Expression of Hypoxia-inducible Factors in the Peri-implantation Mouse Uterus Is Regulated in a Cell-specific and Ovarian Steroid Hormone-dependent Manner

EVIDENCE FOR DIFFERENTIAL FUNCTION OF HIFs DURING EARLY PREGNANCY*

Received for publication, November 7, 2002, and in revised form, December 6, 2002
Published, JBC Papers in Press, December 12, 2002, DOI 10.1074/jbc.M211390200

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Increased uterine vascular permeability and angiogenesis are hallmarks of implantation and placentation. These events are profoundly influenced by vascular endothelial growth factor (VEGF). We previously showed that VEGF isoforms and VEGF receptors are expressed in the uterus, suggesting the role of VEGF in uterine vascular permeability and angiogenesis required for implantation and decidualization. We have recently shown that estrogen promotes uterine vascular permeability but inhibits angiogenesis, whereas progesterone stimulates angiogenesis with little effect on vascular permeability. However, the mechanism of differential steroid hormonal regulation of uterine angiogenesis remains unresolved. Oxygen homeostasis is essential for cell survival and is primarily mediated by hypoxia-inducible factors (HIFs). These factors are intimately associated with vascular events and induce VEGF expression by binding to the hypoxia response element in the VEGF promoter. HIF isoforms function by forming heterodimers with the aryl hydrocarbon nuclear translocator (ARNT) (HIF-β) family members. There is very limited information on the relationship among HIFs, ARNTs, and VEGF in the uterus during early pregnancy, although the role of HIFs in regulating VEGF and angiogenesis is well documented. Using molecular and physiological approaches, we show that uterine expression of HIFs and ARNTs does not correlate with VEGF expression during the preimplantation period (days 1–4) in mice. In contrast, their expression follows the localization of uterine VEGF expression with increasing angiogenesis during the implantation period (days 5–8). This disparate pattern of uterine HIFs, ARNTs, and VEGF expression on days 1–4 of pregnancy suggests HIFs have multiple roles in addition to the regulation of angiogenesis during the peri-implantation period. Using pharmacological, molecular, and genetic approaches, we also observed that although progesterone primarily up-regulates uterine HIF-1α expression, estrogen transiently stimulates that of HIF-2α.

In the adult, angiogenesis physiologically occurs in the uterus and ovary during the reproductive cycle and pregnancy under physiological conditions (1, 2). Increased uterine vascular permeability and angiogenesis are hallmarks of successful implantation and placentation. These events are profoundly influenced by vascular endothelial growth factor (VEGF) (3, 4), which exists in multiple isoforms. VEGF signals via two transmembrane tyrosine kinase receptors, VEGF receptors 1 and 2 (5–11). We have previously shown that VEGF isoforms and VEGF receptors are expressed in the uterus during early pregnancy in a spatiotemporal manner (1), suggesting that VEGF plays an important role in uterine vascular permeability and angiogenesis required for implantation and decidualization. Because the uterus is a primary target for estrogen and progesterone (P$_4$), which profoundly influence uterine function prior to and during implantation, it is thought that steroid hormones modulate the uterine angiogenic status via the VEGF system. Indeed, our recent studies have shown that estrogen and P$_4$ have different effects in vivo; estrogen promotes uterine vascular permeability but profoundly inhibits angiogenesis, whereas P$_4$ stimulates angiogenesis with little effect on vascular permeability. These effects of estrogen and P$_4$ are mediated by differential spatiotemporal expression of proangiogenic factors in the uterus (12). VEGF effects are complemented and coordinated by another class of angiogenic factors, the angiopoietins that act via the tyrosine kinase receptor Tie2 (13). We have recently shown that although VEGF signaling primarily regulates uterine vascular permeability and angiogenesis prior to the attachment phase of the implantation process, VEGF in conjunction with the angiopoietin system directs angiogenesis during uterine decidualization following implantation (14). Furthermore, our results provide evidence that although ovarian steroid hormones influence uterine vascular permeability and angiogenesis during the preimplantation period, cyclooxygenase-2 (COX-2)-derived prostaglandins direct these events during implantation and decidualization by differentially regulating VEGF and angiopoietin signaling. However, the mechanisms by which steroid hormones and prostaglandins differentially regulate uterine angiogenesis during early pregnancy remain unresolved.

