Transcriptional Regulation of Uterine Vascular Endothelial Growth Factor during Early Gestation in a Carnivore Model, Mustela vison*

Flavia L. Lopes1, Joëlle Desmarais, Sandra Ledoux, Nicolas Y. Gévy, Pavine Lefèvre, and Bruce D. Murphy

From the Centre de Recherche en Reproduction Animale, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec J2S 7C6, Canada

Vascular endothelial growth factor (VEGF) is an essential angiogenic signaling element that acts through its two tyrosine kinase receptors, inducing both proliferation of endothelial cells and vascular permeability. Given the importance of vasculogenesis and angiogenesis to early pregnancy, it is of interest to understand the mechanisms regulating vascular development at this stage. We previously demonstrated that VEGF and receptors are up-regulated during embryo implantation in an unique animal model, the mink, a species displaying obligate embryonic diapause. Herein we examined the role of prostaglandin E2 (PGE2) as a regulator of VEGF during early pregnancy and established the mechanisms of this regulation. We demonstrate that activated embryos secrete PGE2 and that expression of PGE synthase in the uterus is dependent upon direct contact with invading trophoblast cells during implantation. Using mink uterine stromal cells transfected with mink VEGF promoter driving the luciferase reporter gene, we show that PGE2 induces promoter transactivation and that this response can be eliminated by blockade of protein kinase A. Treatment with antagonists to PGE2 receptors EP2 and EP4 eliminated the PGE2-induced response in transfected cells. Deletional studies of the promoter revealed that a region of 99 bp upstream of the transcription start site is required for PGE2-induced transactivation. Mutation of an AP2/Sp1 cluster, found within the 99 bp, completely eliminated the PGE2 response. Furthermore, chromatin immunoprecipitation assays confirmed binding of the AP2 and Sp1 transcription factors to the endogenous mink VEGF promoter in uterine cells. PGE2 stimulated acetylation of histone H3 associated with the promoter region containing the AP2/Sp1 cluster. Taken together, these results demonstrate that PGE2 plays an important role in regulating uterine and thus placental vascular development, acting through its receptors EP2 and EP4, provoking protein kinase A activation of AP2 and Sp1 as well as acetylation of histone H3 to transactivate the VEGF promoter.

Prostaglandin E2 (PGE2) is a prostanoid synthesized through the cyclooxygenase pathway characterized by the initial step of formation of prostaglandin H2 from arachidonic acid, catalyzed by the cyclooxygenases 1 and 2 (COX-1 and -2). Formation of PGE2 follows formation of prostaglandin H2 from arachidonic acid and is dependent on the presence of prostaglandin E synthase (PGE synthase). Two isoforms of PGE synthase have been identified; one is a cytosolic form that acts mostly on COX-1-derived prostaglandin H2. The second is a microsomal form, preferentially coupled with the inducible COX-2 induction of PGE2 generation (1). PGE2 exerts its effects following binding to specific receptors containing seven transmembrane domains (2). Four receptor subtypes have been identified to date: EP1, -2, -3, and -4, each activating different intracellular pathways. Knock-out models for each subtype have been investigated, and mice deficient for EP2 presented impaired ovulation and fertilization (3).

The role of prostaglandins in reproductive processes has been extensively investigated. COX-2-deficient mice have impaired ovulation, fertilization, implantation, and decidualization (4). PGE2 is luteoprotective (5); it also plays a role in regulation of immune responses at the site of embryo attachment (6), in ovulation (7), and in the decidualization process in rats (8, 9). Recently, Wang et al. (10) identified PGE2 as the major prostaglandin at implantation sites in hamsters, and expression of microsomal PGE synthase was correlated with expression of COX-2. In mice, both microsomal PGE synthase mRNA and protein were localized in the subluminal stroma surrounding the implanting blastocyst (11). The authors suggested embryonic induction of local PGE synthase, since expression of the enzyme was absent in pseudopregnant females as well as at interimplantation sites in the uterus.

Early pregnancy in mammals is associated with morphological and functional changes in uterine cells, accompanied by vascular remodeling. These changes are required for both placental and embryonic development (12–15). Vascular endothelial growth factor (VEGF) is the major regulator of angiogenesis (16) and is an important factor in regulation of the events of early implantation and establishment of the placenta (17). VEGF is a homodimeric glycoprotein of 40–45 kDa, and, while best known for its potent endothelial cell-specific mitogenic

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1 To whom correspondence should be addressed: McGill University, Montreal Children’s Hospital Research Institute, 2300 Tupper St., Montreal, Quebec H3H 1P3, Canada. Fax: 514-412-4331; E-mail: flavia.lopes@mail.mcgill.ca.

