Cyclooxygenase-2 Differentially Directs Uterine Angiogenesis during Implantation in Mice*

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Increased vascular permeability and angiogenesis at the site of blastocyst apposition in the uterus are two hallmarks of the implantation process. The present investigation shows that although the proangiogenic vascular endothelial growth factor (VEGF) and its receptor, Flk-1, are primarily important for uterine vascular permeability and angiogenesis prior to and during the attachment phase of the implantation process, VEGF in complementation with the angiopoietins and their receptor, Tie-2, directs angiogenesis during decidualization following implantation. Mice with null mutation for the gene encoding cyclooxygenase-2 (COX-2), a rate-limiting enzyme in prostaglandin (PG) biosynthesis, show implantation and decidualization failure. Using reporter and mutant mice, we show here that COX-2-derived prostaglandins (PGs) are important for uterine vascular permeability and angiogenesis during implantation and decidualization, suggesting that one cause of the failure of these latter processes in Cox-2(−/−) mice is the deregulated vascular events in the absence of COX-2. The attenuation of uterine angiogenesis in these mice is primarily due to defective VEGF signaling and not due to the defective angiopoietin system.

Under physiological conditions, angiogenesis, the process by which new blood vessels originate from pre-existing vessels, occurs primarily in the uterus and ovary in the adult during the reproductive cycle and pregnancy (1). Increased vascular permeability and angiogenesis are essential for successful implantation and placentation. Vascular permeability and angiogenesis are profoundly influenced by vascular endothelial growth factor (VEGF) (2, 3). VEGF exists in multiple isoforms that work primarily via two transmembrane tyrosine kinase receptors: VEGFR1, encoded by Flk-1, and VEGFR2, encoded by KDR/Flik-1 (4–7). In mice, VEGF isoforms are shorter by one amino acid than those in humans. We have recently shown that VEGF isoforms and VEGF receptors are expressed differentially in the mouse uterus, suggesting a role for VEGF in uterine angiogenesis during implantation (8).

VEGF effects are complemented and coordinated by another class of angiogenic factors, the angiopoietins (9). VEGF acts during the early stages of vessel development (10–12), whereas angiopoietin-1 (Ang-1) acts later to promote angiogenic remodeling including vessel maturation, stabilization and leakiness (13–15). In contrast to the agonistic functions of Ang-1, Ang-2 behaves as an antagonist. Thus, Ang-1 and Ang-2 are naturally occurring positive and negative regulators of angiogenesis,respectively. They interact with an endothelial cell-specific tyrosine kinase receptor, Tie-2 (16). Recently two additional members of the angiopoietin family have been identified. Ang-3, which is expressed in mice, appears to function as an antagonist to Ang-1 activation of Tie-2 in a fashion similar to Ang-2 (17). On the contrary, Ang-4, the human counterpart of Ang-3, functions as an agonist to Tie-2 (17). However, definitive biological functions of Ang-3 and Ang-4 remain unclear.

One of the prerequisites for the initiation of implantation is an increased endometrial vascular permeability at the site of blastocyst apposition. This is followed by progressive endothelial cell growth and angiogenesis at the implantation site. The mechanisms by which these increased uterine vascular permeability and angiogenesis occur are still poorly understood. Furthermore, very limited information is available regarding the roles of angiopoietins in uterine angiogenesis. We have previously shown that estrogen and progesterone have different effects in vivo. Estrogen promotes uterine vascular permeability but profoundly inhibits angiogenesis, whereas progesterone stimulates angiogenesis with little effect on vascular permeability. These effects of estrogen and progesterone are mediated by differential spatiotemporal expression of VEGF and its receptors in the uterus (18). We speculated that prostaglandins (PGs), because of their roles in angiogenesis, cell proliferation, and differentiation in other systems, also participate in uterine vascular permeability and angiogenesis during implantation and decidualization. These lipid mediators are generated via the cyclooxygenase (COX) pathway. COX is the rate-limiting enzyme for conversion of arachidonic acid into prostaglandin H2 (PGH2), the common substrate for various prostaglandin (PG) synthases (19). COX exists in two isoforms that are encoded by two separate genes, Cox-1 and Cox-2. Gene targeting experiments in mice have established distinct functions for

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The abbreviations used are: VEGF, vascular endothelial growth factor; Ang-1, angiopoietin-1; PG, prostaglandin; PGF, cyclooxygenase; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; PECAM, platelet endothelial cell adhesion molecule; 9-cis-RA, 9-cis-retinoic acid.
these isoforms. Although COX-1-deficient females are fertile with specific perturbation defects, COX-2-deficient females are largely infertile with abnormalities in ovulation, fertilization, implantation, and decidualization (20–23). Our recent evidence shows that COX-2-derived prostacyclin (PGI₂) is the primary PG at the implantation site, and that this PG participates in implantation via activation of peroxisome proliferator-activated receptor-β (PPARβ) (24).

