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The Role of Vascular Endothelial Growth Factor and Estradiol in the Regulation of Endometrial Angiogenesis and Cell Proliferation in the Marmoset

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The present studies explore the roles of vascular endothelial growth factor (VEGF) and estradiol on angiogenesis and stromal and epithelial cell proliferation in the marmoset endometrium during the proliferative phase of the ovulatory cycle. At the start of the proliferative phase, marmosets were 1) treated with vehicle, 2) treated with a VEGF inhibitor (VEGF Trap, aflibercept), 3) ovariectomized, 4) ovariectomized and given replacement estradiol, or 5) treated with VEGF Trap and given replacement estradiol. The uterus was examined 10 d later in the late proliferative phase. Changes in endothelial and epithelial cell proliferation were quantified using a volumetric density method after immunohistochemistry for bromodeoxyuridine to localize proliferating cells, CD31 to visualize endothelial cells, and dual staining to distinguish endothelial cell proliferation. Endothelial proliferation was elevated in late proliferative controls but virtually absent after VEGF Trap. Ovariectomy had a similar inhibitory effect, whereas angiogenesis was restored by estrogen replacement. Estradiol replacement in VEGF Trap-treated marmosets resulted in only a small increase in endothelial cell proliferation that remained significantly below control values. VEGF Trap treatment and ovariectomy also markedly reduced stromal cell proliferation but resulted in increased stromal cell density associated with a reduction in overall endometrial volume. Estrogen replacement in both ovariectomized and VEGF Trap-treated animals restored stromal proliferation rates and cell density. These results show that endometrial angiogenesis and stromal proliferation during the proliferative phase are driven by estradiol and that the effect of estrogen on angiogenesis is mediated largely by VEGF. (*Endocrinology* 149: 4413–4420, 2008)

PHYSIOLOGICAL ANGIOGENESIS, the formation of new blood vessels from preexisting capillaries, is rare in the adult but takes place on a regular cycle in the ovaries and uterus. Vascular endothelial growth factor (VEGF) is a potent stimulator of endothelial cell proliferation and permeability (1). The availability of potent specific antagonists of VEGF such as VEGF Trap (2), allows for the physiological role of VEGF to be investigated by *in vivo* inhibition. In a series of experiments, we have described the role of VEGF in the ovary using the marmoset monkey (*Callithrix jacchus*), a commonly used primate in reproductive research, as a model. Treatment during the 10-d follicular phase resulted in a marked suppression of follicular angiogenesis and follicular development so that ovulation was prevented (3). When administered during the luteal phase, VEGF Trap also markedly suppressed luteal angiogenesis and function (4). These studies established that VEGF is essential for normal follicular and luteal angiogenesis and development.

VEGF and its receptors are expressed in the primate uterus and are likely to be involved in the regulation of uterine angiogenesis and permeability (5, 6). However, the role of VEGF in the primate uterus has yet to be studied by direct experimentation *in vivo*. Such studies carried out in the ovariectomized, estrogen-treated mouse reported that inhibition of VEGF prevented endothelial cell proliferation in one study (7) or had no effect on angiogenesis but inhibited edema and epithelial cell proliferation in another (8). In the immature rat, VEGF immunoneutralization was also shown to block estrogen-induced edema (9) and, in the adult, to prevent implantation (10). The purpose of the present investigation was to examine the changes in the marmoset endometrium after treatment with VEGF Trap with respect to endothelial, stromal, and epithelial cell proliferation. Because normal endometrial function is dependent upon estradiol, and because this steroid has been implicated in the regulation of VEGF (5, 6, 11), we also examined the effects of ovariectomy and estradiol replacement on endometrial angiogenesis. In addition, because VEGF Trap treatment suppresses plasma estradiol by virtue of its inhibitory effects on follicular development, effects of estradiol replacement were also compared in this group.

Materials and Methods

Animals and treatment

Experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986, and approved by the Local Ethical Review Process Committee. In common marmosets (*Callithrix jacchus*), the ovulatory cycle comprises an 8-d follicular phase followed by a 20-d luteal phase, such that follicular recruitment ordinarily takes place during the mid to late luteal phase of the preceding cycle. To synchronize follicular recruitment, selection and ovulation during treatment cycles, marmosets (2–4 yr old), housed as described previously (12), were injected with 1 μg prostaglandin $F_{2\alpha}$ analog (cloprostenol, Planate; Coopers Animal

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Abbreviations: BrdU, Bromodeoxyuridine; VEGF, vascular endothelial growth factor.

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Health Ltd., Crewe, UK) per animal im on d 13–15 of the luteal phase to induce luteolysis. The day of prostaglandin injection was designated follicular d 0. This method of synchronizing follicular recruitment is followed by follicle selection on cycle d 5 and ovulation on d 9–11 (13–15).

To block VEGF, we employed the VEGF Trap (aflibercept) (Regeneron Pharmaceuticals), a recombinant chimeric protein comprising portions of the extracellular domains of the human VEGF receptors 1 and 2 expressed in sequence with the Fc portion of human Ig (2). VEGF Trap binds all isoforms of VEGF-A as well as placenta-derived growth factor. VEGF Trap was at a concentration of 24.3 mg/ml in buffer. In previous studies, we have shown that VEGF Trap (25 mg/kg) given sc every other day on d 0–10 of the cycle or the same dose given once on d 0 are equally efficacious in suppressing follicular angiogenesis (3, 15). In the current study, we used an intermediate schedule of 25 mg/kg sc on d 0, 3, and 7 beginning at the time of prostaglandin $F_{2\alpha}$ analog administration. Animals were killed at 10 d, such that the treatment period covered the duration of the normal proliferative phase. Control animals were studied at the late proliferative phase (d 10) of the normal cycle (n = 6 per group) after treatment with vehicle alone or a control protein, 25 mg/kg recombinant human Fc.