2 The abbreviations used are: PGE, prostaglandin E; COX, cyclooxygenase; VEGF, vascular endothelial growth factor; PKA, protein kinase A; ChIP, chromatin immunoprecipitation; RACE, rapid amplification of cDNA ends.
activity, it also plays a role in increasing vascular permeability (18–21). Prostaglandins are among the factors reported to regulate VEGF (22). VEGF effects on angiogenesis are dependent upon binding to tyrosine kinase receptors, Flt-1 (Fms-like tyrosine kinase, also known as VEGFR-1) and KDR (kinase domain region, also known as VEGFR-2) (23). We have previously demonstrated that VEGF and its receptors are up-regulated during peri-implantation stages of gestation in the mink uterus (24).

Earlier, Kennedy (25) showed that PGE$_2$ is a regulator of increased vascular permeability at implantation sites in rats. Since then, PGE$_2$ has been shown to up-regulate VEGF in a number of tissues, including umbilical cord blood-derived mast cells (26), colon cancer cells (27), endothelial cells (28), human pancreatic cancer cells (29), gastric cancer cells (30), and mouse mammary tumor cells (31). There are few investigations of PGE$_2$ modulation of VEGF transcription, and there are no such investigations pertinent to uterine expression.

In the present report, we have established the pattern of expression of PGE synthase in the uterus during early pregnancy and investigated the role of PGE$_2$ in implantation in an unique mammalian model. We provide the first demonstration that local PGE$_2$ regulates transcriptional activity of the VEGF gene as well as new information bearing on the mechanism of this activation.

**EXPERIMENTAL PROCEDURES**

**Animals and Sample Collection**—All treatment protocols involving the use of animals were approved by the Comité de Déontologie, Faculté de Médecine Vétérinaire, Université de Montréal in accordance with regulations of the Canadian Council of Animal Care. Mink of the Dark and Pastel varieties were purchased and maintained on a commercial farm (A. Richard, St. Damase, Canada). Females were mated twice, 7 days apart, during the first 2 weeks of March, according to standard husbandry procedures. We and others have shown that pro lactin injections terminate obligate embryonic diapause and induce embryo activation and implantation in the mink (32–34), and a standard protocol consisting of daily intramuscular injections of 1 mg/kg prolactin (Sigma) was employed beginning 1 week following last mating and continuing for 12 days. Implantation takes place approximately on the 13th day after the initiation of prolactin injections, verified by the presence of uterine swellings and histological evidence of trophoblast invasion. Pseudopregnancy was induced in non-mated females by two injections of GnRH (10 $\mu$g/kg Factrel; Ayerst, Guelph, Canada) 7 days apart. Uterine tissues were collected at the early stages of implantation from randomly selected females at days 13 and 15 following the first prolactin injections as well as from pseudopregnant animals. As we have previously shown (35), the natural increases in prolactin and consequent progesterone synthesis take place in pseudopregnant animals, and samples were therefore collected and collected at 30 days after induction of the first ovulation. Samples were frozen immediately in liquid nitrogen and stored at $-70$ °C until analyzed.

**Cell Culture**—An immortalized mink uterine stromal cell line (36) was used for the in vitro experiments described herein. Cells were cultured in Dulbecco’s modified Eagle’s medium/...
were excised and purified using a gel extraction kit (Qiagen). Purified cDNA was ligated into a pDrive vector (Qiagen) following the manufacturer’s instructions and further transformed into competent *Escherichia coli* strain XL-1 Blue. Plasmids were isolated with a QIAprep Spin Miniprep kit (Qiagen) and sequenced by automated DNA sequencing for verification (Service d’Analyse et de Synthèse d’Acides Nucléiques de Université Laval, Québec, Canada). PCRs were carried out in a final volume of 50 μl using Tag DNA polymerase (Amersham Biosciences). PCR products were separated in a 1.5% agarose gel and visualized with ethidium bromide.