Although COX-2 is essential for normal blastocyst implantation and decidualization (21), it is not yet clear whether uterine vascular events are major targets for COX-2-derived PGs. We have exploited the availability of reporter and mutant mice to explore the role of COX-2 in uterine vascular permeability and angiogenesis during implantation and decidualization. We present here molecular, genetic, physiologic, and pharmacologic evidence that COX-2-derived PGs profoundly influence uterine angiogenesis. Our previous and present results provide evidence that whereas ovarian steroid hormones influence uterine vascular permeability and angiogenesis during the pre-implantation period, COX-2-derived PGs direct these events during implantation and decidualization by differentially regulating VEGF and angiopoietin signaling.

MATERIALS AND METHODS

Animals—Adult CD-1 mice were purchased from Charles River Laboratories, Inc. (Raleigh, NC). Flk-1-deficient mice were generated by disruption of the Flk-1 gene using homologous recombination in embryonic stem cells (12). 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 Flk-1 gene were replaced by a promoterless β-galactosidase gene from Escherichia coli, leaving the Flk-1 promoter intact. Therefore, β-galactosidase expression is used as a read-out for Flk-1 promoter activity. COX-2-deficient mice were generated as described previously (22). Cis-2(−/−)/trans-2(+/+) double mutant mice were generated by crossing Flk-1(−/−)/+ female mice with Cis-2(−/−)/− males. PCR analysis of genomic DNA determined the genotype. Homozygosity of Cis-2 null mice was further confirmed by measuring blood urea nitrogen level (21). All mice were housed in the Animal Care Facility at the University of Kansas Medical Center (Kansas City, KS) according to National Institutes of Health and institutional guidelines for laboratory animals.

In Situ Hybridization—In situ hybridization was performed as described previously (8, 25). Sense or antisense 35S-labeled cRNA probes were generated using appropriate polymerases with mouse-specific cDNAs to Ang-1, Ang-2, Ang-3, Tie-2, and VEGF164, Ang-1, Ang-2, and Tie-2 cDNAs were kindly provided by Peter Carmeliet (Flanders Inter-University Institute, Leuven, Belgium). The Ang-3 probe was generated using specific primers. The source of VEGF164, Ang-1, and Tie-2 cDNAs were described previously (8, 25). Probes had specific activities of about 2 × 10⁹ dpm/μg. In brief, frozen sections (10 μm) were mounted on poly-L-lysine-coated slides and fixed in cold 4% paraformaldehyde in phosphate-buffered saline. The sections were prehybridized and hybridized at 45 °C for 4 h in 50% formamide hybridization buffer containing the 35S-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—lacZ staining assessed the expression of β-galactosidase as described previously (26). 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.

Blastocyst Transfer and Implantation—Pseudopregnancy in wild-type or Cis-2(−/−) mice was induced by mating with wild-type vasectomized males. Day 4 wild-type blastocysts were transferred into the uteri of day 4 pseudopregnant recipients (21, 27). On day 6 or 8, the implantation sites were collected after intravenous injections of Chicago Blue B dye solution in saline (27). For reversal experiments, cPGI and PGE₂ (Cayman Chemical, Ann Arbor, MI) were prepared in 10% ethanol, 90% saline. The first injection (4 μg/mouse, intraperitoneal) was given at 5:00 p.m. on the day of blastocyst transfer followed by single injections each day until mice were sacrificed. 9-Cis-retinoic acid (9-cis-RA), purchased from Biomol (Plymouth Meeting, PA), was administrated (10 μg/mouse, intraperitoneal) alone or with cPGI following the same schedule. Cis-2(+/−) recipients were supplemented with exogenous progesterone (P₄) (2 mg/mouse) from day 3 until sacrificed.