To determine the effects of total withdrawal of ovarian steroids, marmosets were ovariectomized (n = 4) after sedation with ketamine hydrochloride (Parke-Davis Veterinary, Pontypool, Gwent, UK), anesthesia by Saffan (Alphaxalone/Alpadalone; Schering-Plough Animal Health, Welwyn Garden City, UK) and maintenance of analgesia by buprenorphine (Alstoe Animal Health, Melton Mowbray, UK) im as described previously (16). Ovariectomy was carried out between d 13 and 15 of the secretory phase, and uteri collected 10 d later, the same schedule as for the VEGF Trap treatment.

To determine the effects of estrogen replacement, marmosets were treated with VEGF Trap (n = 6 per group) or ovariectomized as above (n = 4 per group), except that estradiol benzoate was administered in increasing doses to mimic the rising concentrations of plasma estradiol observed during the proliferative phase in intact marmosets (14) based on a modification of a method described previously (17). Estradiol benzoate was supplied in a solution of 5 mg/ml in arachis oil (Intervet UK Ltd., Milton Keynes, UK). This was diluted to obtain a solution of 50 μg/ml in arachis oil and was administered sc once daily in doses increasing from 5 to 20 μg as follows: d 0–4, 100 μl; d 5, 150 μl; d 6 and 7, 200 μl; d 8, 300 μl; and d 9, 400 μl.

To obtain further information on changes in cell proliferation at other stages of the cycle, tissue was collected on d 10–16 of the secretory phase (no treatment, represents d 0 in the main study) and at the mid proliferative phase (d 5) (n = 6 per group).

Blood samples were collected three times per week for 6–8 wk pretreatment and during the study period for progesterone assay as described previously. All animals were injected iv with 20 mg bromodeoxyuridine (BrdU; Roche Molecular Biochemicals, Essex, UK) in saline 1 h before being sedated using 200 μl ketamine hydrochloride (Parke-Davis Veterinary) and 200 μl Saffan. After perfusion with 4% neutral buffered formalin, the uterus was removed immediately, weighed, and kept in 4% neutral buffered formalin for 24 h. The tissues were transferred into 70% ethanol, dehydrated, and embedded in paraffin according to standard procedures. Serial sections were cut so that the endometrial area nearest the center of the uterus was selected for study.

Plasma progesterone throughout the study and plasma estradiol on terminal blood samples were measured using assays described previously (4, 18). Levels of unbound VEGF Trap were measured by ELISA as described previously (19) with plasma being diluted 1:10,000.

Hematoxylin and eosin staining

The uteri were embedded so that longitudinal sections could be cut. Uteri were serially sectioned (5 μm) at least to the middle of the tissue where the lumen was clearly visible and tissue sections placed onto BDH SuperFrost Plus slides (Menzel-Glazer, Menzel GmbH & Co., Braunschweig, Germany). Representative sections were stained with hematoxylin and eosin.

Immunohistochemistry

Methods for immunohistochemical detection of endothelial cells by CD31 and proliferating cells by BrdU were as described previously for the ovary (4). For detection of proliferating endothelial cells, dual staining was obtained by immunohistochemistry with CD31 and BrdU. For CD31 detection, the protocol was followed as described above, but visualization was performed with fast red (Sigma, Poole, UK). Sections were then washed with Tris-buffered saline before the second primary antibody, sheep antibody to BrdU (Fitzgerald, Concord, MA) was added, diluted 1:5000 in normal rabbit serum, and incubated overnight at 4 C. After postincubation washes with Tris-buffered saline, a biotinylated rabbit antisheep secondary antibody (Vector, Peterborough, UK) was added, followed by ABC-AP (Dako, Glostrup, Denmark). After incubation with the ABC-AP complex, slides were transferred to nitroblue tetrazolium buffer before staining with nitroblue tetrazolium for 15 min. The reaction was stopped in water, and the slides were air dried and then cleared in xylene and mounted in Pertex.

Volume fraction measurements

Five anatomic zones were evaluated: the luminal epithelium, glands of the functionalis, the stroma in the functionalis, the glands of the basalis, and the stroma in the basalis. Endothelial cells were identified using CD31 and proliferating endothelial cells by dual staining with CD31 and BrdU. Optimal delineation of the endothelial cells was obtained in the absence of counterstain. The glands of the functionalis and basalis were delineated by their location and shape (20). Volume fraction measurements were performed on sections dual stained with BrdU/CD31 as described previously (20), in three non-overlapping regions, perpendicular to the luminal epithelium extending to the myometrium: the first at the top of the uterus and the second and third on the right and left-hand side of the cross-section, respectively. In each region, successive non-overlapping fields of view were analyzed, totaling 20–30 fields per specimen. A test grid,- comprising 864 points was superimposed on each field, and the number of test points falling on glands (including lumen), proliferating (BrdU-labeled) glandular epithelium, uterine lumen, stroma, proliferating stromal cells, luminal epithelium, proliferating luminal epithelial cells, endothelial cells, and proliferating endothelial cells (identified by colocalizing BrdU and CD31 immunostaining) were counted. The volume fraction occupied by each component was then calculated by expressing the number of points hitting that component as a percentage of the total number of test points applied. In this way, for example, the volume fraction of proliferating (BrdU-labeled) endothelial cells could be expressed as a percentage of the total endothelial cell (CD31-labeled) volume fraction.

