Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2) Functions to Promote Uterine Decidual Angiogenesis during Early Pregnancy in the Mouse

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Implantation of an embryo induces rapid proliferation and differentiation of uterine stromal cells, forming a new structure, the decidua. One salient feature of decidua formation is a marked increase in maternal angiogenesis. Vascular endothelial growth factor (VEGF)-dependent pathways are active in the ovary, uterus, and embryo, and inactivation of VEGF function in any of these structures might prevent normal pregnancy development. We hypothesized that decidual angiogenesis is regulated by VEGF acting through specific VEGF receptors (VEGFRs). To test this hypothesis, we developed a murine pregnancy model in which systemic administration of a receptor-blocking antibody would act specifically on uterine angiogenesis and not on ovarian or embryonic angiogenesis. In our model, ovarian function was replaced with exogenous progesterone, and blocking antibodies were administered prior to embryonic expression of VEGFRs. After administration of a single dose of the anti-VEGFR-2 antibody during the peri-implantation period, no embryos were detected on embryonic d 10.5. The pregnancy was disrupted because of a significant reduction in decidual angiogenesis, which under physiological conditions peaks on embryonic d 5.5 and 6.5. Inactivation of VEGFR-3 reduced angiogenesis in the primary decidual zone, whereas administration of VEGFR-1 blocking antibodies had no effect. Pregnancy was not disrupted after administration of anti-VEGFR-3 or anti-VEGFR-1 antibodies. Thus, the VEGF/VEGFR-2 pathway plays a key role in the maintenance of early pregnancy through its regulation of peri-implantation angiogenesis in the uterine decidua. This newly formed decidual vasculature serves as the first exchange apparatus for the developing embryo until the placenta becomes functionally active. (*Endocrinology* 150: 3845–3854, 2009)

Formation of the uterine decidua during implantation shares many features of corpus luteum formation. Uterine decidualization is characterized by rapid proliferation and differentiation of resident stromal fibroblasts into large epithelioid-like decidual cells (1, 2). Endothelial cells (EC) in close proximity to decidual cells proliferate to form a new dense vascular network in the pregnant uterus (3–5). Similarly, granulosa cell proliferation, differentiation into luteinized cells, and angiogenesis, the formation of vasculature from preexisting vessels, are required for corpora luteum formation and function (6, 7). *In situ* hybridization demonstrates that signaling components of the VEGF/VEGFR-2 pathway are expressed in the decidua during the early postimplantation period (3), and functional studies indicate that VEGF might be involved in the regulation of uterine angiogenesis and implantation in the rodent (8–11) and nonhuman primate (12). VEGF and VEGFR-2 are also expressed during corpus luteum formation in the rodent, nonhuman primate, and human (13, 14). Functional studies using inhibitors of angiogenesis like anti-VEGF antibodies, VEGF Trap (15), or VEGFR-2 blocking antibodies (6, 16) demonstrate that the VEGF/VEGFR-2 signaling pathway plays a key role in the regulation of angiogenesis in corpora lutea (7, 14, 17).

Decidual angiogenesis and maintenance of vasculature in the early postimplantation period is an absolute requirement for

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Abbreviations: BEC, Blood endothelial cells; BrDU, 5-bromo-2′-deoxyuridine; EC, endothelial cell(s); ED, embryonic day; H&E, hematoxylin and eosin; P4, progesterone; PDZ, primary decidual zone; PECAM, platelet endothelial cell adhesion molecule; PROPmice, P4-replaced, ovariectomized, pregnant mice; SDZ, secondary decidual zone; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

normal pregnancy development. It is thought that the newly formed decidual vasculature serves as the first exchange apparatus for the developing embryo until the placenta becomes functionally competent (18). Given the aforementioned similarities between uterine deciduae and corpora lutea, we hypothesized that a key regulator of decidual angiogenesis is the VEGF/VEGFR-2 pathway. To test this hypothesis, we inhibited VEGFR-2 function with DC101, the VEGFR-2 neutralizing antibody that has been successfully used to elucidate the regulation of ovarian angiogenesis (6, 14, 16, 19). Because VEGFR-1 and VEGFR-3 are also expressed in uterine deciduae (3, 20, 21) and are involved in the regulation of vessel formation (22–24), we used specific blocking antibodies to determine whether these receptors have a functional role in the regulation of peri-implantation uterine angiogenesis.

We report that a single peri-implantation injection of an anti-VEGFR-2 blocking antibody disrupts pregnancy development through reduction of angiogenesis in the uterine decidua. Administration of an anti-VEGFR-3 blocking antibody reduces peri-implantation uterine angiogenesis without precluding pregnancy development, whereas blockage of VEGFR-1 has no effect.

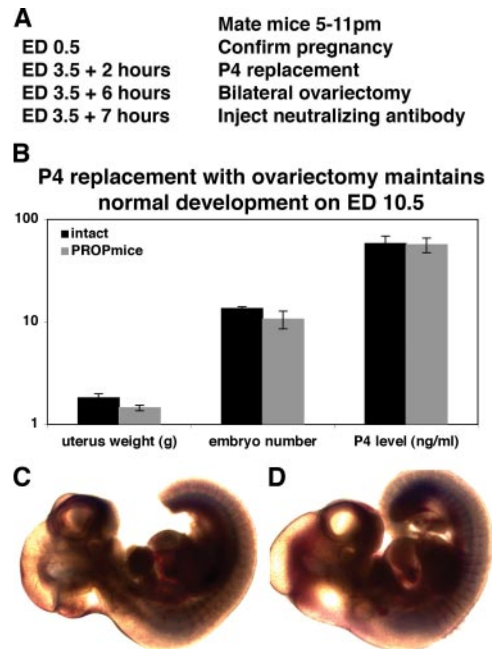

FIG. 1. PROPmice have normal pregnancy development on ED 10.5. A, Experimental design for P4 replaced-ovariectomized pregnant mice (PROPmice). B, Mean uterine weights, numbers of embryos, and P4 levels in the intact mice and PROPmice were similar on ED 10.5 (n = 5 per group; $P > 0.05$ by Student's *t* test). The appearance of ED 10.5 embryos from intact mice (C) was indistinguishable from that of embryos from PROPmice (D).