**Cloning and Sequencing of the Mink VEGF 5′-Untranslated Region and Promoter Regions**—The 5′-flanking region of the mink VEGF gene was cloned by PCR using the Universal Genome Walker Kit (Clontech) from a library constructed from mink genomic DNA. The Expand High Fidelity kit served for amplification. The PCR products were cloned into a pGEM-T vector (Qiagen) for sequencing, which was performed by automated DNA sequencing (Service d’Analyse et de Synthèse d’Acides Nucléiques de Université Laval). Sequence analysis was undertaken using MatInspector (Abteilung Genetek, Braunschweig, Germany) and TF Search (Yukata Akiyama; TF Search: Searching TF Binding Sites). The transcription start site of the VEGF gene was predicted using the program for promoter prediction of the Berkeley Drosophila Genome Project (University of California, Berkeley, CA). To confirm prediction, we employed the 5′/3′-RACE kit (Roche Applied Science) to identify the site of transcription initiation from 2.9 kb of the mink 5′-flanking region. Primers for amplification are described in Table 2.

**Immunohistochemical Analysis of Microsomal PGE Synthase**—Tissues fixed in 4% paraformaldehyde solution were used to demonstrate expression of PGE synthase during the early implantation stages. Sections were rehydrated and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline. Blocking was performed for 1 h using 5% bovine serum albumin in phosphate-buffered saline, and sections were incubated overnight at 4 °C with rabbit anti-human PGE synthase (Cayman, Ann Arbor, MI) diluted 1:150 in 5% donkey serum. Next, sections were washed in phosphate-buffered saline. Blocking was performed for 1 h using 5% donkey serum, followed by a wash in phosphate-buffered saline containing 0.2% Triton X-100 for 10 min at 37 °C. Cells were then washed in phosphate-buffered saline, resuspended in 200 μl of ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and protease inhibitors), and sonicated with a Branson Sonifier 450 (Danbury, CT) at power setting 2 with 10-s pulses at duty cycle 90. ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 16.7 mM NaCl, and protease inhibitors) was used to dilute the chromatin solution 10-fold. Total DNA used for controlling the amount of DNA/sample was purified from one-tenth of the lysate. Each sample was pre-cleared by incubating with 80 μl of salmon sperm DNA/protein A-agarose 50% gel slurry (Upstate Biotechnology, Inc., Lake Placid, NY) for 30 min at 4 °C. Anti-acetyl histone H-3 (5 μg; Upstate Biotechnology), anti-AP2 (5 μg; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Sp1 (5 μg; Santa Cruz Biotechnology), and rabbit IgG (as negative controls) were added and immunoprecipitated at 4 °C overnight. The immunoprecipitate was collected using salmon sperm DNA/protein A-agarose and washed once with buffers in the following order: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 4.0), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 4.0), and protein G-agarose (Amersham Biosciences). The precipitates were washed extensively with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 M LiCl, and 1% SDS, followed by two washes with 1% SDS, 1% Triton X-100, 2 M LiCl. The precipitates were eluted in 200 μl of 1% SDS, 0.1 M Tris-HCl, pH 8.0, and visualized with ethidium bromide.

**TABLE 2**

| Oligonucleotide name | Sequence (5′-3′) |
|----------------------|-----------------|
| 5′RACE-Sp1           | TTGACCCCTTCCTTCCTTGC |
| 5′RACE-Sp2           | CTTTGCAACCGGFPCTTCCTT |
| 5′RACE-Sp3           | GGGAAGATTAAGACGACATTCC |
| ChIP-forward         | CAGGGGTCACGCGCAGATTCCA |
| ChIP-reverse         | CCTCTGCCCTCCTTCACCTA |

**PGE$_2$ Regulation of the VEGF Gene**

**TABLE 2**

| Oligonucleotide name | Sequence (5′-3′) |
|----------------------|-----------------|
| 5′RACE-Sp1           | TTGACCCCTTCCTTCCTTGC |
| 5′RACE-Sp2           | CTTTGCAACCGGFPCTTCCTT |
| 5′RACE-Sp3           | GGGAAGATTAAGACGACATTCC |
| ChIP-forward         | CAGGGGTCACGCGCAGATTCCA |
| ChIP-reverse         | CCTCTGCCCTCCTTCACCTA |
of orthogonal contrasts and the Duncan multiple range test. A probability level of $p < 0.05$ was considered significant.