To assess the effects of the treatment on the area of the endometrial and myometrial compartments, and to determine stromal cell density, a stereological unit consisting of an Axio Imager A1 microscope (Carl Zeiss, Göttingen, Germany) equipped with a video camera (KY-F550; JVC, Yokohama, Japan) and connected to a PC with a computer-assisted stereology system (Image-Pro Plus 6.2 Software; Media Cybernetics, Buckinghamshire, UK) was employed. The system was set up to outline and measure the area of the endometrium and myometrium in a cross-section of the uterus in which the lumen was maximal (*i.e.* approximately the center of the uterus). The endometrial and myometrial areas were outlined (see Fig. 3) and the area calculated using the computer-assisted stereology system. To assess changes in stromal density, the functionalis layer was examined on hematoxylin- and eosin-stained sections, and the slides were analyzed at a ×630 oil immersion objective. The microscope had a motorized stage controlled by the computer for selection of counting frames within an area of interest, in this case the stroma of the functionalis layer where 50 counting frames (45 × 45 μm) were randomly selected. Cell nuclei were counted within each frame, and the stromal density for each animal was taken as the mean of the 50 counts.

Statistical analysis

For volumetric analysis, results were log transformed and analyzed by ANOVA multiple comparison followed by a Bonferroni *post hoc* test. Differences were considered to be significant at $P < 0.05$.

Results

Hormonal changes

Because all treatments were initiated during the secretory phase, plasma progesterone concentrations were elevated at

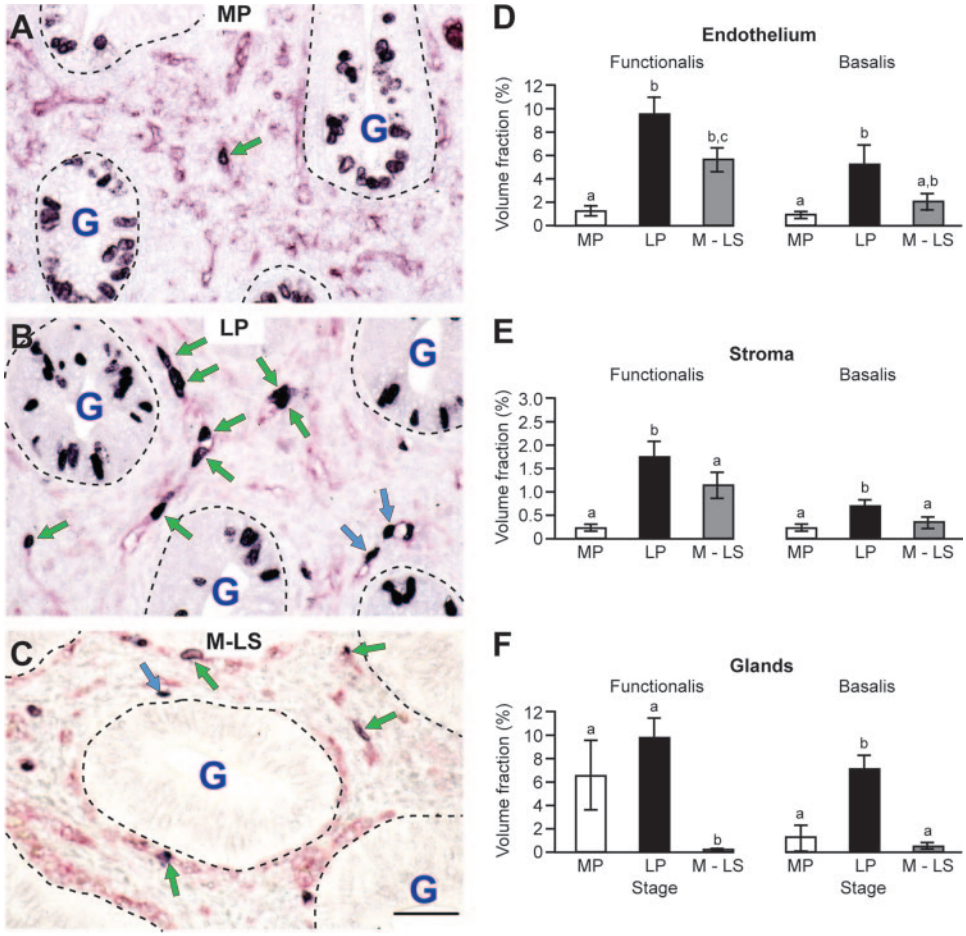

FIG. 1. Functionalis area of endometrium, dual stained for CD31 (*red*) and BrdU (*dark-stained* nuclei) of the endothelium (*green arrows*), stroma/perivascular cells (*blue arrows*) and epithelial cells within glands (G), (*outlined* to distinguish them from stroma) during the mid-proliferative (MP) (A), late proliferative (LP) (B) and mid-late secretory (M-LS) (C) phases of the ovulatory cycle. Volume fraction of proliferating cells is shown for endothelial cells (D), stromal/perivasular cells (E), and epithelial cells of glands (F). Values are means ± SEM; n = 6 per group. Significant differences between stages are denoted by *different letters*. *Scale bar*, 50 μm.

