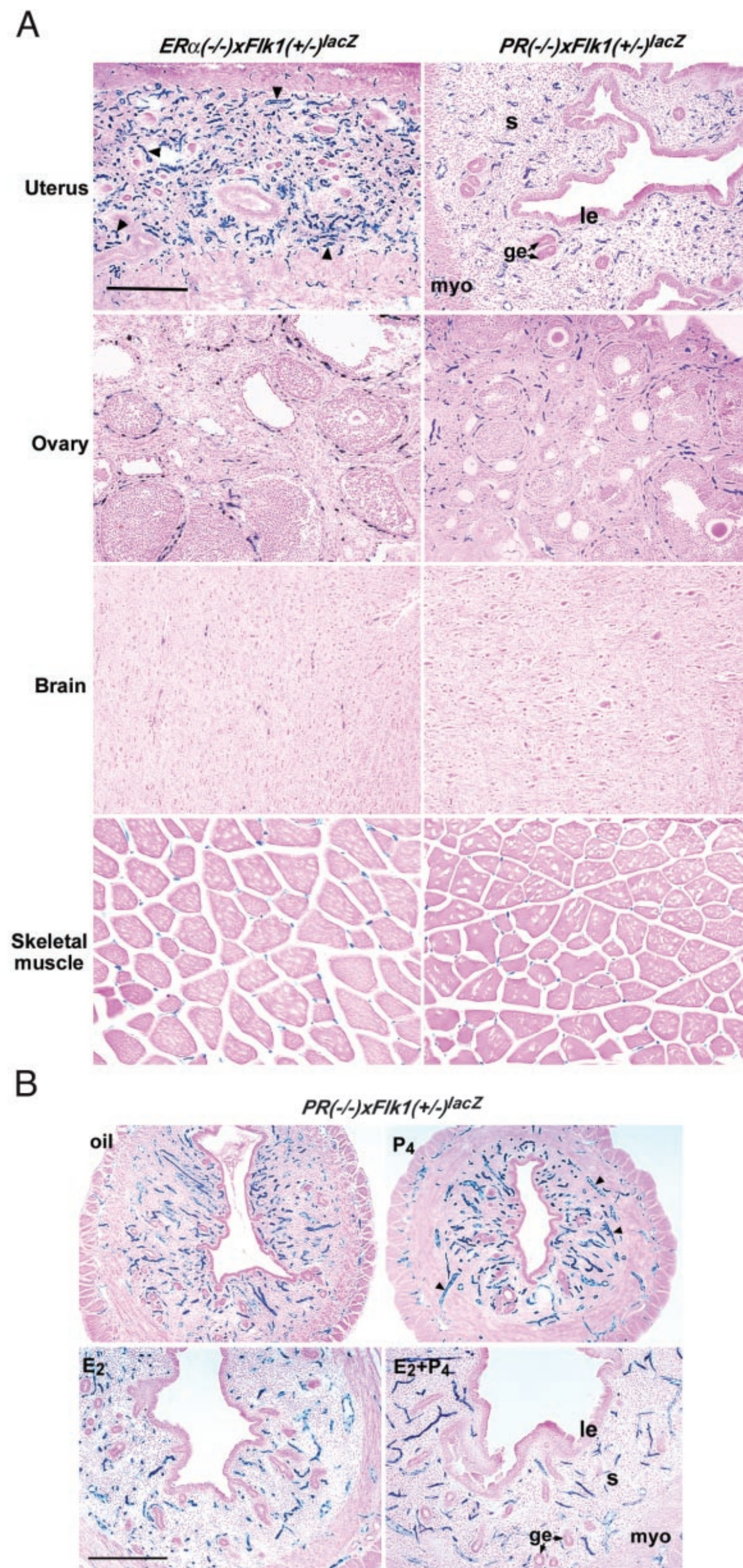

Fig. 6. *lacZ*-Stained Endometrial Blood Vessels in $ER\alpha(-/-) \times Flk1(+/-)^{lacZ}$ or $PR(-/-) \times Flk1(+/-)^{lacZ}$ Double Mutant Mice

A, Photomicrographs of *lacZ*-stained endometrial blood vessels in representative longitudinal uterine sections and cross-sections of the ovary, brain, and skeletal muscle from intact $ER\alpha(-/-) \times Flk1(+/-)^{lacZ}$ or $PR(-/-) \times Flk1(+/-)^{lacZ}$ double mutant mice are shown (*bar*, 60 μm). B, *lacZ*-stained endometrial blood vessels in representative cross-sections of ovariectomized $PR(-/-) \times Flk1(+/-)^{lacZ}$ double mutant mice in response to steroid hormones are shown (*bar*, 60 μm). Ovariectomized mice were injected with oil or steroids once daily for 2 d and killed 24 h after the last injection. *Arrowhead*s indicate the location of *lacZ*-stained blood vessels. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium.