RESULTS

Embryonic Production of PGE$_2$—Embryos collected during the diapause and activation stages were incubated in the presence or absence of mink uterine cells to determine their capability to produce PGE$_2$. Embryos in diapause failed to produce and/or secrete detectable levels of PGE$_2$. The embryos collected following activation by prolactin treatment and that were therefore in active growth (37) produced copious quantities of PGE$_2$ (Fig. 1A), either alone or in co-culture with uterine cells. Uterine cells alone failed to produce this prostaglandin.

Expression of PGE Synthase and EP Receptor mRNA in the Uterus—Uterine tissues collected from implantation and interimplantation sites as well as uteri collected from pseudopregnant females contained mRNA for PGE synthase. The abundance of mRNA for this enzyme was ~7-fold greater in tissues collected from the implantation sites, composed of both embryonic and uterine components, at 3–4 days following implantation compared with all other uterine samples studied (Fig. 1B). EP1 receptor expression was negligible, whereas the receptors of the EP2 and EP4 subtypes were expressed at all stages and locations collected, with no apparent difference among the samples (Fig. 1B). The EP3 receptor mRNA was not detected at any of the sites or stages investigated (data not shown).

Immunohistochemical Localization of PGE Synthase in the Pregnant Mink Uterus—Given the increased expression of the PGE synthase observed at implantation sites (Fig. 1B), we were interested in establishing whether it was of uterine or embryonic origin. We found the PGE synthase protein to be present in the myometrium of all stages evaluated (Fig. 2E). In the endometrium, PGE synthase was localized in the stromal layer immediately surrounding the implanting embryo (Fig. 2, A–C), whereas no significant localization was observed in the uterine tissue opposite to the invading trophoblasts (Fig. 2D). Histological sections from interimplantation sites further confirmed that pattern, in that endome-
trial cells in these regions, lacking direct contact with embryonic tissue, did not express this protein (Fig. 2, E and F).

**Cloning and Sequencing of the 5′-Flanking Region of the Mink VEGF Gene**—A 2.9-kb sequence upstream of the ATG triplet was identified by means of the Genome Walker kit (Clontech) (accession number DQ381737). The transcription initiation site at 1058 bp upstream of the ATG triplet was predicted by means of the promoter prediction program of the Berkeley Drosophila Genome Project and confirmed by 5′-RACE using the RNA of three different mink samples, two of uterine and one of ovarian origin. The VEGF proximal promoter region in the mink bears a high homology to the human (81%) and mouse sequences (72%) (accession numbers AF095785 and U41383, respectively). Matinspector analysis of the mink promoter sequence identified several potential response element sequences previously identified in the human and mouse VEGF promoters, AP1, AP2, and Sp1, among others. Concurring with the human and mouse counterparts, there was no consensus TATA box motif present, whereas an important GC-rich region was found in the proximal promoter region, about 70 bp upstream of the transcription start site.

**PGE2 Induces VEGF Transcription**—We employed the reporter gene luciferase driven by the mink VEGF proximal promoter in mink uterine stromal cells. We observed that PGE2 was capable of inducing a 3-fold induction in transcription of the reporter gene, in response to the doses of 75 and 100 μM (p < 0.05), and a 2-fold induction to the dose of 50 μM, whereas 10 μM resulted in a modest but nonsignificant 50% elevation (Fig. 3A). Similar levels of induction were observed at the three different times tested, 6, 12, and 24 h of PGE2 treatment (Fig. 3B). Furthermore, we tested transfection of other cell types and consequent response to prostaglandin treatment in the form of induction of VEGF transcription. The results indicate that mink VEGF promoter is activated by PGE2 in mink ovarian tumor cells (Fig. 3C) as well as in the human breast cancer cells MCF-7 (Fig. 3D).

**PGE2 Induction of VEGF Transcription Is PKA-dependent**—Mink stromal cells transfected with a 1.5-kb mink VEGF promoter driving the luciferase gene were treated with the cAMP agonists dibutyryl- and chlorophenylthio-cAMP (100 μM, 1 μM), eliciting inductions of the VEGF promoter comparable with those observed following treatment with PGE2 (Fig. 4A). To further investigate the involvement of the PKA pathway in PGE2-induced VEGF transcription, transfected cells were pretreated with the PKA antagonist H89, resulting in complete abolition of the transcriptional activation by PGE2 (Fig. 4B). H89 blockade of PKA likewise abrogated the PGE2 response in mink ovarian tumor cells (not shown).