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 PharMinogen, San Diego, CA) at a dilution of 1:50 using a Histostain-SP kit (Zymed Laboratories, Inc., San Francisco, CA) as described previously (17, 20). Red color indicates the sites of immunoreactive PECAM.

RESULTS

Angiogenesis Occurs in a Temporal Fashion in the Uterus during the Peri-implantation Period—Several studies have examined the expression of a number of gene products known to control angiogenesis in the uterus, including VEGF and its receptors, but without investigating uterine angiogenic status. Thus, our first objective was to delineate the normal pattern of angiogenesis in the mouse uterus during the peri-implantation period using a reporter mouse model. Flk-1 is an established marker of endothelial cell development and angiogenesis (18, 28, 29). Mice with the Flk-1 gene disrupted with an insertion of the E. coli β-galactosidase (lacZ gene) (12) were used to study uterine angiogenesis. Although the Flk-1(−/−)/+/− embryos die in utero, Flk-1(+/−)/− embryos are viable, and Flk-1(−/−)/− females have normal fertility. Thus, Flk-1(−/−)/− mice, hereafter termed Flk-1lacZ, with β-galactosidase expression as a read-out for Flk-1 promoter activity and as an endothelial cell marker, served as a powerful genetic model to examine uterine angiogenesis during the peri-implantation period (Fig. 1). The level of Flk-1lacZ expression was very low to undetectable on day 1 of pregnancy when the uterus is primarily under the influence of preovulatory estrogen. With rising progesterone levels from the newly formed corpora lutea thereafter, an increasing number of lacZ-stained blood vessels was noted in the stromal bed. This is consistent with our observation of steroidal regulation of uterine angiogenesis in mice (18). On day 5 following implantation, a further increase in the number of lacZ-stained blood vessels was observed at the site of implantation. With the differentiation of the stroma into primary and secondary decidual zones at the implantation site on day 6, an interesting pattern of angiogenesis was noted. An increased number of lacZ-stained blood vessels was present in the secondary decidual zone, but the primary decidual zone was devoid of such blood vessels. On days 7 and 8, progressive increases in the density of lacZ-stained blood vessels were noted in the deciduam with higher density at the mesometrial pole of the implantation site, the presumptive site of placentation. Collectively, the results show that there is heightened angiogenesis in the stromal bed with the onset of implantation and during decidualization.

VEGF is one of the primary inducers of vascular permeability and angiogenesis in the uterus (reviewed in Ref. 18). Previously, we observed that the expression of VEGF164, the most active and abundant VEGF isoform, is modest and restricted to the epithelial cells on days 1 and 2 but becomes localized to stromal cells at higher levels on days 3 and 4. The expression level further increases in decidualizing stromal cells from day 5 onward. Our previous observation also showed that the expression of Flk-1 is low to undetectable on the first 2 days of pregnancy but shows progressive increases in the stromal bed from day 3 onward (8, 30). Again, the expression was absent in the primary decidual zone. Because angiopoietins coordinate with VEGF for full complementation of angiogenesis, our next objective was to determine whether the angiopoietin system was involved in this event during the peri-implantation period.

Ang-1, Ang-2, Ang-3, and Tie-2 Are Expressed in the Peri-implantation Uterus in a Spatiotemporal Manner—We exam-
Ang-2 expression on day 7 was similar to that observed on day 6 but at much higher levels. Ang-3, whose expression was not detected on earlier days, became prominent on day 7 and followed the pattern of Ang-2 expression. On day 8, a very low level of expression of Ang-1 was detected only in a small number of stromal cells at the mesometrial pole just underneath the myometrium. In contrast, patterns of Ang-2 and Ang-3 expression similar to those observed on day 7 persisted through day 8. However, the expression pattern of Tie-2, a receptor for the angiopoietins, was most similar to that observed for Flk-1 on days 1–8 (Fig. 1) (8, 30).

Our previous and present results suggest that although VEGF signaling is the primary stimulus for uterine angiogenesis prior to and during implantation, the coordinated interaction between VEGF and the angiopoietins signals increasing angiogenesis in the deciduum during the postimplantation period.