Materials and Methods

Experimental design

The experiments were designed to investigate whether VEGF receptors (VEGFRs) play an important role in the formation and function of uterine decidual blood vessels during early pregnancy development. Seven-to eight-week old female CD1 mice (Charles River Laboratories International, Inc., Wilmington, MA) were mated with adult males from 1700–2300 h. Identification of a vaginal plug the following morning was interpreted as successful mating. 1100 h was considered d 0.5 *post coitum*. On embryonic d (ED) 3.5 + 2 h, four extended-release progesterone (P4) capsules (10 mg P4 per capsule, 21-d release, 4-mm diameter; Innovative Research of America, Sarasota, FL) were placed under the skin of pregnant mice. A bilateral ovariectomy was performed 4 h after placement of the P4 capsules (16) (Fig. 1A). In intact pregnancies, average P4 levels were 29.3 ng/ml and greater than 40 ng/ml on ED 6.5 and 10.5, respectively. P4-replaced, ovariectomized, pregnant mice (PROPmice) with P4 levels greater than 30 ng/ml at the time of death, which made up 95% of the animals, were used for all experiments. For each experiment, a single ip injection of 0.5 ml neutralizing antibodies against either VEGFR-2 (DC101) (14), VEGFR-3 (mF4-31C1) (25), VEGFR-1 (MF-1) (22), or saline was administered on ED 3.75 (Fig. 1A). These neutralizing antibodies, provided by ImClone Systems, Inc. (New York, NY), have been used to inhibit signaling from VEGFRs in murine models of tumor angiogenesis, lymphangiogenesis, atherosclerosis, and ovarian folliculogenesis (23–29). The dosages of VEGFR-neutralizing antibodies were as follows: DC101 (66 mg/kg), mF4-31C1 (132 mg/kg), and MF-1 (132 mg/kg). All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

Dosage and behavior of DC101, mF4-31C1 and MF-1 *in vivo*

Similar to previous studies, no adverse systemic effects were detected after ip administration of rat antimouse antibodies against VEGFR-2 (DC101) (16, 26), VEGFR-3 (mF4-31C1) (25), or VEGFR-1 (MF-1) (22, 27). Dose-response curves using DC101, mF4-31C1, and MF-1 were performed by injecting a single dose (33, 66, 132, or 264 mg/kg) of each antibody on ED 3.75 and evaluating the effects on uterine angiogenesis on ED 7.5. For DC101, angiogenesis was inhibited at 66 mg/kg. This inhibition did not increase in magnitude with higher dosages of DC101. Therefore, a dosage of 66 mg/kg, which is sufficient to inhibit ovarian angiogenesis (19), was chosen for our experiments. For mF4-31C1, an inhibitory response was seen at 132 mg/kg. This did not increase in magnitude with higher dosages. Thus, 132 mg/kg was chosen for our experiments. For MF-1, no significant inhibitory response was seen up to 264 mg/kg. For our experiments, we selected an amount of 132 mg/kg, which is four times the dosage of MF-1 used to inhibit angiogenesis in a tumor model (30).

Similar to previous studies, injection of isotype-matched IgG and saline produced identical results (n = 5), but nonspecific IgG increased background staining in immunohistochemistry (16, 19). Saline was used for all controls.

Blocking antibodies against VEGFRs could penetrate the embryo and disrupt early embryonic development because these receptors are expressed in early pregnancy (31), and homozygous null mutations in VEGFR-1, -2, and -3 are embryonic lethal (32–35). To test whether VEGFR-blocking antibodies bind to VEGFRs in the embryo, PROPmice received ip injections of either DC101, mF4-31C1, or MF-1 on ED 9.5 and were killed on ED 10.5. Cross-sections of ED 10.5 uteri were incubated with rabbit antirat biotinylated secondary antibody (1:100). Specific binding was detected in the uteri but not in the embryos. As a positive control, uterine cross-sections from untreated ED 10.5 mice were incubated with either DC101, mF4-31C1, or MF-1 (all 1:50) followed by the secondary antibody. Binding was detected in the uteri and embryos. As a negative control, ED 10.5 uteri from mice treated with rat IgG were incubated with the secondary antibody. No binding was detected in the uteri or embryos. These experiments demonstrated that our blocking antibodies can bind VEGFRs in both the uterus and embryo, but maternal systemic administration does not result in binding to embryos.

Formation of the decidua

Control mice received ip injections of saline on ED 3.75, whereas treatment group animals received either DC101, mF4-31C1, MF-1, or combined DC101/mF4-31C1. The animals were killed on ED 5.5 to

assess cellular proliferation and on ED 7.5 to evaluate the number of implantation sites, decidual blood vessel density, cellular proliferation, and apoptosis. To assess stromal cell differentiation by alkaline phosphatase staining, ED 6.5 uteri were fixed in 4% paraformaldehyde for 2 h followed by a sucrose gradient. Frozen sections cut at 5 μm were postfixed with 0.2% glutaraldehyde, rinsed in Tris buffer, treated for 10–20 min with 4.5 μl/ml nitro blue tetrazolium chloride (33 mg/ml in dimethylformamide; Sigma- Aldrich, St. Louis, MO), 3.5 μl/ml 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in dimethylformamide; Sigma), 100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, and 0.1% Triton X-100, and counterstained with Nuclear Fast Red (GeneTex, Irvine, CA).

Embryonic development

Peri-implantation

To evaluate whether direct actions of antibodies on uterine deciduae have secondary, indirect effects on embryonic development, saline, DC101, mF4-31C1, or MF-1 were administered to each sample on ED 3.75 and pregnancy was assessed on ED 10.5. The number of implantation sites and embryos and the average embryo weight were determined. Uterine cross-sections were processed to evaluate embryo morphology in each group.

Postimplantation

To test whether these antibodies could directly affect embryonic development, we injected the embryos with VEGFR-blocking antibodies on ED 6.5, a point when embryos start to express VEGFRs and formation of deciduae is almost complete. Control mice received ip injections of saline and treatment group animals each received either DC101, mF4-31C1, or MF-1 on ED 6.5. The animals were killed on ED 10.5, and uteri were processed as described below.

Artificial decidualization

Female CD1 mice were mated with vasectomized males. On d 3.5 of pseudopregnancy, placement of P4 capsules and bilateral ovariectomy were performed as described above. At the time of ovariectomy, one uterine horn was infused with 10 μl of sesame oil (Sigma) to induce artificial decidualization. The noninfused uterine horn served as a control. Animals were randomized into two groups. Controls received ip injections of saline and treatment group animals received the DC101/mF4-31C1 combination. Uteri were removed on d 6.5 of pseudopregnancy, and processed for immunohistochemical evaluation of EC.

Histology, immunohistochemistry, and immunofluorescence

Frozen uteri containing embryos were sliced into 5-μm sections at 50-μm intervals and mounted on glass slides. Hematoxylin and eosin (H&E) staining and immunohistochemistry were performed according to standard procedures (19). The following primary antibodies were used for immunohistochemistry: 1) platelet endothelial cell adhesion molecule (PECAM; biotinylated rat antimouse CD31 antibody; 1:500; BD Pharmingen, San Diego, CA), 2) goat antimouse VEGFR-2 antibody (1:250; R&D Systems, Inc., Minneapolis, MN), 3) goat antimouse VEGFR-1 (1:200; R&D Systems, Inc.), and 4) goat antimouse VEGFR-3 (1:100; R&D Systems, Inc.). Incubation with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) was followed by incubation with avidin and horseradish peroxidase-conjugated biotin (Vectastain Standard ABC Elite kit; Vector). The color reaction was performed with diaminobenzidine tetrahydrocholoride.