d 0. All marmosets responded to treatment with prostaglandin analog or ovariectomy, with a rapid fall in plasma progesterone levels to follicular-phase values, which were maintained for the remainder of the treatment period (data not shown). Stage of cycle in control animals was also confirmed by ovarian histology as described previously (3). At the end of the experimental period, estradiol levels (means ± SEM) were 1965 ± 466 pmol/liter in late proliferative controls and were significantly reduced ($P < 0.01$) by VEGF Trap (230 ± 24 pmol/liter) and ovariectomy (89 ± 28 pmol/liter). Estradiol benzoate treatment restored plasma estradiol to the normal range; 1964 ± 354 pmol/liter in the ovariectomized group and 2009 ± 279 pmol/liter in the VEGF Trap-treated group by the end of the treatment period. All VEGF Trap-treated marmosets exhibited plasma concentrations of free VEGF Trap (not already bound to VEGF) between 100 and 200 mg/liter at 1 d after treatment, and levels remained above 10 mg/liter through d 10. The detection limit of the assay was 0.5 mg/liter, and it has been estimated that effective pharmacological blockade of VEGF in the ovary is maintained until free VEGF Trap levels fall to less than 1–2 mg/liter (21). Thus, levels of unbound VEGF Trap in all treated marmosets remained within the anticipated pharmacologically effective range for the duration of the study.

Volume fraction measurements

BrdU-positive staining was observed in the nuclei of the epithelial cells of the glands, the CD31-positive endothelial cells within the stroma, and CD31-negative stromal cells located adjacent to blood vessels or dispersed within the stroma (Figs. 1, A–C, and 2A). For the purposes of analysis, the latter two groups were combined, and classified as being stromal.

To obtain an overview of changes during the normal cycle, data from the mid-proliferative, late proliferative, and mid-late secretory phase were compared. Angiogenesis was rarely observed in mid-proliferative phase specimens (Fig. 1A) but was common in the late-proliferative phase, especially in the functionalis next to the luminal epithelium (Fig. 1B). Angiogenesis continued to be observed in most specimens during the mid to late secretory phase (Fig. 1C). Quantitative analysis confirmed that endothelial cell proliferation was lowest during the mid-proliferative phase and rose markedly by the late proliferative phase in both the functionalis ($P < 0.001$) and basalis zones ($P < 0.05$) (Fig. 1D). Endothelial cell proliferation in the functionalis zone remained significantly higher ($P < 0.05$) during the secretory phase than in mid-proliferative phase endometrium (Fig. 1D). Stromal cell proliferation was also extremely low in the mid-proliferative phase and rose significantly in both func-

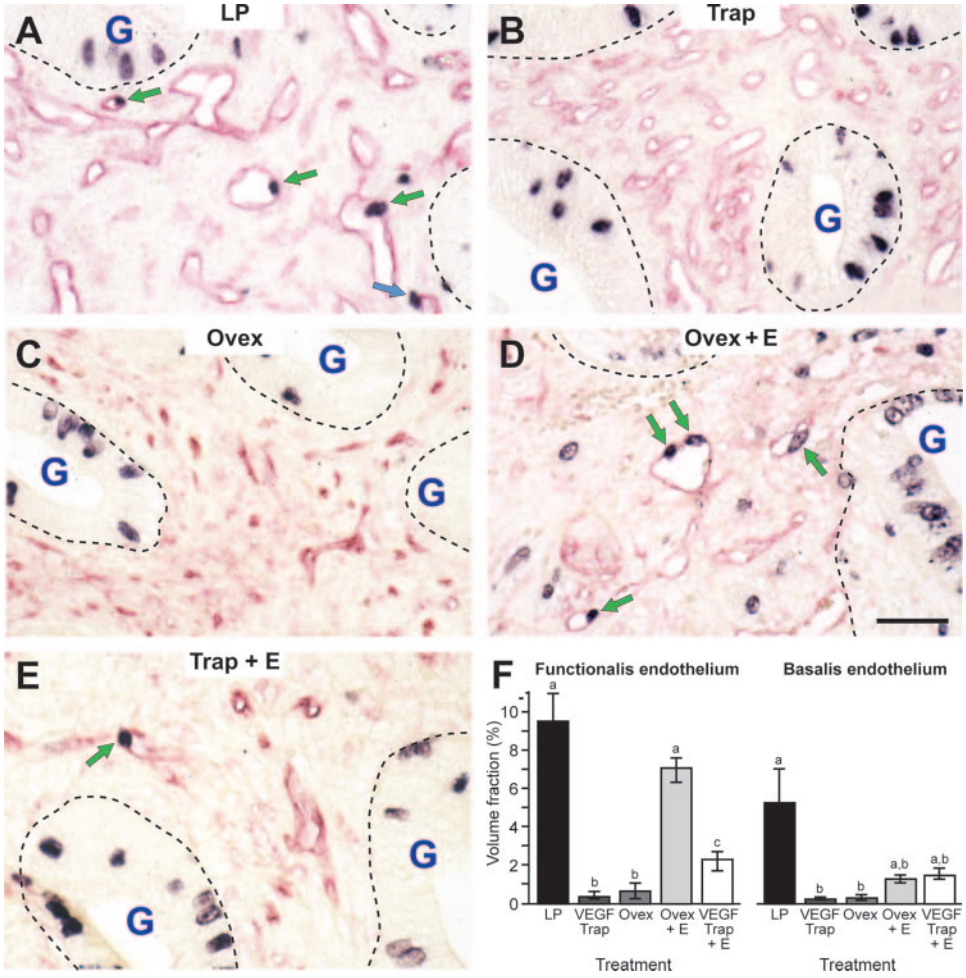

FIG. 2. Functionalis area of endometrium, dual stained for CD31 (*red*) and BrdU (*dark-stained* nuclei) in control late proliferative (LP) (A) phase, after VEGF Trap (Trap) treatment (B), after ovariectomy (ovex) (C), after ovariectomy with estrogen (Ovex + E) replacement (D), and after VEGF Trap with estrogen replacement (Trap + E) (E). Note proliferating endothelial cells (*green arrows*), proliferating perivascular cell (*blue arrow*), and proliferating epithelial cells in glands (G). Histogram (F) shows mean volume fraction of proliferating endothelial cells as a percentage of total endothelial cells within the functionalis and basalis layers. Values are means ± SEM. Significant differences between groups are denoted by *different letters*. *Scale bar*, 50 μm.