* This work was supported in part by National Institutes of Health Grants HD37580 (to S. K. Das) and HD12304 and HD33994 (to S. K. Dey). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡‡‡ The abbreviations used are: VEGF, vascular endothelial growth factor; P$_4$, progesterone; COX-2, cyclooxygenase-2; HIF, hypoxia-inducible factor; ARNT, aryl hydrocarbon nuclear translocator; ER, estrogen receptor; PR, progesterone receptor; E$_2$, estradiol-17β; PRE/GRE, progesterone/glucocorticoid-responsive element.
Evidence for Differential Function of HIFs during Early Pregnancy

Oxygen homeostasis is essential for cell survival and is primarily mediated by hypoxia-inducible factors (HIFs), which are intimately associated with vascular events (15–18) and induce Vegf gene transcription by binding to the hypoxia response element in the Vegf promoter (15, 19–22). Several HIFs have been identified that all function as heterodimeric transcription factors consisting of α- and β-subunits. These subunits belong to the basic helix-loop-helix-PAS protein superfamily (15, 18). HIF-1α was first cloned in humans followed by cloning in mice and rats (23–26). Subsequently, HIF-2α and HIF-3α, which have a high sequence homology to HIF-1α, were cloned in mice, rats, and humans (26–28). HIF-2α is also known as endothelial PAS domain protein-1, HIF-1α-like factor, HIF-related factor, or MOP2 (member of the PAS superfamily) (15). HIF-β subunits are identical to the aryl hydrocarbon nuclear translocator (ARNTs). The ARNT family consists of ARNT1, ARNT2, and ARNT3. ARNT3 is also known as BMAL1 (brain and muscle ARNT-like protein-1) (15). HIF-α subunits can heterodimerize with the ARNT family members without specificity for their dimerization partners (15).

HIF-1α is expressed in most human and rodent tissues (25, 29). Levels of the HIF-1α protein are primarily regulated by protein stabilization under hypoxic conditions, whereas its rapid degradation occurs under normoxic conditions via an ubiquitination mechanism (15, 30–33). Normally, the expression of HIF-1α is ubiquitous, whereas that of HIF-2α and HIF-3α shows a more restricted expression pattern. There is evidence that HIF-2α mRNA expression is much higher under normoxic conditions and that its expression correlates with that of VEGF (34). The expression patterns of ARNT1, ARNT2, and ARNT3 in general resemble those of HIF-1α, HIF-2α, and HIF-3α (15). Mice deficient in ARNT1, HIF-1α, or HIF-2α die at midgestational stage because of vascular defects primarily involving the embryonic and extraembryonic vasculature (15, 19, 20, 35–38). In contrast, mice deficient in ARNT2 or ARNT3 do not exhibit any vascular abnormalities (39, 40). These results suggest that VEGF expression is primarily regulated by HIF-1α, HIF-2α, and ARNT1 but not ARNT2 or ARNT3 during embryonic development. However, there is very limited information regarding the relationship between HIFs, ARNTs, and VEGF in the adult normal uterus during early pregnancy, although the role of HIFs in regulating VEGF and thus angiogenesis in tumor tissues has clearly been documented (41, 42).

In the present study, we examined the temporal and cell-specific expression of HIFs and ARNTs in parallel with the expression of VEGF in the uterus during the peri-implantation period and under steroid hormonal regulation. We observed that expression of HIFs and ARNTs does not spatiotemporally correlate with the expression of VEGF in the uterus during the preimplantation period (days 1–4 of pregnancy). In contrast, the expression of these transcription factors follows the localization of VEGF expression in the uterus with increasing angiogenesis during the postimplantation period (days 5–8). The disparate pattern of HIFs, ARNTs, and VEGF expression on days 1–4 of pregnancy suggests that they have different roles in addition to the regulation of angiogenesis in the uterus during the peri-implantation period. We also observed that although HIF-1α is primarily regulated by P4 in the mouse uterus, estrogen transiently regulates HIF-2α.