To study coexpression using immunofluorescence staining, the following combinations were used: 1) biotinylated rat antimouse CD31 (1:500; BD Pharmingen) in combination with VEGFR-1, -2, or -3 as described above; 2) coexpression of VEGFR-1/VEGFR-2: rat antimouse MF-1 (1:50; ImClone) with goat antimouse VEGFR-2 (1:250; R&D); 3) coexpression of VEGFR-2/VEGFR-3: goat antimouse VEGFR-2 (1:250; R&D) with rat antimouse VEGFR-3 (1:50; eBioscience, San Diego, CA); and 4) coexpression of VEGFR-1/VEGFR-3: goat antimouse VEGFR-1 (1:200; R&D) with rat antimouse VEGFR-3 (1:50; eBioscience). Sections were incubated with secondary antibodies and signals were visualized with fluorescent dye conjugates of streptavidin: AlexaFluor488 and AlexaFluor594 (Molecular Probes, Eugene, OR). After staining for one receptor, avidin-biotin-blocking solution (Vector) was used to prevent nonspecific staining while detecting expression of the second receptor. Images were photographed with an Axioscope 2 plus microscope camera (Zeiss, Göttingen, Germany) and Axiocam HRC digital (Zeiss). Double images were created with Adobe Photoshop Version 6.0 (Adobe Systems, Inc., San Jose, CA).

Cellular proliferation and apoptosis

One hour before they were killed, animals were injected with 5-bromo-2′-deoxyuridine (BrDU; 1 ml/100 g animal weight; Zymed Laboratories, Inc., San Francisco, CA). Proliferating cells were identified with a biotinylated BrDU antimouse primary antibody (Zymed kit). To detect proliferating EC, double staining for BrDU and PECAM was used (19). Apoptotic cells were detected using ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA).

RT-PCR

Decidual tissue isolated from the embryo, extraembryonic structures, and myometrium was obtained from implantation sites on ED 6.5. Three hundred nanograms of total RNA extracted using TRIzol (Invitrogen, Carlsbad, CA) and treated with DNase I (Invitrogen) were reverse-transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Primers to amplify VEGFA, VEGFC, and VEGFD were designed. Standard PCR with 25 amplification cycles was performed. PCR products were sequenced to confirm identity.

Serum P4

Blood was obtained from all animals by cardiopuncture immediately before they were killed. Serum P4 levels were measured using a competitive chemiluminescent immunoassay (Diagnostic Products Corp./Siemens, Los Angeles, CA).

Data analysis

Quantitative analysis of EC, stromal cells, cellular proliferation, and apoptosis was performed using an image analysis system linked to an Axioscope 2 plus microscope. The data were processed with AxioVision software version 4.5 (Zeiss). On ED 7.5, the area of decidua in a uterine cross-section was measured (in square millimeters). The percentage of the decidua occupied by blood vessels was calculated by dividing the total area of PECAM staining by the area of decidua multiplied by 100. The intense ring of PECAM staining around the embryo, the embryo, and the peripheral muscularis layer were excluded.

To assess proliferation, the decidua of a uterine cross-section was outlined. The image analysis system was configured to measure the number of *brown*-stained nuclei (*i.e.* BrDU-positive cells) using the segmentation part of the program. An adjacent section, stained with hematoxylin, was used to count the total number of the cells. Proliferation indices were calculated on ED 5.5 and 7.5 by dividing the number of BrDU-positive cells by the total number of cells multiplied by 100. This strategy was used to quantify apoptosis and stromal cell differentiation.

Statistics

Statistical analyses were performed using the Statistical Package for Social Science version 15.0 (SPSS, Inc., Chicago, IL). Data are presented as mean ± SE. Unpaired Student's t test and ANOVA with Bonferroni correction were used to compare sample means. $P < 0.05$ was considered a statistically significant difference.

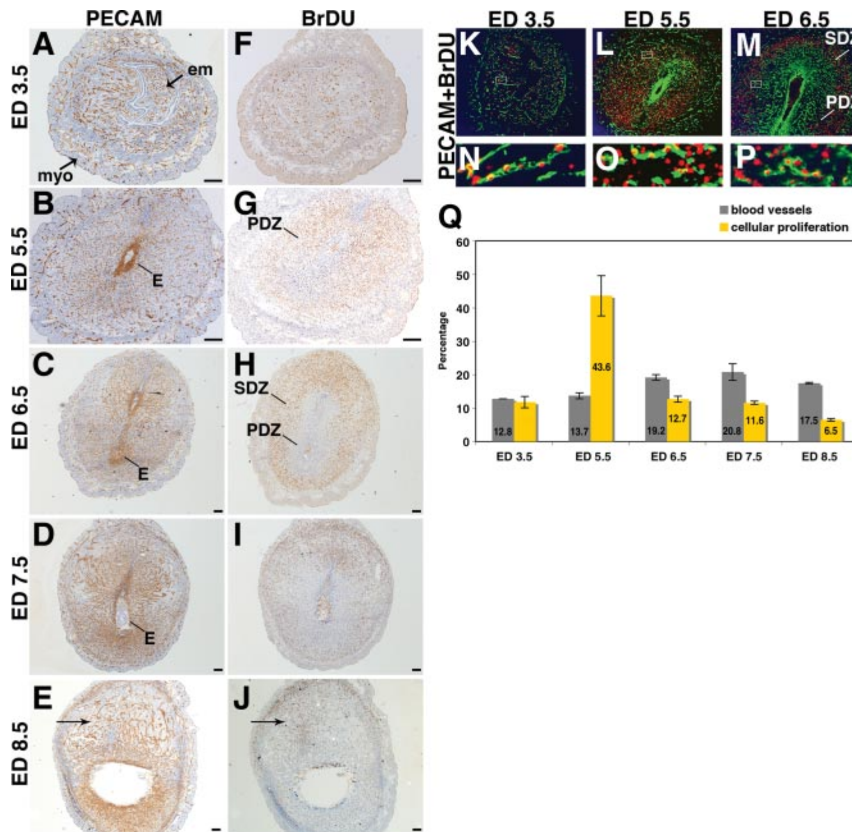

FIG. 2. Uterine angiogenesis and cellular proliferation during the peri-implantation period are dynamic processes with reproducible patterns. A–E, BEC, detected by PECAM expression, are shown as *brown* staining in representative cross-sections of pregnant uteri from PROPmice. BEC are prominent in both the endometrium (em) and myometrium (myo) on ED 3.5 (A). Uterine vascular density peaks between ED 6.5 and 7.5 (C and D) and declines to reach its nadir on ED 8.5 (E). The reduction in uterine vascular density is most pronounced at the site of placenta formation (*arrow* in E). F–J, BrDU expression is shown as *brown* staining in representative cross-sections of pregnant uteri from PROPmice. On ED 5.5 (panel G), cellular proliferation, indicated as BrDU-positive cells, occurs in the area around the embryo, the PDZ. In contrast, on ED 6.5 (H), cellular proliferation is prominent in the SDZ. Cellular proliferation peaks on ED 5.5 (G) and declines on ED 6.5 (H) and 7.5 (I) to reach its nadir on ED 8.5 (*arrow* in J). The reduction in cellular proliferation is most pronounced at sites of placenta (*arrow* in J). Immunofluorescent double staining of uterine cross-sections for BEC (PECAM, *green*) and proliferating cells (BrDU, *red*) demonstrates that many of the proliferating cells in the decidua are BEC (K–P). Proliferating BEC are concentrated in the PDZ on ED 5.5 (L) and in the SDZ on ED 6.5 (M). *Yellow* staining cells, highlighted by *insets* (N–P), represent active decidual angiogenesis. Q, Quantification of peri-implantation blood vessels and cellular proliferation. All experiments were performed a minimum of three times. *Scale bars*, 200 μm (for A, B, F, G, and K–M) and 100 μm (for C–E and H–J). E, Embryo location.