tionalis ($P < 0.001$) and basalis ($P < 0.05$) layers by the late proliferative phase (Fig. 1E). In the glands of the functionalis in the mid-proliferative phase, the cell proliferation index was highly variable and not significantly different from the peak levels seen in the late proliferative phase (Fig. 1F). In the basalis glands, cell proliferation was significantly greater in the late proliferative compared with mid-proliferative phase ($P < 0.01$) (Fig. F). In the secretory phase, cell proliferation was significantly reduced in the glands of both the functionalis and basalis ($P < 0.001$) (Fig. 1, C and F).

In the main study, abundant dual staining of proliferating endothelial cells was evident in late proliferative controls (Fig. 2A). In marked contrast, endothelial cell proliferation was virtually absent in the uteri of animals treated with VEGF Trap (Fig. 2B) or ovariectomized (Fig. 2C). Abundant endothelial cell proliferation was evident in the functionalis of ovariectomized marmosets that received estrogen (Fig. 2D), but estrogen replacement appeared to produce only a small increase in endothelial cell proliferation in VEGF Trap-treated animals (Fig. 2E). Quantitative analysis revealed that when treatment groups were compared with late proliferative phase controls, endothelial cell proliferation was significantly lower in VEGF Trap-treated and ovariectomized animals, being virtually absent both in the functionalis ($P < 0.001$) and basalis zones ($P < 0.05$) (Fig. 2F). After estrogen replacement, angiogenesis was fully restored in the functionalis in the ovariectomized animals (Fig. 2F). In contrast, endothelial cell proliferation was reduced in estrogen-treated animals given VEGF Trap compared with controls ($P < 0.05$), although this value was higher than in animals given Trap alone ($P < 0.01$) (Fig. 2F). In the basalis zone, estrogen replacement produced small increases in endothelial proliferation in both ovariectomized and VEGF Trap-treated groups (Fig. 2F). Despite the near total inhibition of endothelial cell proliferation after VEGF Trap or ovariectomy, vascular density within the stroma was not decreased (see Fig. 2, B and C). The likely cause of this paradox was the coincident reduction in stromal volume produced by these treatments. The mean fraction volume of endothelial cells as a percentage of total stromal cells showed no significant difference among groups.

Histology

The gross appearance of the uterus was altered in VEGF Trap-treated and ovariectomized animals, being reduced in size relative to controls. Area measurements confirmed that both the endometrium ($P < 0.01$) and the myometrium ($P < 0.05$) were significantly smaller in the ovariectomized animals than late proliferative controls (Fig. 3, B and C). Al-

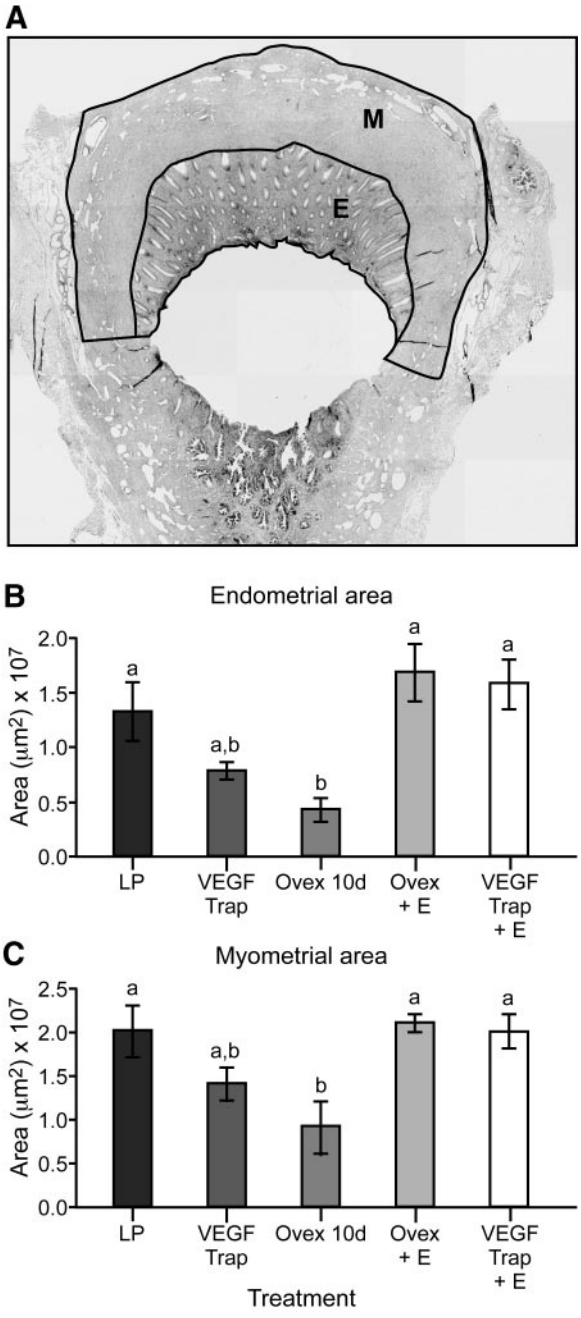

FIG. 3. A, Marmoset uterus showing a typical outline used to calculate myometrial (M) and endometrial (E) areas. The areas of both the endometrium (B) and myometrium (C) were significantly reduced by ovariectomy and fully restored by estrogen replacement. Significant differences between groups are denoted by *different letters*.

though both endometrial and myometrial areas were also reduced in VEGF Trap-treated animals, these intermediate values were not statistically different from those of ovariectomized animals or controls. Estrogen replacement restored both endometrial and myometrial areas to normal in both groups (Fig. 3, B and C).