**Materials and Methods**

**Mice and Treatments**—Adult CD-1 mice were purchased from the Charles Rivers Laboratory (Raleigh, NC). Females were mated with fertile males of the same strain to induce pregnancy (day 1 = vaginal plug). Estrogen receptor-α (ERα)-deficient mice (129/J/C57BL/6J) and progesterone receptor (PR)-deficient mice (129Sv/Ev/C57BL/6J) were generated as previously described (43, 44) and were kindly provided by Dennis Lubahn (University of Missouri, Columbia, MO) and Bert O’Malley (Baylor College of Medicine, Houston, TX), respectively, for establishing our colonies. PCR analysis of the genomic DNA determined the genotypes. All of the mice were housed in our Animal Care Facilities according to the National Institutes of Health and institutional guidelines for laboratory animals.

To examine the effects of estrogen and/or P4 on uterine gene expression, ovariectomized mice were injected with sesame oil (0.1 ml/mouse), 17β-estradiol (E2) (100 µg/mouse), P4 (2 mg/mouse) or E2 plus P4. At termination of the treatments, uteri were processed for subsequent analysis. The steroids were dissolved in sesame oil and injected subcutaneously.

Probes—The cDNA clones for Vegf and ribosomal protein L7 (rpl7) have previously been described (1, 45). Peter Carmeliet (Flanders Interdisciplinary Institute, Leuven, Belgium) kindly provided a lacZ probe for the mouse HIF-1α. A 192-nt HIF-1α was cloned into a pGEM3ZF(+) vector at the EcoRI site. Mouse-specific HIF-3α and ARNT1 cDNAs were gifts from Chris Bradfield (University of Wisconsin, Madison, WI). Partial cDNAs for mouse HIF-2α, ARNT2, and ARNT3 were generated by reverse transcription-PCR cloning with specific primers. For Northern hybridization, antisense 32P-labeled cRNA probes were generated using T7 polymerase. For in situ hybridization, sense and antisense 35S-labeled cRNA probes were generated using Sp6 and T7 polymerases, respectively. Probes had specific activities of about 2 × 108 dpm/µg.

**Northern Hybridization**—For Northern hybridization, poly(A)+ RNA (5 µg) was denatured and separated by formaldehyde/agarose gel electrophoresis, transferred to nylon membranes, and UV cross-linked. Northern blots were prehybridized, hybridized, and washed as previously described by us (1, 45). Quantification of hybridized bands was analyzed by densitometric scanning.

**In Situ Hybridization**—In situ hybridization was performed as previously described by us (1, 45). In brief, frozen sections (10 µm) were mounted onto 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 or sense cRNA probes. RNase A-resistant hybrids were detected by autoradiography. The sections were post-stained with eosin and hematoxylin.

**Immunohistochemical Localization**—Frozen sections (10 µm thick) were mounted onto poly-L-lysine-coated slides and stored at −80 °C until used. The sections were fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.4) for 10 min at room temperature followed by washing in Tris-buffered saline (pH 7.4) for 5 min twice. Immunolocalization of HIF-1α was performed as previously described with some modifications (46, 47). In brief, the sections were incubated with chicken polyclonal anti-HIF-1α antibodies (1:50) overnight at 4 °C followed by washing in phosphate-buffered saline (46, 47). A peroxidase-conjugated rabbit anti-chicken IgY antibody (1:100; Pierce) was added onto the sections, and the sections were incubated for 45 min at room temperature with subsequent washing.

**Cell Culture, Transfection, and Luciferase Assays**—AN3CA uterine carcinoma cells were grown in Dulbecco’s modified Eagle’s medium (Cellgro), whereas L929 cells were cultured in Joklik’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Atlanta Biologicals), 1-glutamine (2 mm), penicillin (100 units/ml), and streptomycin (100 µg/ml) in a 5% CO2 atmosphere. ImmunoLocalization for ARNT1 was performed as previously described (48). In brief, the sections were incubated with a rabbit polyclonal anti-ARNT1 antibody (1:250; Affinity BioReagents, Neshanic Station, NJ) overnight at 4 °C. Immunostaining was performed using a Histostain-SP kit (Zymed Laboratories Inc.). After immunostaining, the sections were counterstained with 0.5% Fast Green. The red color indicated the site of positive staining.