Results

Normal embryonic development is maintained after P4 replacement and bilateral ovariectomy

PROPmice were used to evaluate the effect of blocking VEGFR-1, -2, and -3 on uterine function during the peri-implantation period. To demonstrate that pregnancy progresses normally in this experimental model, PROPmice and intact pregnant controls received a single ip injection of saline on ED 3.75. On ED 10.5, there were no differences in uterine weights, numbers of embryos, gross embryo morphology, or maternal serum P4 levels between PROPmice and intact controls (Fig. 1).

Patterns of angiogenesis, cellular proliferation, and stromal cell differentiation are maintained in PROPmice

Blood endothelial cells (BEC) expressing PECAM are prominent throughout the endometrium and myometrium of the uterus on ED 3.5 before ovariectomy. After implantation, vascular density increases with the peak decidual vascular density between ED 6.5 and 7.5 (Fig. 2Q). Subsequently, vascular density declines with reduction most pronounced at sites of placenta formation (Fig. 2, A–E).

Intense staining for PECAM was noted in the uterine decidua directly surrounding the embryo on ED 5.5 and 6.5 (Fig. 2, B and C). This PECAM staining was observed in intact mice (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) and has been noted by others (36). It does not show a vascular pattern or expression of VEGFRs, markers of EC. Thus, this PECAM staining does not reflect typical blood vessels. Some of the staining for PECAM might be due to the presence of leukocytes and/or apoptotic cells (supplemental Fig. 2).

Cellular proliferation in the decidua peaks on ED 5.5 (Fig. 2Q). The amount of cellular proliferation declines more than 50% on ED 6.5. On ED 8.5, there are few BrDU-positive cells in the decidua. The remaining proliferating cells are located at sites of placenta formation (Fig. 2, F–J). Whereas cellular proliferation occurs throughout the decidua, including the primary decidual zone (PDZ), on ED 5.5, cellular proliferation is restricted to the secondary decidual zone (SDZ) on ED 6.5 (Fig. 2, G and H). This pattern of proliferation in the decidua is characteristic of pregnancy in intact animals (37). Double staining with PECAM and BrdU identifies proliferating BEC among the population of the dividing cells on ED 3.5, 5.5, and 6.5 (Fig. 2, K–P).

To demonstrate that PROPmice undergo a "typical" decidual reaction, stromal differentiation was assessed by alkaline phosphatase staining on ED 6.5 (supplemental Fig. 3). These data indicate that decidual vascular events, cellular proliferation, and stromal differentiation in PROPmice are similar to that of intact pregnant animals.

Expression of VEGFRs and VEGFR ligands in peri-implantation uteri of PROPmice

VEGFRs are expressed in the uterus during the peri-implantation period. However, each receptor has a unique pattern of expression (Fig. 3). After implantation, VEGFR-1 and VEGFR-3 are both expressed in the PDZ (Fig. 3, B–E and L–O). Whereas the subset of cells expressing VEGFR-1 shows a circular peri-embryonic staining pattern, staining for VEGFR-3 is restricted to the mesometrial pole. VEGFR-2 expression is prominent in the PDZ on ED 5.5, but on ED 6.5