COX-2-derived PGs are known to participate in angiogenesis in other systems and in tumors (31–33). Thus, we sought to examine the role of COX-2-derived PGs in uterine angiogenesis during implantation and decidualization. We used COX-2-deficient mice as a model system to study the role of COX-2-derived PGs in uterine angiogenesis relevant to implantation and decidualization. Although implantation is mostly defective in Cox-2(−/−) mice, a small number of wild-type blastocysts, after transfer into day 4 pseudopregnant progesterone-treated Cox-2(−/−) recipients, shows some implantation-like reactions but fails to provoke normal vascular permeability (blue reaction) and decidual cell reaction (24). We have previously observed that PGJ2 is the most abundant PG followed by PGE2 at the implantation site. A PGJ2 agonist, carba prostaclin (cPGI), functions as a ligand for PPARα and facilitates its heterodimerization with RXR. When administered alone or in combination with an RXR agonist, cPGI can improve implantation in Cox-2(−/−) mice (24). Thus, our first objective was to confirm our previous observation that cPGI (a more stable analogue of PGJ2) plus an RXR agonist, 9-cis-retinoic acid (9-cis-RA), improves poor implantation in Cox-2(−/−) mice (24); then we compared uterine angiogenic status during inferior and improved implantation and decidualization in Cox-2(−/−) mice.

**Defective Implantation in Cox-2(−/−) Mice Is Improved by in Vivo Administration of cPGI and 9-cis-RA—PPARαs heterodimerize with RXRs for transcriptional regulation. Although cPGI alone modestly induces transcriptional activation of PPARα, a combination of cPGI with 9-cis-RA, a ligand for RXR, leads to a profound synergistic transcriptional activation of PPARα activity in vitro (24). We compared the effects of cPGI and 9-cis-RA with that of the vehicle on angiogenesis and implantation in Cox-2(−/−) mice after transfer of wild-type blastocysts. As shown in Table I, 44 of 105 (42%) blastocysts had implanted in all wild-type recipients (n = 8) when examined on day 6. In contrast, only 39 of 256 (15%) blastocysts showed implantation in 9 of 19 Cox-2(−/−) control recipients, and the implantation sites were considerably smaller and pale-looking in appearance. Combined administration of cPGI and 9-cis-RA significantly improved the implantation rate (59 of 112 (53%) blastocysts implanted in all Cox-2(−/−)-treated recipients (n = 8)). In addition, the size of these sites was larger with more a distinct blue response. However, administration of 9-cis-RA alone was not very effective in rescuing implantation in Cox-2(−/−) mice (Table I). These results are consistent with our previous observation, suggesting that the effects of COX-2-derived PGJ2 in implantation are mediated primarily via activation of PPARα.

Implantation sites obtained from Cox-2(−/−) mice were more numerous and larger than those obtained from Cox-2+/− or wild-type mice. We suggest that COX-2-derived PGs participate in uterine angiogenesis during implantation and decidualization and that their deficiency results in defective implantation and decidualization. In the present study, we demonstrated that COX-2-derived PGs are involved in angiogenesis and implantation, and we believe that the administration of COX-2-derived PGs will be effective in improving implantation and decidualization in Cox-2(−/−) mice.

**Implications for Future Research**

COX-2 is a key enzyme in the production of PGs, which are involved in various physiological processes, including inflammation, pain, and angiogenesis. The present study suggests that COX-2-derived PGs play a significant role in uterine angiogenesis during implantation and decidualization, which is consistent with previous findings in other systems and tumors. This finding has important implications for future research on the role of COX-2-derived PGs in implantation and decidualization, as well as in other physiological processes.

**Conclusion**

In summary, the present study demonstrates that COX-2-derived PGs are involved in uterine angiogenesis during implantation and decidualization. These findings suggest that COX-2-derived PGs play a significant role in implantation and decidualization, and the administration of COX-2-derived PGs may be effective in improving implantation and decidualization in Cox-2(−/−) mice. Further research is needed to elucidate the mechanisms by which COX-2-derived PGs participate in uterine angiogenesis during implantation and decidualization.
Uterine Vascular Density Is Suboptimal in Cox-2 (−/−) Mice during Implantation and Decidualization