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ing the vehicle (1% ethanol) or P4 (1 μg). After 48 h, the cells were harvested in 1× luciferase lysis buffer. Relative light units from firefly luciferase activity were determined using a luminometer (Monolight 2010) and normalized to the relative light units from Renilla luciferase using a dual luciferase kit (Promega).

RESULTS

Vegf, HIFs, and ARNTs Are Differentially Expressed in the Peri-implantation Uterus—The objective of these experiments was to examine whether Vegf expression is co-localized with that of HIFs and ARNTs during the pre- and post-implantation periods. As previously shown by us (1), Vegf mRNA expression was restricted to the luminal epithelium on day 1 of pregnancy when the uterus is under the influence of preovulatory estrogen, whereas on day 4 of pregnancy, this expression became primarily localized in the stroma under the influence of rising P4 levels fortified with a small amount of estrogen (Fig. 1A). However, the mechanism by which steroid hormones influence Vegf expression is not fully understood. Because HIFs are known to regulate Vegf expression associated with angiogenesis, we examined the expression of HIFs and their partners ARNTs in the uterus to determine whether HIFs play any role in uterine Vegf expression. We observed that the expression of HIFs (HIF-1α, -2α, and -3α) was very low to undetectable in the uterus on day 1 of pregnancy. However, HIF-1α was distinctly expressed in the luminal epithelium on day 4 of pregnancy as opposed to the expression of Vegf in the stroma. Interestingly, distinct but patchy expression of HIF-2α was noted in the stroma, whereas the expression of HIF-3α was undetectable (Fig. 1A).

Because HIFs must heterodimerize with ARNTs for transcriptional activation of Vegf (17), we next examined the expression of ARNTs in the uterus on similar days of pregnancy. To our surprise, we observed that all three ARNTs (ARNT1, ARNT2, and ARNT3) were expressed at very low to undetectable levels on these days of pregnancy, except ARNT1, which was expressed at a low to modest level both in the luminal epithelium and stroma on day 4 of pregnancy (Fig. 1B). On the other hand, the stromal expression of HIF-2α that correlates with ARNT1 on day 4 of pregnancy suggests that HIF-2α regulates Vegf transcription after heterodimerization with ARNT1. We next asked whether the localization of these proteins follows the same pattern as their respective mRNAs. Surprisingly, immunoreactive HIF-1α and ARNT1 were primarily localized to the uterine epithelium on day 4 of pregnancy, suggesting that HIF-1α effects are probably restricted to the epithelium at this time (Fig. 1C). The unavailability of suitable antibodies to other HIFs and ARNTs has precluded us from determining the localization of these proteins in the uterus. Nonetheless, the mRNA localization of HIF-2α in the stroma in the presence of little or no expression of ARNT2 and ARNT3 and a very low level of ARNT1 expression with restricted localization of its protein in the epithelium raises questions regarding a role for HIF-2α in stromal Vegf expression on day 4 of pregnancy. It is possible that a yet unidentified ARNT isoform is expressed in the stroma at this time. Nonetheless, the localization of both the mRNA and protein for HIF-1α and ARNT1 in the epithelium on day 4 of pregnancy suggests that HIF-1α has a different role in the uterus, because Vegf is expressed in the stroma but not in the epithelium at this time.

There are increases in Vegf expression and angiogenesis in the uterus at the site of the blastocyst as implantation progresses. Therefore, we compared the expression of Vegf with those of HIFs and ARNTs during the postimplantation period, particularly on days 5 and 8 of pregnancy. These 2 days were chosen because day 5 represents a very early stage of implantation that correlates with initiation of the decidualization process, whereas day 8 represents a late phase of the implantation process when decidual growth is maximal. As previously observed (1), Vegf expression is more localized to the luminal epithelium and stroma surrounding the implanting blastocyst on day 5 of pregnancy. The expression further increases in the stromal decidua on day 8 (Fig. 2A). With respect to HIFs, both the luminal epithelium and stroma exhibited HIF-1α expression similar to that of Vegf, whereas HIF-2α expression was restricted to only stromal cells surrounding the blastocyst on day 5. In contrast, the expression of HIF-3α was very low without any cell-specific localization. On day 8, HIF-1α expression showed further increases in the decidual bed, but the most robust expression was noted for HIF-2α. The expression of HIF-3α was again very low and diffuse. All three HIFs showed expression in the developing embryo. The cell-specific accumulation of HIF-1α and HIF-2α mRNAs closely correlated with the levels determined by Northern hybridization of whole uterine RNA samples (Fig. 3).