**EP2 and EP4 Antagonists Block the PGE2-induced Up-regulation of VEGF Transcription**—After verifying that PGE2 stimulates VEGF through a PKA-dependent pathway (Fig. 4), we sought to confirm that the response observed in VEGF transcription was dependent upon ligand binding to the receptors previously known to elicit increases in cAMP. Antagonists to the receptors EP1, EP2, and EP4 were added to the transfected

**FIGURE 2. Immunohistochemical characterization of PGE synthase in the mink uterus.** A–C, PGE synthase is localized principally to the stromal cells surrounding the implanting trophoblast cells (arrows). D, absence of PGE synthase localization in the endometrium tissue on the mesometrial (opposite) side of the uterus to the invading embryo. E and F, VEGF localizes at the vessels and the myometrium (white arrows), but not in the endometrial cells, in samples collected from interimplantation sites. Bars, 500 μm.
stromal cells 1 h prior to treatment with PGE₂. Although ligand binding to EP1 does not elicit PKA-dependent responses, the antagonist for EP2 also blocks EP1. We therefore tested an EP1-specific antagonist to control for the blocking of this receptor. The EP1-specific antagonist had no significant effect on the transcription of the reporter gene driven by the mink VEGF promoter (Fig. 5). The antagonist impairing both EP2 and EP1 receptors significantly blocked the transactivation of the VEGF promoter (Fig. 5; \( p < 0.05 \)). A significant, if less pronounced, attenuation was observed following treatment with the EP4 antagonist (Fig. 5; \( p < 0.05 \)). Complete abrogation of promoter activation was observed when the cells were treated with antagonists for EP1/EP2 and EP4 (Fig. 5).

**AP2 and Sp1 Mediate PGE₂-induced Responses in the Mink VEGF Promoter**—We carried out promoter deletion analysis to identify the region(s) of the promoter transactivated following PGE₂ treatment (Fig. 6). The two longest constructs used, containing 1289 and 708 bp, respectively, resulted in comparable levels of induction following PGE₂ treatment. The third construct, spanning only 99 bp upstream of the transcription initiation site resulted in slightly lower induction by PGE₂, although not significantly different from the two longer versions. Loss of response to PGE₂ was not observed until only 51 bp remained upstream of the transcriptional site, and a further deletion of 34 bp completely eliminated basal promoter activity (Fig. 7A; \( p < 0.05 \)). Within the 99 bp remaining upstream of the transcription, an AP2 and an Sp1 binding site were predicted by MatInspector. We then effected a mutation to render these sites unable to bind these transcription factors. Given their proximity and nucleotide overlap, a single mutation was employed to interfere with both sites. Subsequent transfection trials demonstrated that mutation of these sites completely eliminated the PGE₂-induced promoter transactivation (Fig. 7B; \( p < 0.05 \)).

**AP2 and Sp1 Interact with the VEGF Promoter in Response to PGE₂**—Following the mutation of the AP2/Sp1 cluster and loss of PGE₂-induced promoter response, we sought to verify if treatment with PGE₂ could induce the binding of these two transcription factors to the endogenous mink VEGF promoter in the uterine stromal cell line by ChIP assay. Confluent cells were serum-starved for 20 h prior to the addition of PGE₂. Following 6 h of PGE₂ treatment, cells were cross-linked and immunoprecipitated with the AP2 and Sp1 antibodies. Cross-linking was reversed, and the immunoprecipitated DNA was then amplified by PCR using primers spanning the proximal promoter region (including the transcription start site). Confirming the deletional studies, binding of both transcription factors to the VEGF promoter region in uterine cells was induced by treatment with PGE₂ (Fig. 8A). A 4-fold induction of AP2 binding to the VEGF promoter region was observed following PGE₂ treatment, whereas PGE₂ doubled the binding of Sp1 to the VEGF promoter.

**PGE₂ Induces Histone Modifications**—To investigate whether PGE₂ also plays a role in covalent modification of histones, we performed a ChIP assay on PGE₂-treated cells with an antibody recognizing histone H3 acetylated on lysine 14. Mink stromal cells were serum-starved for 20 h and then were treated with 75 \( \mu \)M PGE₂ for 6 h. Cells were then cross-linked and immunoprecipitated. Cross-linking was reversed, and the immunoprecipitated DNA was then amplified by PCR using primers spanning the proximal promoter region.