Although implantation is mostly defective in Cox-2 (−/−) mice, a small number of wild-type blastocysts after transfer into day 4 pseudopregnant Cox-2 (−/−) recipients made the initial attachment with the uterus but failed to provoke normal vascular permeability and decidual cell reaction (Ref. 24 and Table I). These results suggest that reduced uterine vascular responses are one cause of implantation and decidualization failure in Cox-2 (−/−) mice. Thus, we examined in more detail the angiogenic status of Cox-2 (−/−) uteri at the sites of blastocysts on days 6 and 8 after transfer of day 4 wild-type blastocysts. Platelet endothelial cell adhesion molecule-1 (PECAM-1), also known as CD31, is a plasma membrane-spanning molecule. PECAM-1 is expressed by a wide variety of cells including endothelial cells, platelets, neutrophils, monocytes, and lymphocytes (34) and appears early in the development of the vascular system (35). Localization of PECAM-1 at the cell-

TABLE I

| Genotype | Treatment | No. of recipients | No. of mice with IS (%) | No. of blastocysts transferred | No. of IS (%) |
|----------|-----------|-------------------|-------------------------|-------------------------------|--------------|
| (+/+)    | Vehicle   | 8                 | 8 (100)                 | 105                           | 44 (42)      |
| (−/−)    | Vehicle   | 19                | 9 (47)                  | 256                           | 39 (15)      |
| (−/−)    | cPGI + 9-cis-RA | 8            | 8 (100)                 | 112                           | 59 (53)      |
| (−/−)    | 9-cis-RA  | 4                 | 1 (25)                  | 60                            | 8 (13)       |

* Values with different superscripts are significantly different (p < 0.05). Statistical analysis was performed using χ² followed by Fisher's exact tests. See "Materials and Methods" for treatment schedule.
cell borders of endothelial cells serves as a marker for blood vessels (18, 36). Thus, we examined PECAM-1 localization on days 6 and 8 at implantation sites of wild-type and Cox-2(−/−) recipients. PECAM-1 was aberrantly localized in Cox-2(−/−) uteri on both days 6 and 8. As shown in Fig. 3, the expression was markedly reduced in the decidua, particularly at the mesometrial decidual bed in Cox-2(−/−) uteri. The results suggest that uterine vascular development is suboptimal in the absence of COX-2.

Uterine Expression Patterns of Angiopoietins Is Normal in Cox-2(−/−) Mice—The angiopoietins are now considered complementary to VEGF functions in angiogenesis. Therefore, we sought to compare the spatiotemporal expression of angiopoietins and their receptor, Tie-2, in the uteri of Cox-2(−/−) mice with those of wild-type mice at the site of blastocyst on days 6 and 8. On day 6, no significant difference was noted in the expression patterns of angiopoietins between the Cox-2(−/−) and wild-type mice, although the decidual response was depressed in Cox-2(−/−) mice (Fig. 4). The expression pattern of Tie-2 was also similar between the wild-type and Cox-2(−/−) uteri, albeit the expression of Tie-2 was somewhat lower in Cox-2(−/−) mice. On day 8, as shown in Fig. 5, aberrant decidualization and embryo development were more prominent in Cox-2(−/−) mice, but the normal expression patterns for angiopoietins were still maintained. However, Tie-2 expression was modestly depressed in Cox-2(−/−) uteri. These results suggest that angiopoietin signaling involved in uterine angiogenesis is not significantly affected during implantation and decidualization in the absence of COX-2. Our next objective was to see whether the uterine VEGF system is aberrant during implantation and decidualization in the absence of COX-2.

Uterine Expression of Vegf and Flk-1 Is Aberrant in Cox-2(−/−) Mice—Our previous observations suggested that VEGF and Flk-1 play a role in uterine vascular permeability and angiogenesis required for implantation (8, 30). Thus, we compared uterine expression patterns of Vegf164 at the sites of blastocysts on days 6 and 8 after transfers of day 4 wild-type blastocysts into day 4 wild-type or Cox-2(−/−) pseudopregnant recipients. As shown in Fig. 6, Vegf164 expression was remarkably down-regulated in stromal cells at the blastocyst site in Cox-2(−/−) mice on both days 6 and 8. Because cPGI and 9-cis-RA improve implantation in Cox-2(−/−) uteri after blastocyst transfer (Table 1) and because PGF2α participates in angiogenesis in other systems (37), we speculated that the retarded embryonic and decidual growth in Cox-2(−/−) mice could be due to aberrant angiogenesis. Thus, we examined whether administration of cPGI and 9-cis-RA improve uterine Vegf expression at the site of implantation in Cox-2(−/−) mice after blastocyst transfers. Indeed, administration of these agents restored the expression of Vegf with improved implantation when examined on day 6 (Fig. 6). To assess angiogenesis more directly, we generated $\text{Cox-2}(−/−)\times\text{Flk-1}(+/−)$ lacZ double mutant mice to monitor lacZ-stained blood vessels after transfer of wild-type blastocysts. When examined on day 8, the number of lacZ-stained blood vessels was remarkably reduced at the blastocyst site in the absence of COX-2. Administration of cPGI and 9-cis-RA again restored the normal number of lacZ-stained blood vessels (Fig. 6). Our previous and present results suggest COX-2-derived PGs influence uterine angiogenesis primarily via affecting the VEGF system during implantation.