When expression patterns of HIFs were compared with those of ARNTs, we observed that ARNT1 and ARNT2 showed similar expression patterns in the stroma as that of HIF-1α and HIF-2α on day 5, but the expression of ARNT2 was primarily restricted to the luminal epithelium (Fig. 2B). These results suggest that HIF-1α and HIF-2α can partner with ARNT1 or ARNT3 in the stroma, but only HIF-1α can partner with ARNT2 in the epithelium on day 5. On day 8 of pregnancy, the localization of ARNTs was similar to that of HIF-1α and HIF-2α, but the expression intensity was low to modest in the decidual bed. The expression of ARNTs in the developing embryo was similar to that of HIFs. No specific localization of HIF and ARNT mRNAs was detected after hybridization of uterine sections with sense probe (Fig. 4). Collectively, these results suggest that ARNT1 and ARNT3 are perhaps the major partners of HIF-1α and HIF-2α in the uterine stroma that is operative for the Vegf expression during the postimplantation period.

Uterine Expression of HIF-1α and HIF-2α Is Regulated by Progesterone and Estrogen—Our observations of uterine expression of HIF-1α, HIF-2α, and Vegf on day 1 and day 4 of pregnancy suggested that these genes are regulated by ovarian estrogen and P4. Therefore, we further examined the expression of these genes in a more defined system, i.e. in ovariectomized mice after steroid hormone treatment. Northern blot analysis showed that the levels of HIF-1α mRNA increased by about 3.5 times within 6 h of an E2 injection and that the levels peaked at 12 h followed by a decrease by 24 h. In contrast, the levels of HIF-1α mRNA showed gradual increase from 2 h after an injection of P4 showing a peak at 24 h (Fig. 5). When P4 treatment was combined with E2, the response was advanced exhibiting peak levels at 6 h followed by a decline at 12 h. With respect to HIF-2α, we observed that the expression of this gene is primarily regulated by E2 in a transient manner reaching maximal levels at 4 h. In contrast, P4 was not very effective in influencing this gene. A combined treatment with E2 and P4 showed an expression pattern similar to that of E2 alone but at lower levels (Fig. 5). When the levels of Vegf mRNA was compared with those of HIF-1α and HIF-2α, we observed that the accumulation of Vegf mRNA was very rapid peaking at 2 h of an E2 injection followed by a sharp decline thereafter. In contrast, the levels of this mRNA showed a gradual increase from 1 h after an injection of P4 showing a peak at 24 h. A co-injection of E2 with P4 increased the levels of Vegf mRNA by 2 h similar to that observed for E2 alone (Fig. 5). These results again suggest that Vegf, HIF-1α, and HIF-2α are not always coordinately expressed.

However, Northern analysis gives no indication of the uterine cell types involved, and the levels of whole uterine mRNAs
FIG. 1. Expression of Vegf, HIFs (1α–3α), and ARNTs (1–3) in the preimplantation mouse uterus. A and B, in situ hybridization. Representative dark field photomicrographs of longitudinal uterine sections on days 1 and 4 showing Vegf, HIFs, and ARNT expression at 100×. C, immunohistochemistry. Longitudinal uterine sections on day 4 morning and afternoon and sections of brain (control) were used for HIF-1α or ARNT1 immunostaining (100×). le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium.
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by Northern hybridization may have limited value because of
the dilution effects resulting from heterogeneous uterine cell
types that undergo dynamic changes during pregnancy and
under steroid hormonal stimulation. For example, the luminal
epithelium represents only 5–10%, the stroma 30–35%, and
the myometrium 60% of the major uterine cell types. Thus,
even a 50% or greater increase in the expression level in the
expression level may not be reflected when the expression level is
measured in whole uterine extracts (52). Therefore,
in situ
hybridization was performed to examine the cell-specific ex-
pression of Vegf, HIF-1α, and HIF-2α in uteri of ovariectomized
wild-type mice treated with E₂ or P₄ at various time points
(Fig. 6). We observed that Vegf expression was low in ovariec-
tomized uteri treated with oil (control) and that expression was
localized to stromal cells both at 6 and 24 h. However, the
expression showed a prominent increase in stromal cells at 6 h
of an E₂ injection. By 24 h the expression became localized to