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The restricted expression of *Vegf* in the uterine epithelium with a very low or undetectable level of expression in the stroma during the late phase of estrogenic stimulation suggests that VEGF is not readily available in the stroma for increased angiogenesis. In contrast, our observation of increased vessel density in P_4-treated *Flk1(+/−)^lacZ^* mice, as well as increased accumulation of *Vegf* mRNA in stromal cells and *Flk1* mRNA in stromal endothelia of wild-type mice after P_4 treatment, suggests that this steroid stimulates uterine angiogenesis. This steroid participates in this process via its nuclear receptor, PR, because the vessel density is severely compromised in uteri of *PR(−/−)* × *Flk1(+/−)^lacZ^* double mutant mice with manifestation of predominantly E actions, and reversal of P_4 effects by a PR antagonist RU-486 can be observed in wild-type mice.

P_4 has recently been shown to attenuate *in vitro* proliferation of human endothelial cells derived from various tissues (40). This P_4 effect appears to be mediated via PR, because these cells express PR, and a PR antagonist compromises this P_4 effect. Furthermore, using *PR(−/−)* mice, these investigators provided evidence that P_4 also interferes with re-endothelialization of injured aortae, again suggesting the inhibitory role of P_4 in endothelial cell proliferation (40). The discrepancy between this study and our present investigation is most likely due to the differences in experimental designs and parameters used and cell types studied. Vázquez *et al.* (40) studied the influence of P_4 on proliferation of human dermal and coronary endothelial cells and of mouse brain endothelial cells in culture, whereas our study examined the effects of P_4 and/or E2 on endometrial angiogenesis in mice *in vivo*. They did not examine the effects of P_4 on proliferation of PR-expressing human endometrial endothelial cells. We speculate that ovarian steroids influence endothelial cell functions with respect to angiogenesis in a tissue-specific manner. Because the uterus is a major target for E and P_4, and because heterogeneous cell types of the uterus respond differently to these hormones in a dynamic manner, we suspect that uterine endothelial cells respond to these steroids differently from cells in extrauterine sites. It is also highly possible that angiogenic factors generated by the action of steroid hormones on uterine cell types act on endothelial cells for angiogenesis in a paracrine manner. However, such paracrine effects are absent in endothelial cells in culture.

P_4-induced angiogenesis in the uterus is physiologically meaningful, because this steroid is an essential hormone for the initiation and maintenance of pregnancy in all mammals examined, and uterine angiogenesis is an essential component during pregnancy. Although E and P_4 are critical to implantation and pregnancy maintenance in mice and rats, P_4 alone is sufficient for these events in several species including hamsters, guinea pigs, rabbits, and pigs (reviewed in Ref. 41). This suggests that P_4 is a prime regulator of uterine angiogenesis during early pregnancy in these species. Furthermore, uterine expression of proangiogenic factors is very low on d 1 of pregnancy in mice when the uterus is under the dominance of preovulatory ovarian E secretion. However, dramatic increases in the expression of these factors are observed from d 4 onward, with rising P_4 levels by the newly formed corpora lutea (2, 4). Thus, perhaps a balance between negative and positive influences resulting from coordinated interactions between E and P_4 during early pregnancy determines the normal angiogenic status of the uterus during early pregnancy in mice.

Our results suggest that E-induced early endometrial vascular permeability and stromal edema are mediated by an early induction of VEGF and FLK1 in the stromal bed, whereas the attenuated uterine angiogenesis during the late estrogenic growth phase is the result of suppressed *Vegf*, *Nrp1*, and *Flk1* expression in the stromal bed. In contrast, P_4-induced uterine angiogenesis is executed by late and sustained induction of *Vegf* and *Flk1* in conjunction with *Nrp1* (Fig. 7). Our results also suggest that an increased vascular permeability is not always a prerequisite condition for increased uterine angiogenesis. In conclusion, we provide here the first evidence for the regulation of angiogenesis in a physiologically relevant adult organ system *in vivo*. This information will be important for comparing normal physiological angiogenesis with the process during pathological conditions such as uterine adenocarcinoma, endometriosis, and dysfunctional uterine bleeding. In this respect, it is interesting to note that xenografts resulting from a human endometrial cancer cell line overexpressing ERα showed reduced tumor growth and angiogenesis (42).