**FIGURE 3.** VEGF promoter activity is increased following PGE₂ treatment in mink uterine and ovarian cell lines and in MCF-7 mammary tumor cells. Cells were transfected with 1.5 kb of the mink VEGF promoter driving the luciferase reporter gene. A, transfected mink stromal cells were treated for 12 h with doses of PGE₂ (10, 50, 75, and 100 \( \mu \)M), 8, cells were treated with 75 \( \mu \)M of PGE₂ for different times (6, 12, and 24 h). C, transfected mink ovarian tumor cells. D, MCF-7 cells were treated for 12 h with 75 \( \mu \)M PGE₂. The quantification represents mean ± S.E. of triplicate transfection experiments. Different superscripts represent significant differences in means (\( p < 0.05 \)).
imal promoter region (including the transcription start site). Treatment with PGE$_2$ was effective in inducing acetylation of histone H3 2-fold in comparison with nontreated controls (Fig. 8B). The region amplified by PCR corresponds to the promoter region containing the binding sites for AP2 and Sp1, shown here to be involved in PGE$_2$ transactivation of the mink VEGF promoter (Figs. 7, A and B, and 8A).

**DISCUSSION**

Extensive angiogenesis is paramount to successful maintenance of gestation (40), providing the increase in blood supply to the implanting and rapidly developing embryo. VEGF has been demonstrated to be one of the major angiogenic factors inducing proliferation and migration of endothelial cells as well as permeability in vessels in a wide array of normal and pathogenic mammalian tissues (16).

The early investigations of Kennedy (41) demonstrated the importance of prostaglandins in the process of embryo implantation in rodents, and confirmation came in the form of complete abrogation of implantation in mice bearing null mutation of the COX-2 gene (4). We established the occurrence of COX-2 expression by both the trophoblast and uterine stroma at the site of implantation in the unique carnivore model we employ in the current study, the mink (42). Investigations by Matsumoto et al. (17) on the COX-2-deficient mouse have indicated a functional link between prostaglandin synthesis and VEGF expression in mouse implantation. We previously demonstrated that, in the mink uterus, VEGF mRNA and protein are up-regulated around the time of implantation (24), indicating an important role for this growth factor in this species and the rationale for this investigation.

In the present study, we provide the first evidence to place PGE$_2$ as an important regulator of VEGF transcription during the peri-implantation period in mammals. First, we show that the mink embryo that has escaped from diapause is an important source of PGE$_2$. Further, we have demonstrated the presence of the PGE synthase, the enzyme responsible for synthesis of PGE$_2$ from prostaglandin H$_2$, and have shown that it is regulated in the uterus during early stages of pregnancy, providing a second source of PGE$_2$. Moreover, we have unequivocally placed the expression of PGE synthase in the stroma surrounding the invading trophoblastic layer and have demonstrated its absence from the interim-implantation endometrium. This concurs with a similar phenomenon in mice where PGE synthase mRNA and protein occur in the subliminal stroma surrounding the implanting blastocyst (11). The PGE synthase expression we observed appears to coincide with expression of COX-2, which is likewise localized at the sites of trophoblast invasion, particularly in the necks of the uterine glands during early implantation (42). This co-localization places the presence of the
substrate for PGE₂ in a timely manner to allow for PGE₂ synthesis by the PGE synthase.

In the present study, we were interested in investigating potential regulators of VEGF and the mechanisms involved in this regulation. PGE₂ was our choice, given its effects on VEGF expression in other tissues, the secretion of PGE₂ by the embryo, and the presence of PGE synthase at the site of implantation. By employing a homologous in vitro system using immortalized mink uterine stromal cells with the mink VEGF promoter driving the reporter gene luciferase, we show that PGE₂ induces expression of VEGF as demonstrated. PGE₂ can act through four different G-coupled receptors, EP₁–EP₄, each employing its own second messenger system (2). EP₁ acts through activation of the phospholipase C system and by inducing a rise in intracellular calcium levels (43), whereas EP₂ and EP₄ both induce responses through activation of the PKA pathway, and both have been involved in PGE₂-induced regulation of VEGF (31, 44, 45). Whereas multiple EP₃ isoforms have been identified to date, and they can act through different signaling pathways associated with G₁, G₃, and G₄ activation (2), the absence of detectable message for this receptor subtype in uterine and embryonic tissue suggests that it is not involved in early implantation processes studied herein.