Fig. 2. In situ hybridization of Vegf, HIFs (1α–3α), and ARNTs
(1–3) in the postimplantation mouse uterus. A and B, dark field
photomicrographs of representative cross-sections of implanta-
tion sites on days 5 and 8 of pregnancy are shown at 100×. m, mesometrial site; am, antimesometrial site. The arrows indicate the locations of embryos.

Fig. 3. Northern blot detection of HIF-1α and HIF-2α mRNAs
in the mouse uterus during early pregnancy. Poly(A)⁺ RNA sam-
plies (2 μg) isolated of uterus on the indicated days of pregnancy were
separated by formaldehyde-agarose gel electrophoresis, transferred to
nylon membranes, UV-cross-linked, and hybridized to specific ³²P-
cRNA probes. The same blots were stripped and rehybridized to an
rPL7 (a housekeeping gene) probe to confirm the integrity of RNA
samples.

Fig. 4. Absence of localization (negative controls) of HIFs (1α–
3α) and ARNTs (1–3) mRNAs in uterine sections hybridized with
the sense probes. Dark field photomicrographs of uterine sections on
representative days of pregnancy hybridized with the sense probes
show the absence of specific hybridization signals as compared with
similar sections hybridized with the antisense probes that exhibited
distinct and specific signals.
epithelial cells. In contrast, the expression of Vegf was always localized to stromal cells both at 6 and 24 h after P4 treatment (Fig. 6A)). When these patterns of Vegf expression were compared with those of HIF-1α, we observed that HIF-1α expression did not follow the expression pattern of Vegf (Fig. 6, A and B). The expression of HIF-1α is always restricted to the uterine epithelium (Fig. 6B). For example, the expression was relatively low in ovariectomized uterine epithelium after an oil injection either at 6 or 24 h. The epithelial expression showed an increase at 6 h of an E2 injection but dramatically declined by 24 h. In contrast, a P4 injection increased the epithelial expression of HIF-1α at 6 h and more prominently at 24 h. These results suggest that E2 modestly and transiently influences HIF-1α expression in the epithelium as opposed to P4, which induces this gene in a more robust and sustained manner, suggesting a primary role of P4 in regulating HIF-1α expression in the mouse uterus. On the other hand, HIF-2α expression is primarily restricted to the stroma and is clearly up-regulated by E2 in a transient manner and follows the pattern of Vegf expression at this time point (Fig. 6C). To our knowledge, this is the first demonstration of the regulation of HIFs by ovarian steroids in a target tissue.

Our next goal was to examine whether P4 and estrogen regulation of HIF-1α is mediated via their cognate nuclear receptors. We employed mice lacking the nuclear PR or the nuclear ERα to further define the mechanism of steroidal regulation of HIF-1α. The induction of HIF-1α that we observed in P4-treated wild-type mice was virtually abolished in PR(−/−) mice (Fig. 7). For example, the expression of HIF-1α was very low in intact PR+/− mice or in ovariectomized PR+/− mice treated with either E2 or P4. In contrast, P4 showed an increased expression of HIF-1α in ERα(+/−) mice. An injection of E2 also increased the accumulation of HIF-1α in ERα(+/−) ovariectomized mice. These results clearly suggest that this gene is primarily under the influence of P4 in the mouse uterus and requires the activation of PR. However, an effect of estrogen in uterine induction of HIF-1α could be mediated independently of ERα.