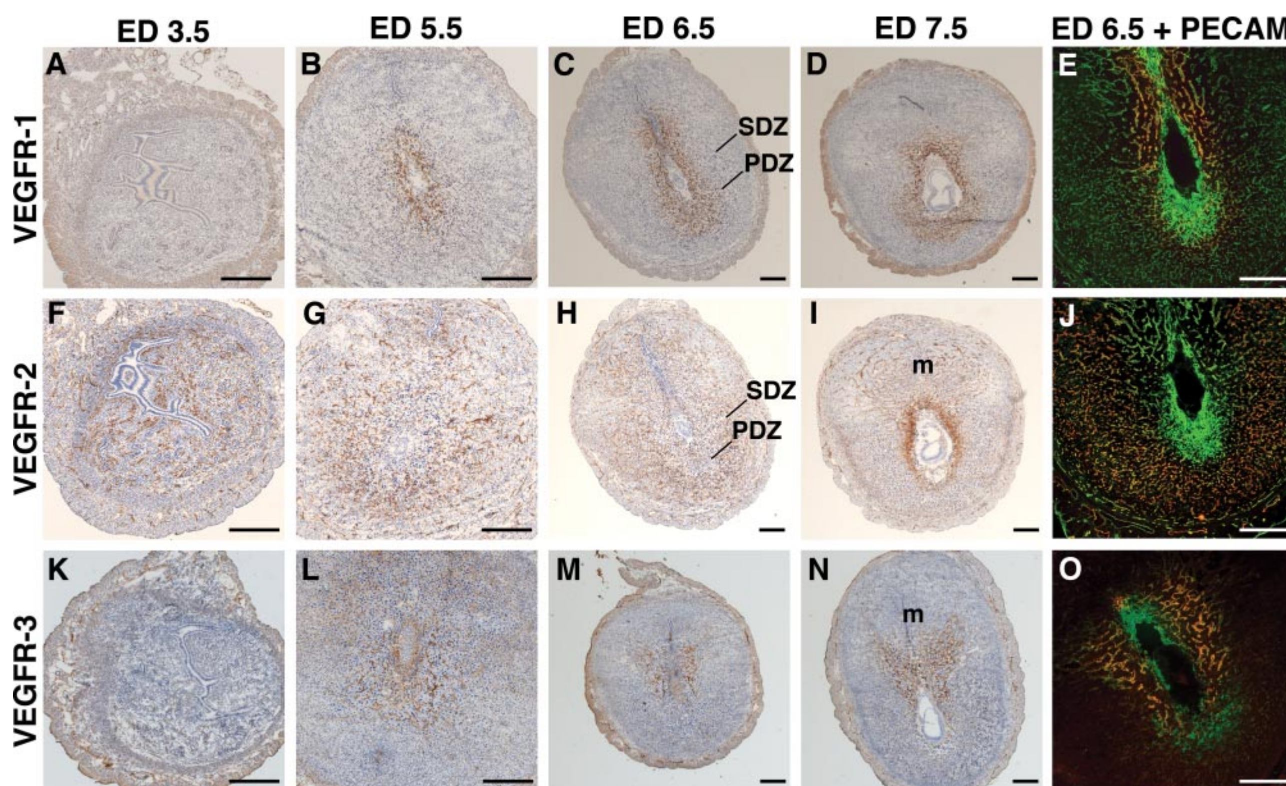

FIG. 3. VEGFR-1, -2, and -3 have unique expression patterns in the peri-implantation uterus and distinct populations of newly formed peri-implantation BEC express VEGFR-1, -2, and -3. On ED 3.5, VEGFR-2 (F) expression is easily detected throughout the endometrium and myometrium, whereas VEGFR-1 (A) and VEGFR-3 (K) expression is scant. VEGFR-2 is expressed on cells in both the PDZ and SDZ. On ED 5.5, VEGFR-2 (G) expression is most intense directly adjacent to the embryo. On ED 6.5, VEGFR-2 (H) expression is primarily in the SDZ with weaker expression in the PDZ. VEGFR-1 (B–D) and VEGFR-3 (K–N) are expressed in the PDZ directly surrounding the implanted embryo. The expression patterns of VEGFR-1 and VEGFR-3 on ED 6.5 (C, H) and ED 7.5 (D, M) differ. On ED 7.5, there is prominent expression of both VEGFR-2 (m; I) and VEGFR-3 (m; N) in the mesometrial pole; however, the patterns of expression differ. Immunofluorescent double staining for PECAM (*green*) and VEGFR-2 (*red*) shows significant coexpression in the SDZ on ED 6.5 (J). Yellow staining cells in the PDZ on ED 6.5 (E), represent the subset of BEC (*green*) expressing VEGFR-1 (*red*). Yellow staining cells in the PDZ (O) represent the subset of BEC (*green*) expressing VEGFR-3 (*red*). All experiments were performed a minimum of three times. *Scale bars*, 200 μm (A, B, E–G, J–L, O) and 100 μm (C, D, H, I, M, N).

staining for VEGFR-2 is more prominent in the SDZ (Fig. 3, F and G). This pattern of expression is similar to the pattern for cellular proliferation, suggesting vascular proliferation (Fig. 2, G and H). Although both VEGFR-2 and VEGFR-3 are expressed in the mesometrial pole on ED 7.5, the subset of cells expressing these receptors differs (Fig. 3, D and N). Immunofluorescent double labeling demonstrated that VEGFRs are coexpressed with PECAM (Fig. 3, E, J, and O), and VEGFR-1 and VEGFR-3 are coexpressed with VEGFR-2 (supplemental Fig. 4). RT-PCR was used to detect mRNA expression of VEGFR ligands, VEGFA, VEGFC, and VEGFD, on ED 6.5 (supplemental Fig. 5).

Effect of VEGFR-2 (DC101), VEGFR-3 (mF4-31C1), or VEGFR-1 (MF-1) neutralization on blood vessel development, cellular proliferation and apoptosis

After DC101, mF4-31C1, and MF-1 treatment, the percentages of the decidua occupied by blood vessels were 9.1 ± 1.0%, 13.1 ± 0.7%, and 17.2 ± 1.1%, respectively, whereas they were 20.8 ± 0.9% in the controls (Fig. 4G). Neutralization of VEGFR-2 and VEGFR-3, but not VEGFR-1 significantly reduced the percentage of the decidua occupied by blood vessels ($P < 0.05$). To determine whether blocking VEGFR-2 and VEGFR-3 would have an additive effect, we administered a combination DC101/mF4-31C1 treatment. Compared with DC101 alone, there was no statistically significant difference in the percentage of the deciduae occupied by blood vessels (7.2 ± 0.9%) after DC101/mF4-31C1 treatment (Fig. 4G).

Histological analyses of uterine cross-sections demonstrated that treatment with DC101 significantly reduced decidual vasculature and stromal cell differentiation (controls 43.2 ± 4.7%; DC101 22.9 ± 2.4%) (supplemental Fig. 3) and arrested embryonic development. Embryonic structures were absent after DC101 treatment. In contrast, a normal-appearing, intact embryo was detected after mF4-31C1 treatment (Fig. 4). The attached embryo was noted on ED 5.5 and 6.5 after DC101 treatment (supplemental Fig. 6). Thus, we concluded that the reduction in decidual vasculature was not due to failed implantation.

To investigate the mechanism(s) responsible for the reduction in decidual blood vessels, cellular proliferation was assessed on ED 5.5 and 7.5, and apoptosis was assessed on ED 7.5. Data for ED 5.5, the time of peak proliferation in untreated animals, and ED 7.5 show similar trends. Neutralization of VEGFR-2 or VEGFR-3 significantly reduced cellular proliferation in the decidua ($P < 0.05$) (Fig. 4G). In control animals, 43.6 ± 0.6% of the decidual cells were proliferating. The percentages of proliferating cells after DC101, mF4-31C1, MF1, and DC101/mF4-