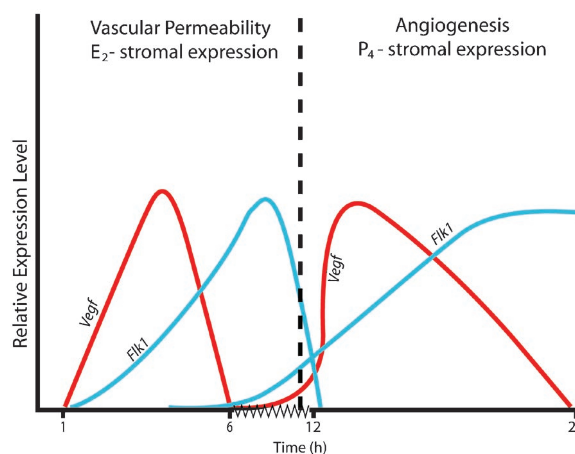

Fig. 7. A Scheme Depicting Uterine Vascular Permeability and Angiogenesis in Response to Steroids

This scheme suggests that in the stromal bed, E2 rapidly, but transiently, induces *Vegf* and *Flk1*, whereas P_4 induces these genes in the stroma in a more delayed and sustained manner.

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MATERIALS AND METHODS

Animals and Treatments

Adult CD-1 mice were purchased from the Charles River Laboratories, Inc. (Raleigh, NC). Flk1-deficient mice were generated by disruption of the *Flk1* gene using homologous recombination in embryonic stem cells (17). A targeting vector was constructed in which the translated portion of the first coding exon and the proximal part of the first intron of the *Flk1* gene were replaced by a promoterless β-*galactosidase* gene from *E. coli*, leaving the *Flk1* promoter intact. Therefore, β-galactosidase expression is used as a read-out for *Flk1* promoter activity. ERα-deficient mice (129/J/C57BL/6J) and PR-deficient mice (129SvEv/C57BL/6) were generated as previously described (31, 43) and were kindly provided by Dennis Lubahn (University of Missouri, Columbia, MO) and Bert O'Malley (Baylor College of Medicine, Houston, TX), respectively, for establishing our colonies. $ER\alpha(-/-) \times Flk1(+/-)^{lacZ}$ double mutant mice were generated by crossing $Flk1(+/-)^{lacZ}$ females with $ER\alpha(+/-)$ males, whereas $PR(-/-)/Flk1(+/-)^{lacZ}$ double mutants were generated by crossing $Flk1(+/-)^{lacZ}$ females with $PR(-/-)$ males. PCR analysis of the genomic DNA determined the genotypes. All mice were housed in the Animal Care Facility at the University of Kansas Medical Center (Kansas City, KS) according to NIH and institutional guidelines for laboratory animals.

To examine the effects of E and/or P_4 on uterine expression of proangiogenic genes or uterine angiogenesis (*lacZ* staining), ovariectomized mice were injected with sesame oil (0.1 ml/mouse), estradiol-17β (E2) (100 ng/mouse), P_4 (2 mg/mouse), E2 and P_4, E2 and ICI 182,780 (an ER antagonist), or P_4 and RU486 (a PR antagonist). ICI 182,780 and RU486 were injected at a dose of 500 μg and 1 mg per mouse per day, respectively. In another set of experiments, ovariectomized mice were implanted sc with SILASTIC brand implants (0.1 $\times$ 1 cm) filled with E2, or implants (0.1 $\times$ 2 cm) filled with P_4, for 4 d. At termination of the treatments, uteri were processed for subsequent analysis. For systemic injections, steroids and antagonists were dissolved in sesame oil and injected sc.

Probes

The cDNA clones for *Vegf*, *Flk1*, *Nrp1*, and *rpL7* have been previously described (2, 4). For Northern hybridization, antisense ^{32}P-cRNA probes were generated, whereas for *in situ* hybridization, sense or antisense ^{35}S-cRNA probes were generated using appropriate polymerases. Probes had specific activities of about 2 $\times$ 10^9 dpm/μg.

Northern Hybridization

For Northern hybridization, total RNA (6.0 μg) or poly(A)$^+$ RNA (2.0 μg) was denatured and separated by formaldehyde/agarose gel electrophoresis, transferred to nylon membranes, and UV cross-linked. Northern blots were prehybridized, hybridized, and washed as previously described (2, 4). Quantification of hybridized bands was analyzed by densitometric scanning.