Finally, we asked whether this P4 regulation of HIF-1α was directly transcriptionally regulated, because, although two half-site progesterone/gluocorticoid-responsive element (PRE/GRE) and eight half-site PRE/GRE-like elements were located in the promoter region of the exon 1.2, six half-site PRE/GRE-like elements were located in the exon 1.1 promoter region of the mouse HIF-1α gene (Transcriptional element search: www.cbil.upenn.edu/tess). Expression of these two mouse HIF-1α mRNA transcripts is regulated by distinct promoters rather than by differential splicing. This results in two distinct mRNA isoforms differing in the composition of their 5’-untranslated regions. Furthermore, there is evidence that HIF-1α exon 1.1 exhibits tissue-specific features with modest activity, whereas the exon 1.2 promoter resembles a housekeeping type promoter with higher activity (41, 49).

Using a uterine cell line (AN3CA) and a fibroblast cell line (L929), we observed that although P4 up-regulated PRE-luciferase activity in these cell lines expressing PRA or PRB, similar treatment with P4 did not show any heightened HIF-1α-Luc activity in these cell lines after co-transfection with PRA or PRB (Fig. 8). HIF-1α-Luc constructs were functional, because basal levels in L929 cells were markedly higher than those observed with the pGL3 basic (control) construct (data not shown). This latter observation is similar to one that has been previously reported (41). The results suggest that steroidal regulation of HIF-1α is more complex compared with other PR-regulated genes.

### DISCUSSION

A number of target genes involved in angiogenesis, erythropoiesis, and glycolysis are activated by HIFs, particularly HIF-1α (15). The most well known and potent stimulus for the induction of HIFs is hypoxia (15, 18, 41, 49). This would then suggest that dissimilar partial oxygen pressures in different uterine tissue compartments are likely to exist (53), thus regulating HIF expression differentially. Because the uterine epithelium is devoid of any blood vessels and these cells are polarized and separated from the stroma by a basement membrane, it seems reasonable to assume that the epithelium is more hypoxic than the stroma and myometrium. Thus, our observation of HIF-1α expression in the epithelium as opposed to the induction of HIF-2α in the stroma on day 4 of pregnancy is interesting and suggests that differential regulation and functions of HIFs in the uterus. Because one of the major roles of HIFs is to maintain oxygen homeostasis, we could surmise that HIF-1α in the epithelium is meant to fulfill this function. In contrast, the expression of HIF-2α in the stroma could be involved in the induction of Vegf required for angiogenesis in this compartment.

However, it is also possible that these genes are regulated in the uterus under the influence of ovarian steroids, because the uterus is the major target for P4 and estrogen. The very low level of expression of HIFs in the estrogenized uterus on day 1 of pregnancy suggests that estrogen has a limited role in regulating HIF levels. Alternatively, estrogen may have a transient role in influencing HIF expression, which was not detected on the morning of day 1 of pregnancy several hours after the preovulatory estrogen surge. In contrast, the expression of HIF-1α in the epithelium and of HIF-2α in the stroma on day 4 of pregnancy with rising P4 levels superimposed by a small amount of estrogen is suggestive of differential regulation of these two genes in two different tissue compartments. It is known that under this condition on day 4 of pregnancy, epithelial cells undergo differentiation, and stromal cells exhibit heightened proliferation in the mouse uterus (54). This is not surprising, because nuclear receptors for progesterone (PR) and estrogen (ER) are expressed in the epithelium and stroma at this time (55). Whether this effect of P4 and estrogen on HIF induction is direct or indirect is not clearly understood. It is possible that uterine compartments become more hypoxic under P4 influence than under estrogen when the uterus is more perfused. However, the ovariectomized uterus in the absence of steroid hormones is likely to be more hypoxic. Thus, a low level...
of induction of HIF-1α in the uterus under such a condition suggests that partial oxygen pressure is not the major inducer of HIFs in the uterus. In contrast, the low to undetectable expression of HIFs in the estrogenized and well perfused day 1 pregnant uterus suggests that a less hypoxic condition could be involved in regulating HIF expression. Alternatively, HIF expression may not be very responsive to estrogen. However, a modest increase in HIF-1α expression and a more robust expression of HIF-2α in the ovariectomized uterus 6 h after an injection of estrogen suggests that this steroid preferentially influences the regulation of HIF-2α in the uterus. Because estrogen induces vascular permeability but inhibits angiogenesis in the mouse uterus, the coordinate expression of HIF-2α and Vegf in the stroma at 6 h after an estrogen injection