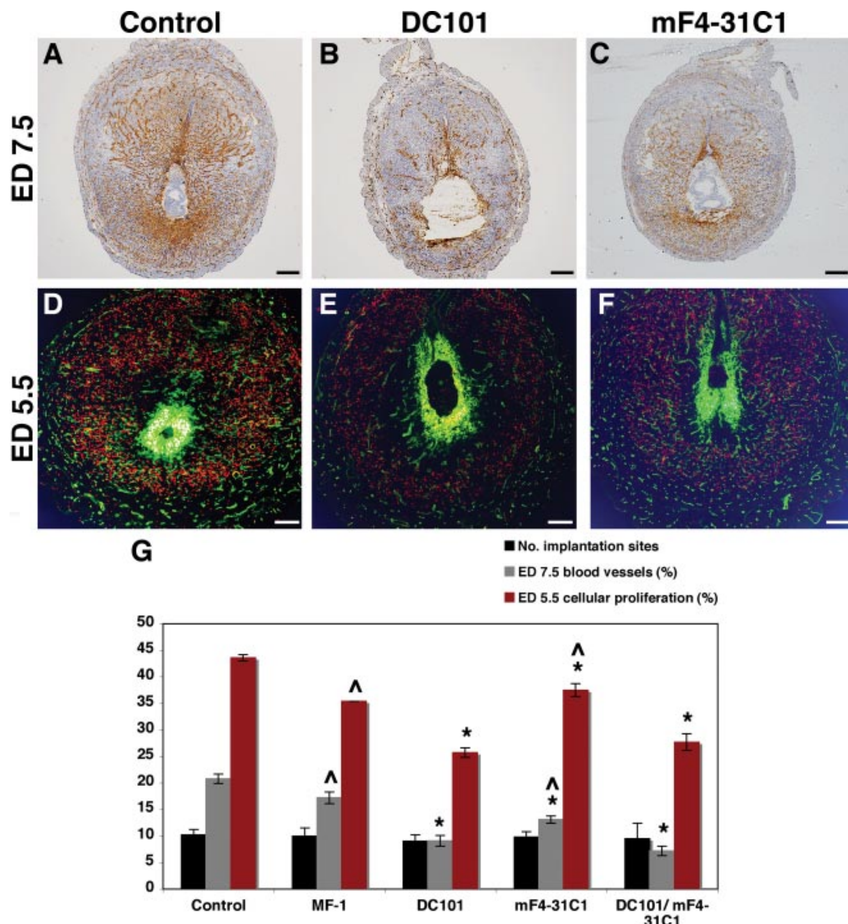

FIG. 4. Inhibition of VEGFR-2 during the peri-implantation period disrupts embryonic development because of a reduction in peri-implantation uterine blood vessels and cellular proliferation. Mice treated with the DC101 or mF4-31C1 have significantly reduced uterine vascular density (B, C, G) and cellular proliferation (E–G) compared with controls (A and D; n = 5 per group). Uterine vascular density and cellular proliferation after MF-1 treatment were statistically identical to untreated PROPmice controls (G). Note the decrease in cellular proliferation (BrDU, *red*) and BEC proliferation [BrDU (*red*) + PECAM (*green*)] in the decidua of DC101- (E) and mF4-31C1- (F) treated mice on ED 5.5. *, $P < 0.05$ compared with PROPmice controls. ^, $P < 0.05$ compared with DC101 or DC101/mF4-31C1 treatment. *Scale bars*, 100 μm (A–C) and 200 μm (D–F).

31C1 combination treatment were 25.7 ± 0.9, 37.5 ± 1.2, 35.4 ± 0.01, and 27.7 ± 1.6%, respectively. The reduction in cellular proliferation with DC101 or DC101/mF4-31C1 combination therapy was significantly greater than with mF4-31C1 treatment ($P < 0.05$). Representative uterine cross-sections on ED 5.5 for DC101 and mF4-31C1 are presented (Fig. 4, E and F). After treatment with DC101 or mF4-31C1, the percentages of proliferating cells and proliferating BEC (Fig. 4, E and F) were reduced. On ED 7.5, 0.12% of decidual cells were apoptotic in the controls. None of the treatments significantly increased the percentage of apoptotic cells (<0.3%) when compared with controls. We concluded that a reduction in cellular proliferation, not an increase in apoptosis, is the primary mechanism by which VEGFR neutralization leads to decreased decidual vasculature.

Effect of VEGFR-2 (DC101), VEGFR-3 (mF4-31C1), or VEGFR-1 (MF-1) neutralization on embryonic development

Administration of DC101, but not mF4-31C1 or MF-1, to PROPmice on ED 3.75 disrupted pregnancy development evaluated on ED 10.5 (Fig. 5). Each single ip injection of DC101, mF4-31C1, or MF-1 resulted in a uterus with a normal number of implantation sites. The gross and microscopic appearances of uteri from mice treated with mF4-31C1 or MF-1 were indistinguishable from those of the controls (Fig. 5). Uteri from DC101-treated mice appeared dark, possibly consistent with hemorrhaging in the implantation sites. H&E staining of uterine cross-sections confirmed abnormal embryonic development, because no normal embryonic structures were detected (Fig. 5G). Effects of DC101 on embryonic development were seen as early as ED 6.5 (supplemental Fig. 6).

In contrast, neutralization of VEGFR-2 starting on ED 6.5 did not affect pregnancy development on ED 10.5. Statistically identical numbers of embryos (controls 10.7 ± 1.2; DC101 13.7 ± 0.9) and embryo weights (controls 0.138 ± 0.01 g; DC101 0.110 ± 0.02 g) were noted. Thus, the timing of the antibody injections is critical (16).

Neutralization of VEGFR-2 reduces uterine vasculature in a model of artificial decidualization

An intraluminal injection of 10 μl sesame oil was used to induce artificial decidualization in pseudopregnant PROPmice. Decidual morphology and peri-implantation blood vessel development were evaluated on ED 7.5 (Fig. 6, A and B). DC101/mF4-31C1 combination therapy reduced decidual vasculature and led to abnormal-appearing, small deciduae (Fig. 6, D–F). These results are similar to those observed after DC101 treatment in PROPmice (Fig. 4B). This finding indicates that the VEGF/VEGFR-2 pathway might have a regulatory role in angiogenesis during artificial decidualization.

Discussion

Reduction of angiogenesis in the uterine decidua during the early postimplantation period with the anti-VEGFR-2 blocking antibody, DC101, decreases decidual vascular density and stromal cell differentiation. These changes disrupt embryonic development. Although blockage of the VEGFC/VEGFR-3 pathway reduces decidual vasculature and proliferation, the progression of pregnancy is not precluded. In contrast, the administration of an anti-VEGFR-1 antibody has no affect on peri-implantation angiogenesis, vascular density, or pregnancy development. Thus, the VEGF/VEGFR-2-signaling pathway regulates the formation of new vasculature in the peri-implantation period, which is essential for the creation of a functional decidua.