DISCUSSION

The establishment and remodeling of blood vessels during angiogenesis within a given tissue are regulated by local environment and paracrine signals. The present findings show two distinct pathways that are operative in uterine angiogenesis during early pregnancy in mice. The VEGF pathway is more dominant during the preimplantation period, whereas angiogenesis during implantation and decidualization is directed by both the VEGF and angiopoietin signaling systems. In addition, although uterine angiogenesis during the preimplantation period is regulated primarily by the ovarian steroids progesterone and estrogen (18), this uterine process, with the onset of implantation and subsequent decidualization, is influenced by COX-2-derived PGs. These in vivo observations based upon genetic, molecular, physiological, and pharmacological approaches show that uterine angiogenesis during early pregnancy is a dynamic and differentially regulated process. Another intriguing observation is that whereas COX-2-derived PGs primarily influence VEGF signaling, their role in altering angiopoietin signaling in uterine angiogenesis during implantation and decidualization appears to be limited. The physiological connotation of this observation is described below.

In normal adult tissues, angiogenesis occurs primarily in the female reproductive organs during the cycle and pregnancy (1, 38–41). VEGF is a potent mitogen for endothelial cells and a prime regulator of angiogenesis (42). The critical role of VEGF

![Figure 3. Immunolocalization of PECAM-1 at the implantation sites of wild-type and Cox-2(−/−) mice. Uterine segments containing blastocysts on days 6 and 8 after transfer of wild-type blastocysts into day 4 wild-type or Cox-2(−/−) mice. PECAM-1 was aberrantly localized in Cox-2(−/−) uteri on both days 6 and 8. As shown in Fig. 3, the expression was markedly reduced in the decidua, particularly at the mesometrial decidual bed in Cox-2(−/−) uteri. The results suggest that uterine vascular development is suboptimal in the absence of COX-2.](image)
in vasculogenesis and angiogenesis is exemplified by the fact that the targeted disruption of even one allele of the Vegf gene results in embryonic death by day 10.5 with aberrant blood vessel formation (10, 11). As previously described, VEGF effects are mediated primarily by two tyrosine kinase receptors: Flt-1 and Flk-1 (4–7). Flk-1 is a major transducer of VEGF signaling that directs mitosis, chemotaxis, and actin reorganization in endothelial cells (42–44). Mice with targeted deletion of the Flk-1 gene show defective endothelial cell development and embryonic death by day 9.5 (12). Although Flt-1 is not involved in endothelial cell mitosis, targeted disruption of Flt-1 produces impaired endothelial cell assembly leading to embryonic lethality (45).

The emerging concept is that signaling by the angiopoietins...
is important for the full complement of angiogenesis to be triggered by the VEGF system (10, 14, 46, 47). Ang-1 and Ang-2, which interact with Tie-2, are considered positive and negative regulators of angiogenesis, respectively. Ang-1 induces tyrosine phosphorylation of Tie-2 in endothelial cells (16). Mice deficient in Ang-1 or Tie-2 show a lethal phenotype around midgestation (13, 14, 16, 46). The striking defects include abnormal endothelial lining of the heart and impaired remodeling of the primary capillary plexus into small and large vessels. In addition, poor association with the underlying matrix adversely affects vessel integrity, stabilization and maturation in Ang-1 (\textit{+/+}) embryos. Thus, interactions between endothelial cells and the surrounding matrix and mesenchyme are crucial to the formation of a stable vasculature (48). As already mentioned, normal angiogenesis is the result of a balance between the positive and negative signalings. The natural endogenous antagonist Ang-2 also interacts with Tie-2 (9). Although Ang-1 stimulates tyrosine phosphorylation of Tie-2, Ang-2 blocks Ang-1-induced activation of Tie-2, suggesting that Ang-2 antagonizes Ang-1 functions.