In Situ Hybridization

In situ hybridization was performed as previously described (2, 4). In brief, frozen sections (10 μm) were mounted onto (poly)L-lysine–coated slides and fixed in cold 4% paraformaldehyde in PBS. The sections were prehybridized and hybridized at 45 C for 4 h in 50% formamide hybridization buffer containing the ^{35}S-labeled antisense cRNA probes. RNase A-resistant hybrids were detected by autoradiography. Sections were post-stained with eosin and hematoxylin. Sections hybridized with the sense probes did not result in any positive hybridization.

lacZ Staining and Quantification

The expression of β-galactosidase was assessed by *lacZ* staining as previously described (44). In brief, small pieces of tissues were fixed in 0.2% paraformaldehyde solution followed by infusion in 30% sucrose at 4 C overnight. Tissues were embedded in OCT and snap-frozen. Frozen sections were mounted onto glass slides and stained overnight at 37 C using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate. Sections were counterstained with eosin. Uterine area occupied by *lacZ*-stained blood vessels was quantitated. Random sections of uteri were used for *lacZ* staining; digital images were obtained, and measurements were made using the Scion Image program (Scion Corp., Frederick, MD). For consistency of measurements, the total uterine stromal area was defined by subtracting the section area occupied by the uterine luminal and myometrial layers, and the percentage of uterine stromal area occupied by *lacZ*-positive vascular structures was measured for each section.

Immunohistochemical Localization of PECAM

Frozen longitudinal uterine sections (10 μm) were subjected to immunostaining using a rat-anti-mouse monoclonal antibody to PECAM (PECAM-1, BD PharMingen, San Diego, CA) at a dilution of 1:50 using a Histostain-SP kit (Zymed Laboratories, Inc., San Francisco, CA) as previously described (45). Red color indicates the sites of immunoreactive PECAM.

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REFERENCES

1. Folkman J 1995 Angiogenesis in cancer, vascular, rheumatoid and other diseases. Nat Med 1:27–31
2. Chakraborty I, Das SK, Dey SK 1995 Differential expression of vascular endothelial growth factor and its receptor mRNAs in the mouse uterus around the time of implantation. J Endocrinol 147:339–352
3. Hyder SM, Stancel GM 1999 Regulation of angiogenic growth factors in the female reproductive tract by estrogen and progesterone. Mol Endocrinol 13:806–811
4. Halder JB, Zhao X, Soker S, Paria BC, Klagsbrun M, Das SK, Dey SK 2000 Differential expression of VEGF isoforms and $VEGF_{164}$ in vascular permeability and angiogenesis during implantation. Genesis 26:213–224
5. Dvorak HF, Nagy JA, Feng D, Brown LF, Dvorak AM 1999 Vascular permeability factor/vascularendothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. Curr Top Microbiol Immunol 237:97–132
6. Ferrara N, Davis-Smith T 1997 The biology of vascular endothelial growth factor. Endocrine Rev 18:4–25