Further analysis of hematoxylin- and eosin-stained sections revealed that reductions in uterine volume evident in ovariectomized and VEGF Trap-treated animals were associated with decreased cell size and increased cell density. Representative sections from the functionalis zone are shown in Fig. 4. In the uteri of late proliferative controls, stromal cells were large and contained abundant cytoplasm, and the glandular epithelium exhibited the round or oval appearance characteristic of estrogen stimulation (Fig. 4A). After VEGF Trap treatment or ovariectomy, the epithelial cells appeared more densely packed, and cytoplasmic volume appeared reduced relative to controls (Fig. 4, B andC). These changes were even more marked in the stroma of both treatment groups (Fig. 4, B and C). These observed decreases in cell size, and consequent increases in packing density, were reversed by estrogen replacement (Fig. 4, D and E).

BrdU labeling showed that proliferation the epithelium of the functionalis glands was high in controls but was also taking place after ovariectomy or VEGF Trap treatment and also were unaffected by estrogen replacement (Fig. 4, F–J). Quantification revealed no significant differences among groups in the epithelial cell proliferation index in the functionalis (Fig. 4K). In the basalis glands, epithelial cell proliferation was significantly reduced in the VEGF Trap-treated group compared with the controls, and proliferation was restored by estrogen replacement.

Stromal cell proliferation was significantly lower ($P < 0.001$) in VEGF Trap-treated and ovariectomized animals in both the functionalis and basalis zones and was restored by estrogen in both groups (Fig. 4, F–J, M, and N). Despite the clear inhibition of stromal cell proliferation, the number of stromal cells per unit area was increased in both VEGF Trap and ovariectomized animals relative to controls ($P < 0.001$). Treatment of both VEGF Trap and ovariectomized groups with estrogen resulted in significant reductions in stromal density ($P < 0.01$) compared with VEGF Trap and ovariectomy alone (Fig. 4O).

With respect to the luminal epithelial cells, intense proliferation was observed at the late proliferative phase and was significantly reduced ($P < 0.05$) in the ovariectomized group. Proliferation rates were not significantly different from the control for any other treatment group (data not shown), and there were no significant changes in the volume fraction of epithelial cells among the different groups (data not shown).

Discussion

This study provides the first description of the effects of VEGF inhibition on the primate uterus and shows that treatment of marmosets with a potent inhibitor of VEGF over the course of the proliferative phase results in a near complete inhibition of endothelial cell proliferation. In addition, stromal cell proliferation was also markedly suppressed, whereas the stroma itself became condensed. Thus, inhibition of VEGF in the intact marmoset results in an inhibition of endometrial angiogenesis. However, pharmacological inhibition of VEGF in intact animals also inhibits ovarian follicular angiogenesis, leading to reductions in plasma concentrations of estradiol (3, 19). Estrogen is known to stimulate the synthesis of VEGF in the endometrium and may similarly influence the expression of numerous other factors associated with angiogenesis and maintenance of vascular function in primates (6) and rodents (22). Indeed, ovariectomy also inhibited angiogenesis and produced other morphological changes in the uterus