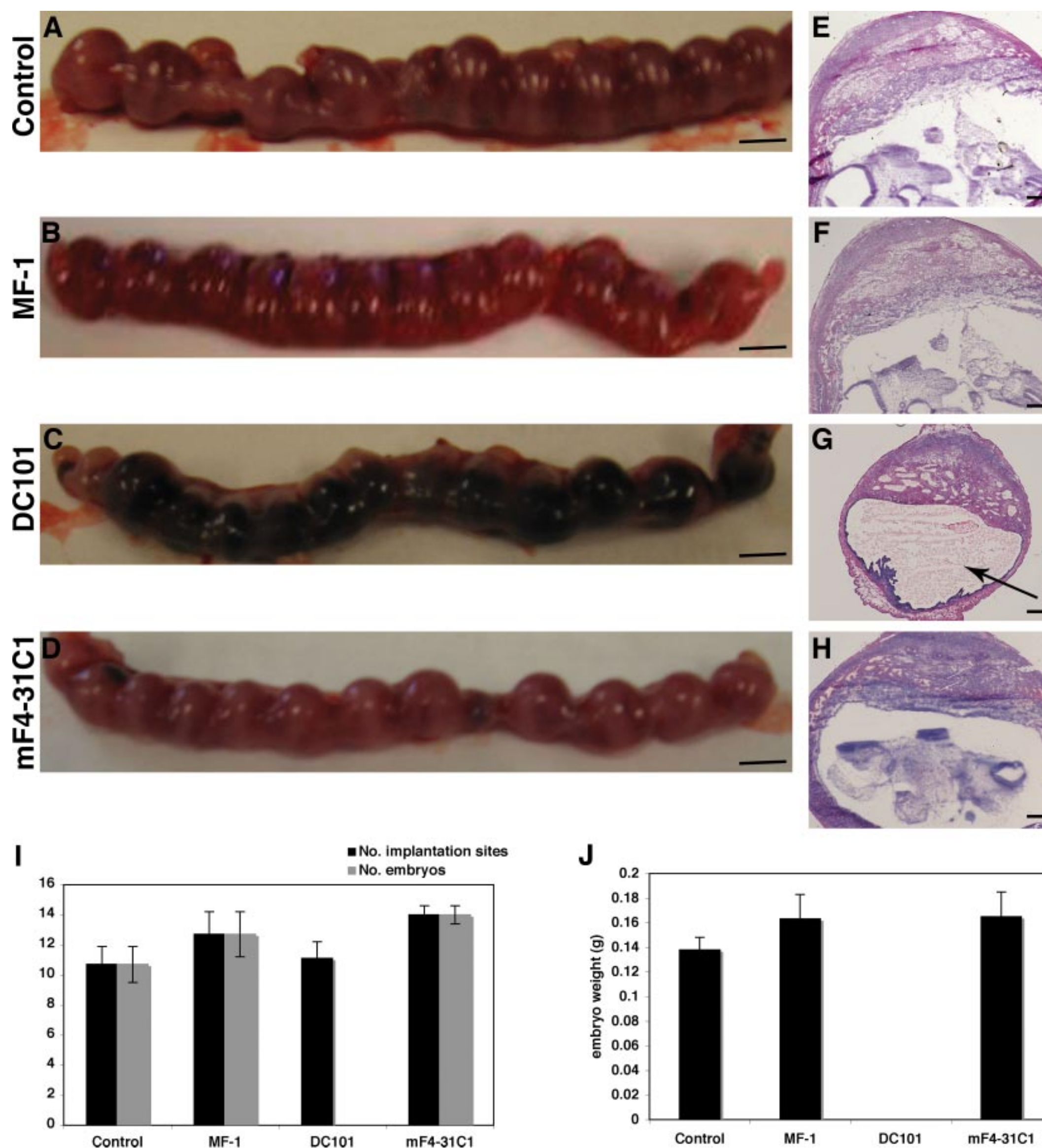

FIG. 5. Inhibition of VEGFR-2 but not VEGFR-1 or VEGFR-3 during the peri-implantation period disrupts pregnancy development evaluated on ED 10.5. A–D, Intact uteri containing embryos on ED 10.5. Uteri from isotype-matched, IgG-injected PROPmice controls (A), anti-VEGFR-1 (MF-1)-treated mice (B), anti-VEGFR-2 (DC101)-treated mice (C), and anti-VEGFR-3 (mF4-31C1)-treated mice (D) contain similar numbers of implantation sites (I). The gross appearance of DC101-treated mice (C) reveals hemorrhage in the implantation sites. E–H, Cross-sections of uteri on ED 10.5 from PROPmice controls (E), MF-1- (F), DC101- (G), and mF4-31C1- (H) treated mice. Note the empty lumen in DC101-treated mice (G; *arrow*). The number of implantation sites, the number of embryos, and the embryo weights were statistically identical ($P > 0.05$) in all groups except DC101 treatment (I and J; n = 5 per group). *Scale bars*, 5 mm (A–D) and 100 μm (E–H).

VEGFA- and VEGFC/VEGFD-dependent signaling pathways are essential to ovarian function (7, 16, 24) and embryonic development (31–33, 35). Both the embryo (18) and corpus luteum (38) participate in the process of decidualization. To evaluate whether VEGFR-dependent signaling pathways are active during decidual angiogenesis, it was important to ensure that potential effects of VEGFR-blocking antibodies were restricted to the uterus. To achieve this, we used PROPmice with time-specific, systemic administration of VEGFR-blocking antibodies (16). Antibodies were administered on ED 3.75, 1 h after ovariectomy and after the attachment reaction and decidualization had been initiated (5). We avoided the effects of the antibodies on embryonic development by injecting the VEGFR-blocking antibodies 3 days before VEGFR expression was initiated in the embryo (31). Validity of such an experimental approach comes from gene knockout models, which demonstrate that absence of either VEGFR-1 (31), -2 (35), or -3 (32) does not interfere with implantation or early embryonic development. Because the biological action of a single antibody injection generally lasts for about 3 days (28), it is possible that after administration on ED 3.75, biologically relevant antibody concentrations are present on ED 6.5, the point when the VEGFRs start to be expressed in the embryo. However, blocking antibodies did not penetrate the embryo (16). To further exclude such a possibility we injected VEGFR-blocking antibodies on ED 6.5 and 9.5. In both cases, embryonic development progressed normally when pregnancy was evaluated on ED 10.5. In addition, after systemic administration of DC101 or mF4-31C1 on ED 9.5, neither was detected by immunohistochemical analysis in the ED-10.5 embryos. Thus, the antibodies did not significantly penetrate the embryo

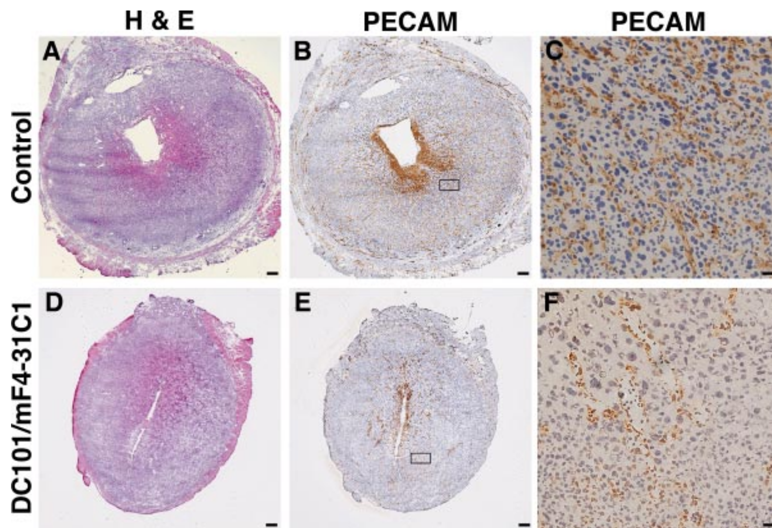

FIG. 6. Inhibition of VEGFR-2 and VEGFR-3 reduces uterine vasculature induced by artificial decidualization. Representative cross-sections of uteri from pseudopregnant PROPmice on d 6.5 of pseudopregnancy were stained with H&E (A and D) and PECAM (B and C). When compared with pseudopregnant control mice (B and C), the uterine vasculature was reduced after DC101/mF4-31C1 treatment (E and F). *Insets* (C and F) correspond to boxes in panels B and E and highlight differences in vascular density. Each experiment was performed three times. *Scale bars*, 100 μm (A–E) and 100 mm (C and F).

to block vascular development directly. In summary, three factors permitted examination of peri-implantation decidual angiogenesis: the combination of PROPmice, the timing of the VEGFR-blocking antibody injection, and the lack of antibody penetration to the embryo.