We demonstrated the presence of EP₂ and EP₄ mRNA in the mink uterine tissues during early stages of implantation and placentation, as well as in the pseudopregnant uterus, but no variation in expression and thus no pregnancy-specific regulation of these receptors was present. This suggests that regulation of the ligand PGE₂ rather than up-regulation of the receptors is responsible for the effects seen in VEGF expression. The fact that these two receptors induce cAMP-dependent responses strongly argues that the increase in VEGF transcription is cAMP-dependent.

Intracellular pathways involving cAMP most usually involve phosphorylation of cAMP-response element-binding protein (46), but we were unable to identify classic cAMP-response elements within the proximal promoter region of the mink VEGF gene, although responses involving transcription factors to which no putative binding sequences were predicted are quite possible. We present compelling evidence to indicate that the cis-acting AP₂ and Sp₁ elements are the downstream effectors of the PGE₂ induction of the VEGF promoter. Mutation of these overlapping regions for AP₂ and Sp₁ was sufficient to induce loss of transcription induction by PGE₂, without the loss of basic pro-
PGE₂ Regulation of the VEGF Gene

**PGE₂ Regulation of the VEGF Gene**

**A.** Control  |  PGE₂  
---|---
AP2  
SP1  
Negative control  
Input  

**B.** Control  |  PGE₂  
---|---
Ac-H3  
Negative control  
Input  

FIGURE 8. PGE₂ treatment induces AP2 and Sp1 interaction with the VEGF promoter as well as histone modification in mink uterine cells. Mink stromal cells were serum-starved for 24 h prior to the addition of PGE₂ (75 μM) for 6 h. Immunoprecipitation was performed using antibodies against AP2 and Sp1 (A) and acetylated histone H3 (B). Following chromatin precipitation, 500 bp of the proximal promoter region of VEGF was amplified by PCR. Input control was established by amplification of an equivalent amount of DNA that had not been previously subject to immunoprecipitation. Negative control for antibody specificity was established by precipitation with rabbit IgG.

The use of ChIP has enabled us to verify that PGE₂ increases binding of these two transcriptional factors to the proximal promoter region of VEGF. This concurs with the studies of human smooth muscle cells of the airway, which have placed Sp1 downstream of cAMP in the induction of VEGF by PGE₂ (45). Further, the AP2/Sp1 cluster has previously been shown to be involved in transcriptional regulation of VEGF (47, 48). The partnership between AP2/Sp1 is also implicated in transcriptional regulation of another gene, CYP17, following increased levels of cAMP (49).

The region we show controlling transcription lies in a GC-rich region of the mink VEGF promoter, from −71 to −53. This again concurs with previous findings in which a homologous GC-rich region appears to regulate VEGF promoter activation by transforming growth factor-α through the AP2 consensus site in humans (50). This has also been demonstrated by ultraviolet B radiation through AP2/Sp1 in humans (48). Similarly, p42/p44 mitogen-activated protein kinase modulates VEGF expression through AP2/Sp1 binding in hamsters (47), and platelet-derived growth factor does so through Sp1 in humans (51). Taken together, the results in humans and hamsters and our findings with the mink VEGF promoter argue for the conclusion that this promoter region, along with the transcription factors AP2 and Sp1, plays a central role in the regulation of VEGF transcription.

In addition to recruitment of cis-acting elements, chromatin modifications are also required in order to allow transactivation of promoter regions. Among these modifications, histone acetylation has been associated with active chromatin and consequent access of transcription factors to promoter regions (52). In the mink model, PGE₂ provoked acetylation of H3 localized to the proximal promoter region, spanning the area containing the response elements for AP2 and Sp1. This demonstrates that PGE₂ induces activation of the transcription factors in conjunction with induction of chromatin restructuring to bring about promoter binding and transactivation.

In summary, the current study provides the first evidence that PGE₂ of embryonic and endometrial provenance regulates the expression of VEGF at implantation sites in the uterus of the mink. This indicates that, in this unique model of decidua-free, endothelial-chorial placentation, there is dependence on the presence of local prostaglandin synthesis for establishment of the vascularity required for maintenance of early gestation.

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