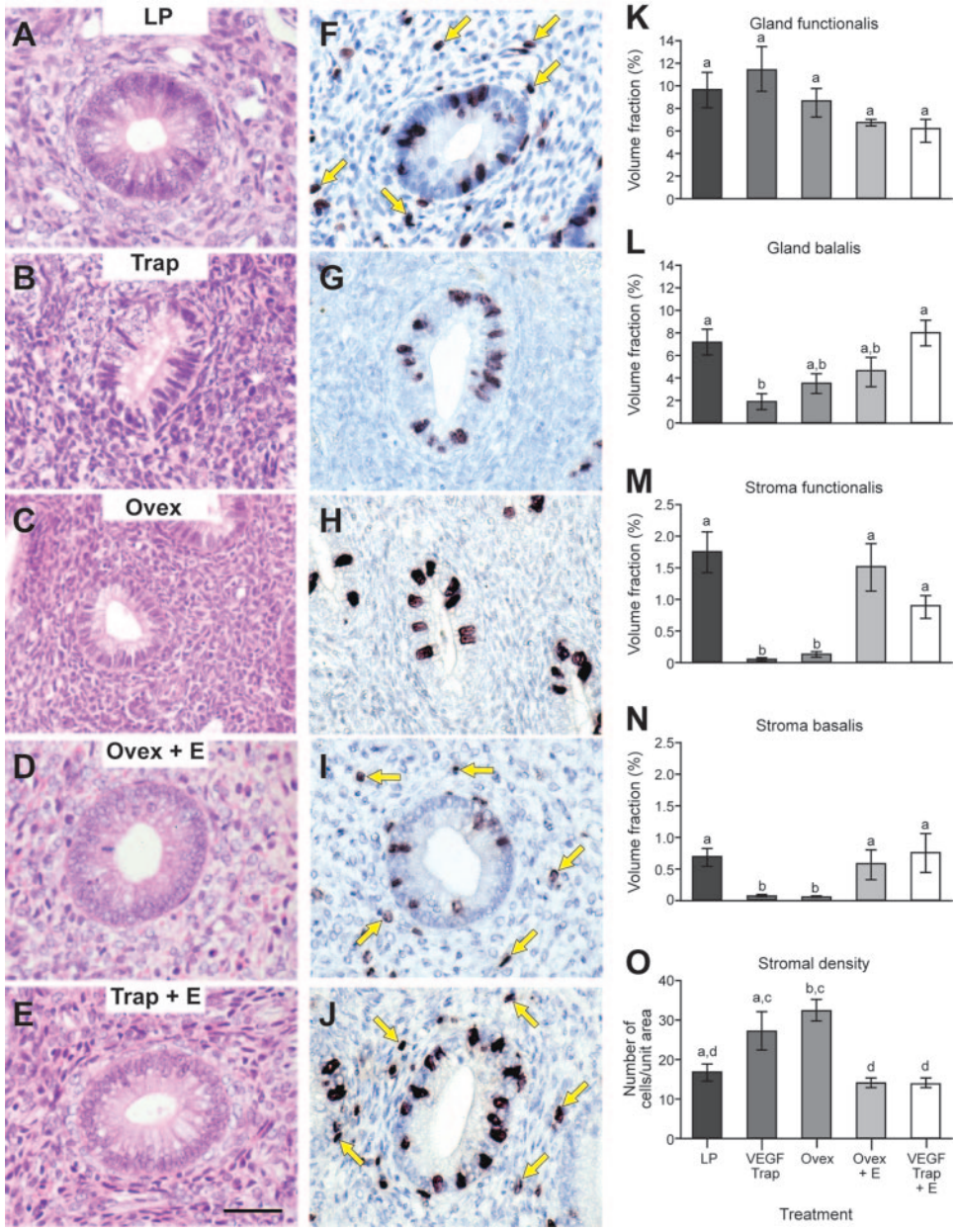

FIG. 4. Hematoxylin- and eosin-stained sections of functionalis of control late proliferative phase (LP) (A), after VEGF Trap (Trap) treatment (B), after ovariectomy (Ovex) (C), after ovariectomy with estrogen replacement (Ovex + E) (D), after VEGF Trap with estrogen replacement (Trap + E), and in corresponding BrdU-stained sections (F–J). Note that VEGF Trap treatment and ovariectomy reduce epithelial cell size in the glands and increase cell density of the stroma; effects reversed by estrogen replacement. In all groups, epithelial cell proliferation (dark-stained nuclei) is present in glands. Note that stromal proliferation in the control (*dark-stained* nuclei, *yellow arrows*) is lost after VEGF Trap treatment or ovariectomy but is restored by estrogen replacement. Quantification of cell proliferation (K–O) shows that VEGF Trap decreased glandular proliferation in the basalis glands, and this was reversed by estrogen replacement. Stromal proliferation was suppressed in both the functionalis and basalis glands after VEGF Trap or ovariectomy, and this effect was reversed by estrogen replacement. Quantitative analysis also confirmed that cell density in the stroma (O) was increased by VEGF Trap treatment or ovariectomy, and this effect was reversed by estrogen replacement. Values are means ± SEM. Significant differences between groups are denoted by *different letters*. *Scale bar*, 50 μm.

that resembled those seen after VEGF inhibition. Therefore, we also assessed the effects of estrogen replacement on these parameters in animals that were treated with VEGF Trap or ovariectomized. All morphological and proliferative changes in the uterus that resulted from ovariectomy were substantially normalized by estrogen replacement. Estrogen replacement also fully restored stromal proliferation and stromal cell density largely to control levels. However, in animals treated with VEGF Trap, estrogen replacement had only a minor stimulatory effect on endothelial cell proliferation, demonstrating that VEGF plays a major and indispensable role in the final pathway that regulates angiogenesis in the primate endometrium.

Evidence from earlier studies using a variety of experimental models have suggested that exposure to estradiol during the proliferative phase drives VEGF production and angiogenesis in the uterus (6, 23, 24). Our quantitative studies in the marmoset confirm that there is indeed a significant increase in endothelial cell proliferation between the mid-proliferative (d 5) and late proliferative phases (d 10) and that this increase in angiogenesis is dependent upon estrogen and mediated by VEGF. However, there is little direct evidence indicating that there is a cyclic peak in uterine angiogenesis during the late proliferative phase in humans; rather, the endometrium in women has been reported to exhibit a relatively stable rate of endothelial cell proliferation (25). However, it has been acknowledged that this negative finding might be attributable to technical difficulties inherent in human studies such as individual variability in hormonal cycles or variability inherent in the use of small biopsies (26–29). To establish a more precise relationship between steroid exposure and changes in endometrial angiogenesis, Nayak and

Brenner (5) have used an ovariectomized rhesus monkey model in which the endocrine milieu of the menstrual cycle was reproduced by sequential treatment with progesterone and estradiol implants. After progesterone withdrawal, an estrogen-dependent peak of angiogenesis was identified at d 7–8 of the proliferative phase (5). The rise in angiogenesis between d 5 and 10 of the normal cycle in the marmoset observed in the present study further supports the view that an estrogen-induced peak in uterine angiogenesis, occurring during the mid to late proliferative phase, is a characteristic feature of the reproductive cycle in primates. Although there are differences in the duration of the proliferative phase between the marmoset and macaques or human, the pattern and changes in steroid hormone receptor localization in the endometrium are quite similar (16). The clear identification of this cyclic rise in uterine angiogenesis suggests that the marmoset will prove a valuable model for future studies designed to evaluate the roles of other putative angiogenic factors.