During implantation, a profound P4-dependent increase in cellular proliferation occurs in the stromal tissue surrounding the avascular embryo (38). In our model, cellular proliferation on ED 5.5 occurs throughout the developing decidua including the PDZ adjacent to the embryo, whereas on ED 6.5 proliferation is more profound in the SDZ, similar to previously published reports (37). Many of the dividing cells identified in the decidua are proliferating EC as has been reported for pregnancies with intact ovaries (4). The identification of proliferating EC in the antimesometrial area indicates that angiogenesis is not restricted to the mesometrial region as has been previously speculated (3, 39). Angiogenesis occurs in all areas surrounding the embryo during uterine decidualization. As a result of this process, the embryo is temporarily surrounded by a dense vascular network on ED 6.5 and 7.5. As noted in intact pregnancies (40), EC are evenly distributed in the PDZ and SDZ. Based on lack of expression of smooth muscle actin (41), this newly formed peri-embryonic vasculature consists of immature vessels of the capillary type (supplemental Fig. 7). In summary, the decidual vascular events observed in our model are similar to events occurring in intact, pregnant animals.

We demonstrate specific expression patterns for VEGFRs in the newly formed decidual vasculature. BEC in PDZ and SDZ differ in the amount of VEGFR-2 expressed. VEGFR-2 colocalizes with PECAM+ BEC in the SDZ, whereas weaker VEGFR-2 staining of PECAM+ BEC was observed in the PDZ. Although *in situ* hybridization revealed similar findings in the SDZ (3), the finding of PECAM+ BEC in the PDZ was surprising because it was previously reported that this zone is devoid of blood vessels (36). This discrepancy might be attributable to differences in the sensitivity of the methodologies used to detect blood vessels: immunohistochemistry (Douglas N. C., H. Tang, and R. C. Zimmermann, unpublished data) *vs*. LacZ staining in Flk-1^{lacZ} mice (36) and *in situ* hybridization (3, 36). The profound reduction in vascular formation that resulted from VEGFR-2 neutralization was not unexpected because nearly all newly formed decidual vasculature expresses VEGFR-2.

Double staining with antibodies against VEGFR-3 and the EC marker PECAM reveals consistent coexpression, whereas coexpression of LYVE-1, a lymphatic marker, and VEGFR-3 is rare (our unpublished observations). In addition, VEGFR-3 consistently coexpresses with VEGFR-2. These observations suggest that, in the murine decidua, the VEGFR-3-expressing tubular structures noted in the PDZ are simple blood vessels, not lymphatics. It has been recently demonstrated that VEGFR-3+ blood vessels are common in ovarian follicles of superovulated mice (24), tumor angiogenesis (23), and sprouting angiogenesis in the retina (24). The finding that inhibition of VEGFR-3 only partially blocked angiogenesis is consistent with the localization of this receptor to vasculature in the PDZ. It is possible that VEGFR-2 activation compensated for the inactivation of VEGFR-3 on some of the vessels that express both receptors. The effects of VEGFR-3 neutralization demonstrated that the embryo can tolerate, within limits, disturbances of vascular formation in uterine deciduae. In contrast, the neutralization of VEGFR-1 had no effect on decidual angiogenesis. VEGFR-1 acts primarily through recruitment of bone marrow-derived endothelial and hematopoietic cells to areas of active angiogenesis (27, 30, 42). It is unclear whether VEGFR-1 has a specific role during decidual angiogenesis or if receptor expression merely points to the origin of these EC. mRNA expression of the VEGFR ligands supports our findings that VEGFR-2- and VEGFR-3-signaling pathways are active during peri-implantation uterine angiogenesis (3).

Significant time-limited proliferation of epithelial or epithelial-like cells and EC occurs in maternal tissue under physiological conditions such as formation of corpora lutea and formation of uterine deciduae in early pregnancy. Regulation of luteal angiogenesis involves glycoprotein hormones such as LH, which originate in the pituitary gland and stimulate VEGF production in luteinized granulosa cells (7). In contrast, steroid hormones like P4 originate in the ovary and are involved in the regulation of decidual angiogenesis. Because expression of P4 receptors in uterine EC is very low, and *in vitro* stimulation of such cells results in minimal to no proliferation (43), it is likely that P4-dependent angiogenesis works by indirect means through P4 receptors active in fibroblasts or decidual cells (10, 38, 44). In the presence of comparable serum P4 levels, uterine EC proliferation after implantation is significantly higher than EC proliferation before implantation (4). This observation makes it likely that implantation of the embryo provides a stimulus that significantly enhances angiogenesis through formation of the decidua. Because early pregnancy development proceeds normally in

VEGFR knockout mice (33), it is unlikely that the nature of the stimulus is a specific proangiogenic factor produced by the embryo. In this context, it is important to recall that mechanical stimulation (*e.g.* sesame oil) of hormonally primed uteri results in uterine decidualization and blood vessel development in the absence of an embryo (18, 45). Artificial decidualization induces VEGF and VEGFR expression (3). Blocking VEGFR-2 and VEGFR-3 provides the most marked reduction in vasculature (see Fig. 4 and Ref. 24). To initiate an investigation of the role of VEGFRs in artificial decidualization, we administered the DC101/mF4-31C1 combination to pseudopregnant mice. Our results indicate that neutralization of VEGFR-2 and VEGFR-3 pathways inhibits angiogenesis under such conditions. Thus, mechanical stimuli can substitute for an embryo in the initiation of VEGFR-mediated decidual angiogenesis. Based on these observations, it is clear that P4 is a prerequisite for peri-implantation angiogenesis, but another natural or artificial stimulus is needed to actually induce decidual angiogenesis that involves the VEGFR family. As a result of these stimuli, decidual cells become a major source of stromal VEGF production, which explains the robust expression of this compound in early pregnancy (3). Because implantation and artificial decidualization are similar to inflammatory processes, we speculate that resident uterine white blood cells like neutrophils and macrophages, some of which are likely to be derived from bone marrow, might become activated through such decidualization stimuli and serve as a second source of VEGF (46–49). Indeed, uterine dendritic cells are required for early vascular events associated with implantation (50), and bone marrow-derived white blood cells are critical for angiogenesis in cancer (42). Therefore, inhibition of VEGFR-2 function might not only have blocked local sprouting of blood vessels but could have also interfered with the recruitment and function of bone marrow-derived VEGFR-2-positive cells.

Our observations in the mouse are clinically significant because they elucidate the involvement of VEGF-dependent angiogenesis in the uterus during early pregnancy. Our findings suggest that abnormal peri-implantation angiogenesis due to dysfunction of the VEGF/VEGFR-2 signaling pathway could be a cause of early miscarriage (21, 51). Therefore, one should use agents like cabergoline (52), which could disrupt decidual vascularization when used to treat ovarian hyperstimulation syndrome, or cyclooxygenase-2 inhibitors (36) used to treat pelvic pain with caution during early pregnancy. On the other hand, our findings might explain why cyclooxygenase inhibitors are helpful in treating dysfunctional uterine bleeding (53, 54).

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