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7. Carmeliet P, Rerreira V, Breier G, Pollefeyt S, Kieckens L, Harpal K, Eberhardt C 1996 Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439
8. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, Orquote-Shea KS, Powell-Braxton L, Hillan KJ, Moore MW 1996 Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442
9. Tischer E, Mitchell R, Hartmann T, Silva M, Godspodarowicz D, Fiddes JC, Abraham J 1991 The human gene for vascular endothelial growth factor. J Biol Chem 266: 11947–11954
10. Peters KG, Devries C, Williams LT 1993 Vascular endothelial growth factor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. Proc Natl Acad Sci USA 90:8915–8919
11. Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M 1990 Nucleotide sequence and expression of a novel human receptor type tyrosine kinase (flt) closely related to the fms family. Oncogene 5:519–524
12. Millauer B, Wizigmann VS, Schnurch H, Martinez R, Moller NPH, Risau W, Ullrich A 1993 High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 72:835–846
13. Quinn TP, Peters KG, De Vris D, Ferrara N, Williams LT 1993 Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc Natl Acad Sci USA 90:7533–7537
14. Fong GH, Rossant H, Gertsenstein M, Breitman ML 1995 Role of Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376:66–70
15. Waltenberger J, Claesson WL, Siegbahn A, Shibuya M, Heldin CH 1994 Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem 269:26988–26995
16. Yoshida A, Anand-Apte B, Zetter BR 1996 Differential endothelial migration and proliferation to basic fibroblast growth factor and vascular endothelial growth factor. Growth Factors 13:57–64
17. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Dreitman ML, Schuh AC 1995 Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376:62–66
18. Kawakami A, Kitsukawa T, Takagi S, Fujisawa H 1995 Developmentally regulated expression of a cell surface protein, Neuropilin, in the mouse nervous system. J Neurobiol 29:1–17
19. Takagi S, Kasuya Y, Shimizu M, Matsuura T, Tsuboi M, Kawakami A, Fujisawa H 1995 Expression of cell adhesion molecule, neuropilin, in the developing chick nervous system. Dev Biol 170:207–222
20. He Z, Tessier-Lavigne M 1997 Neuropilin is a receptor for the axonal chemorepellant semaphorin III. Cell 90:739–751
21. Kalodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD 1997 Neuropilin is a semaphorin III receptor. Cell 90:753–762
22. Neufeld G, Tessler S, Gitay-Goren H, Cohen T, Levi BZ 1994 Vascular endothelial growth factor and its receptors. Prog Growth Factor Res 5:89–97
23. Soker S, Takashia S, Miao HQ, Neufeld G, Klagsbrun M 1998 Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92:736–745
24. Kawasaki T, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, Yagi T, Fujisawa J 1999 A requirement for neuropilin-1 in embryonic vessel formation. Development 126: 4895–902
25. Kitsukawa T, Shimono A, Kawakami A, Kondoh H, Fujisawa H 1995 Overexpresssion of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. Development 121:4309–4318
26. Hyder SM, Nawaz Z, Chiappetta C, Stancel GM 2000 Identification of functional estrogen response elements in the gene coding for the potent angiogenic factor vascular endothelial growth factor. Cancer Res 60:3183–3190
27. Astwood EB 1939 Changes in the weight and water content of the uterus of the normal adult rat. Am J Physiol 126:162–170
28. Huet YM, Dey SK 1987 Role of early and late oestrogenic effects on implantation in the mouse. J Reprod Fertil 81:453–458
29. Harris J, Gorski J 1978 Evidence for a discontinuous requirement for estrogen in stimulation of deoxyribonucleic acid synthesis in the immature rat uterus. Endocrinology 103:240–245
30. Spaziani E 1975 Accessory reproductive organs in mammals: control of cell and tissue transport by sex hormones. Pharmacol Rev 2:207–286
31. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA Jr, Shyamala G, Conneely OM, O'Malley BW 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev 9:2266–2278
32. Paria BC, Tan J, Lubahn DB, Dey SK, Das SK 1999 Uterine decidual response occurs in estrogen receptor-α-deficient mice. Endocrinology 140:2704–2710
33. Hewitt SC, Korach KS 2000 Progesterone action and responses in the α ERKO mouse. Steroids 65:551–557
34. Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML, Rossant J 1993 Flk-1, an flt-related receptor tyrosine kinase, is an early marker for endothelial cell precursors. Development 18:489–498
35. Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K, Nishikawa S-I 2000 Flk-1positive cells derived from embryonic stem cells serve as vascular progenitors. Nature 408:92–96
36. Huet-Hudson YM, Chakraborty C, Dey SK, Suzuki Y, Andrews GK, Dey SK 1990 Estrogen regulates synthesis of EGF in mouse uterine epithelial cells. Mol Endocrinol 4:510–523
37. Pavelock K, Brass KM, Ouafik L, Osol G, May V 2001 Differential expression and regulation of vascular endothelial growth factor receptors neuropilin-1 and neuropilin-2 in rat uterus. Endocrinology 142:613–622
38. Das SK, Taylor JA, Korach KS, Paria BC, Dey SK, Lubahn DB 1997 Estrogenic responses in estrogen receptor-alpha deficient mice reveal a distinct estrogen signaling pathway. Proc Natl Acad Sci USA 94:12786–12791
39. Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresh DA 1999 Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol Cell 4:915–924
40. Vázquez F, Rodríguez-Manzaneque JC, Lydon JP, Edwards DP, O'Malley BW, Iruela-Arispe ML 1999 Progesterone regulates proliferation of endothelial cells. J Biol Chem 274:2185–2192
41. Dey SK 1996 Implantation. In: Adashi, EY, Rock JA, Rosenwaks Z, eds. Reproductive endocrinology, surgery and technology. New York: Lippincott-Raven Press; 421–434
42. Ali SH, O'Donnell AL, Balu D, Pohl MB, Seyler MJ, Mohamed S, Mousa S, Dandona P 2000 Estrogen receptor-α in the inhibition of cancer growth and angiogenesis. Cancer Res 60:7094–7098
43. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162–11166
44. Hogan BLM, Beddington R, Costantini F, Lacy E 1986 Manipulating the mouse embryo, a laboratory manual. New York: Cold Spring Harbor Laboratory Press; section H, pp. 373–375
45. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK 1997 Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91:197–208

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