That estrogen replacement restored angiogenesis in ovariectomized marmosets further solidifies the evidence that estrogen, produced by developing follicles, normally drives a cyclic pattern of angiogenesis in the uterus. Furthermore, gene expression studies in primate endometrium and effects of pharmacological inhibition of VEGF in rodents have strongly suggested that this effect of estrogen is mediated by VEGF. The present study has confirmed that VEGF plays a major role in mediating estrogen-induced angiogenesis in the uterus. However, it should be noted that treatment with VEGF Trap did not completely abrogate angiogenesis in marmosets that also received exogenous estrogen. There are several possible explanations for this finding. First, although VEGF Trap inhibits all isoforms of the VEGF-A family as well as placental growth factor, it does not inhibit VEGF-C or VEGF-D, which are also produced in the endometrium albeit at low levels (30). Second, we cannot exclude the possibility that at times of peak expression, levels of VEGF in the endometrium may result in increased local consumption of VEGF Trap. Third, and perhaps more likely, other putative angiogenic factors are present within the endometrium (31), and these may be able to sustain a low level of angiogenesis even in the face of VEGF inhibition. *In vivo* support for the idea that other angiogenic factors may act in the uterus comes from a recent study of hamster endometrial tissue grafted ectopically into the dorsal skin-fold chambers; here it was reported that combined inhibition of VEGF, fibroblastic growth factor, and platelet-derived growth factor was more effective in blocking angiogenesis than inhibition of VEGF alone (32). Finally, estrogen may have a direct stimulatory effect upon endometrial endothelial cells on which estrogen receptor β is expressed in the primate (33), including the marmoset (16).

Although VEGF inhibition or ovariectomy almost completely abrogated endothelial cell proliferation in the endometrium, these treatments did not result in a reduction in vascular density. This observation is in accord with that of Heryanto *et al.* (7), who also found that whereas angiogenesis inhibitors prevented uterine angiogenesis in estrogen-treated ovariectomized mice, vascular density was not decreased. This seemingly paradoxical effect is likely attributable to the marked reduction in stromal volume associated with the treatment such that the existing blood vessels supply a substantially reduced volume of tissue and hence appear more densely packed, as in the case for the stromal cells themselves (7). Thus, although the vascular density in the uterus was not reduced, the total endothelial mass is likely to be decreased.

Apart from inhibiting endothelial cell proliferation, it is likely that the pharmacological blockade of local VEGF results in additional changes in the structure and function of the uterine vasculature. Taking effects on the marmoset ovary as an example, VEGF inhibition induces an increase in the expression of the tight junctional protein claudin 5 (34), an up-regulation of hypoxia-inducible factor-1α (35), a decrease in expression of mRNA for VEGF receptors (4), and an acute increase in endothelial cell death in the recently formed blood vessels in the corpus luteum (21). VEGF inhibition has also been shown to suppress the estrogen-induced increase in uterine permeability in rodents (7–11). These findings, together with the fact that VEGF inhibition has been shown to reduce vascular permeability in diverse vascular beds across species, strongly suggest that VEGF also plays a pivotal role in modulating vascular function and permeability in the primate uterus, including regulation of tight junctions (24). Indeed, decreases in the permeability of uterine vessels may have contributed to the overall decrease in uterine volume and increase in stromal cell density observed in ovariectomized and VEGF Trap-treated marmosets.

Glandular epithelial cell proliferation in the basalis zone and luminal epithelial cell proliferation were partially inhibited by ovariectomy or treatment with VEGF Trap compared with the late proliferative phase controls. This is to be expected because estrogen is the established stimulator of this process (29). However, the apparent absence of effects of VEGF Trap treatment or ovariectomy on epithelial cell proliferation in the functionalis zone was somewhat surprising. Taken together, these results suggest that in the marmoset, proliferation in the functionalis zone may be less dependent on the high levels of estradiol normally present during the proliferative phase, requiring only residual amounts of estradiol or other estrogens available from the circulation or by local conversion of steroids within the endometrium. It should be noted that reduction in size of the epithelial cells and luminal area in the absence of reduction in nuclear area in ovariectomized and VEGF Trap-treated animals compared with controls could result in an overestimation of volume fraction of proliferating cells with the quantitative method used.

In conclusion, our findings show that endometrial angiogenesis and stromal proliferation during the proliferative phase are driven by estradiol and that the effect of estradiol on angiogenesis is mediated by VEGF. It is widely recognized that angiogenesis is of profound importance in uterine function and that defects in the molecular regulation of the normal process are likely to be involved in common uterine pathologies and infertility (11, 36). The ability to manipulate endometrial angiogenic factors should have major relevance with respect to the understanding of the etiology and developments of novel treatments for conditions such as heavy menstrual bleeding, breakthrough bleeding, and endometriosis (11, 36–41). The identification of a rise in angiogenesis in the late proliferative phase in the marmoset provides a target for investigation of effects of manipulation of

other putative angiogenic factors and suggests that the marmoset will prove a valuable model. Furthermore, a marmoset model of endometriosis has recently been developed (42), increasing the potential value of this species in understanding the regulation of endometrial function in health and disease.

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