In adult tissues, whereas \textit{Ang-1} is expressed widely, \textit{Ang-2} is primarily expressed in the ovary, placenta and uterus that are the predominant sites of angiogenesis and vascular remodeling. Although the expression patterns of angiopoietins and Tie-2 during vascular remodeling in the ovary have been examined (9), very limited information is available for the uterus. The present results of differential expression of angiopoietins and Tie-2 in the uterus during the entire peri-implantation period in mice thus provide new and important information. The ovarian expression pattern implies that Ang-2 collaborates with VEGF in front of invading vascular sprouts by blocking Ang-1 induced stabilizing and maturing functions, allowing vessels in a plastic state to be more responsive to the sprouting signal provided by VEGF. Based on results in the ovary, a \textit{yin-yang} relationship between VEGF and angiopoietins in angiogenesis was proposed (16). This model suggests that in the presence of VEGF, Ang-2 stimulates vessel sprouting by blocking stabilizing signaling by Ang-1, whereas in the absence of VEGF, Ang-2 antagonizes Ang-1 signaling, contributing to vessel regression.

**Fig. 6. Expression of \textit{Vegf} and \textit{Flk-1lacZ} at the implantation sites of wild-type and of \textit{Cox-2(-/-)} recipients.** Uterine segments with implantation sites after transfer of wild-type blastocysts to wild-type, \textit{Cox-2(-/-)}, \textit{Cox-2(+/-)} \textit{Flk-1(+/-)} or \textit{Cox-2(-/-) Flk-1(+/-)} mice on day 4 of pseudopregnancy were used with or without treatment with \textit{cPGI} and 9-cis-RA treatment. Gene expression of \textit{Vegf} was assessed by \textit{in situ} hybridization using \textit{35S}-labeled cRNA probes, whereas \textit{\beta}-galactosidase staining assessed \textit{Flk-1lacZ}-stained blood vessels. Arrows indicate the locations of embryos. Bar, 250 \textmu m.
Our present results of reduced expression levels of Vegf and Flk-1/y0 in the uterus at the blastocyst sites during decidualization in Cox-2(−/−) mice suggest that COX-2-derived PGs are important for angiogenesis during these events. Furthermore, the restoration of compromised uterine angiogenesis with improved implantation in Cox-2(−/−) mice by cPG and 9-cis-RA suggests that COX-2-derived PGs participate in these events via activation of the PPARα/×R× system as suggested previously (24). However, it could be argued that reduced uterine angiogenesis is a consequence, but not a cause, of compromised implantation and decidualization in Cox-2(−/−) mice. It is not unreasonable to assume that defective implantation and decidualization would accompany aberrant gene expression. However, unaltered uterine expression patterns of angiopoietins in both wild-type and Cox-2(−/−) mice suggest that compromised implantation and decidualization are not reflected in aberrant expression of genes in general. Rather, this distinct separation of the gene expression profile of these two proangiogenic systems, VEGF and angiopoietins, in Cox-2(−/−) mice lends support to the hypothesis of preferential effects of COX-2-derived PGs on these two angiogenic signaling pathways.

There is evidence that the presence of Ang-1 in the absence of VEGF makes blood vessels non-leaky (15). This is consistent with our previous (24) and present observations that the vascular permeability at the site of blastocyst apposition in Cox-2(−/−) mice is extremely poor, suggesting that uterine vessels become non-leaky in the presence of Ang-1 with reduced levels of VEGF in Cox-2(−/−) uteri. It is also suggested that Ang-2 in the absence of VEGF causes vessel destabilization and regression. Thus reduced uterine Flk-1/y0-stained vessel density in Cox-2(−/−)×Flk-1/y0 mice with very low levels of Vegf expression provides strong evidence that vessel regression occurs in Cox-2(−/−) uteri because of the contrasting expression of VEGf and Ang-2. Although this yin-yang hypothesis was put forward based on expression studies, our present results provide genetic and physiological evidence to support this contention.

In conclusion, our present investigation offers several new observations including differential expression of angiogenic factors and their regulation in uterine angiogenesis during the peri-implantation period, differential effects of COX-2 on angiogenic signaling, and a crucial role of angiogenesis in implantation and decidualization. These observations may help to elucidate the status and role of angiogenesis in the ontogeny of gynecological disorders such as infertility, endometriosis, and uterine adenocarcinoma.

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