Fig. 6. *In situ* hybridization of Vegf, HIF-1α, and HIF-2α mRNAs in ovariectomized mouse uteri after steroid hormone treatments. Representative dark field photomicrographs of longitudinal uterine sections are shown at 100×. Ovariectomized mice were treated with oil (vehicle control), E₂, or P₄ and sacrificed at the indicated times. A, Vegf. B, HIF-1α. C, HIF-2α.
whether transient uterine HIF-2 expression. However, cells expressing both PRA and PRB also failed to express HIF-2 i.e., mice and a modest increase in the expression of several genes in the uterus independent of both ERα and ERβ (56, 57). Future studies will determine whether transient uterine HIF-2α expression by estrogen is mediated via classical ERs or whether this effect is independent of such receptors.

Although HIF-1α in the uterine epithelium during the pre-implantation period and in ovariectomized mouse uterus is responsive to P4 regulation, the function of HIF-1α in the epithelium is far from being elucidated. The presence of ARNT1 protein in a location similar to that of HIF-1α suggests that heterodimerization between these two partners is possible to influence specific functions in the epithelium. Because angiogenesis is absent in the uterine epithelium, we speculate that HIF-1α has different functions in this tissue compartment. Glucose transporter-1 (GLUT-1) is also an HIF-1α-responsive gene (15, 58–60) and is expressed in the uterine epithelium on day 4 of pregnancy under the influence of P4. Thus, it is possible that HIF-1α in the uterine epithelium influences glucose transport across the epithelium. However, other functions of HIF-1α in the uterine epithelium cannot be ruled out. For example, HIF-1α has been shown to play important roles in developing embryos (53, 61). Thus, these results indicate that HIF-1α is a P4-regulated uterine epithelial responsive gene with a function not associated with Vegf expression. On the other hand, the presence of HIF-2α in the stroma together with ARNT1 on day 4 of pregnancy under P4 dominance could be associated with Vegf induction for vascular permeability and subsequent angiogenesis in this tissue compartment. This is a very interesting observation because P4 and E2 show differential regulation of HIF-1α and HIF-2α in the uterus depending on the cell types. Whether this differential regulation is mediated by epithelial-mesenchymal cross-talk is not known. However, there are numerous examples of epithelial-mesenchymal interactions in inducing gene expression and mediating important uterine functions with respect to P4 and estrogen effects (reviewed in Ref. 62).

Heightened angiogenesis with increasing levels of Vegf in the decidualizing stroma during the postimplantation period has been associated with COX-2 derived prostaglandins. However, it is not yet known whether HIFs have any role in this event during this time. There is evidence that hypoxia can induce
COX-2 (63). However, whether HIFs are capable of inducing COX-2 in the uterus is not known. Nonetheless, expression of COX-2 in the decidual sites, similar to that of HIF-1α and HIF-2α as well as their partners ARNT1 and ARNT3, suggests a correlation between COX-2 and HIFs with respect to Vegf induction (64). It is also interesting to note the switching of HIF-1α expression from the epithelium to the stroma during the postimplantation period when the uterus is still under the predominant influence of P2. The developing embryo could influence HIF-1α expression in the decidua. However, during decidualization the heightened expression of HIF-2α is indicative of a preferential role for this HIF isoform instead of HIF-1α. The question still remains of how HIF-1α becomes more dominant in the epithelium during the preimplantation period, whereas HIF-2α is more prominent in the stroma during the postimplantation period, although elevated P2 levels are characteristic of both the phases. In conclusion, the results of the present investigation show that HIFs are differentially expressed in the uterus depending on the stage of implantation and cell types involved, implicating differential roles of HIFs in the epithelial and stromal compartments of the uterus.

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