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Effects of Estradiol-17β on Expression of mRNA for Seven Angiogenic Factors and Their Receptors in the Endometrium of Ovariectomized (OVX) Ewes

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We have previously established an ovariectomized (OVX) ewe model to study how steroid removal and replacement affects uterine blood vessel and tissue growth. Using this model, endometrial expression of mRNA for 14 angiogenic factors (7 genes and their respective receptors) in caruncular (CAR) and intercaruncular (ICAR) endometrium were evaluated by quantitative real time RT-PCR at 0 (control), 2, 4, 8, 16, or 24 h after treating OVX ewes with an estradiol-17β (E2) implant. In CAR and ICAR, compared to 0 h, the mRNA expression of vascular endothelial growth factor (*VEGF*), VEGF receptor (R)1, soluble guanylate cyclase (*GUCY1B3*; the R for nitric oxide [NO]), hypoxia inducible factor (*HIF*)*1α*, and placental growth factor (*PlGF*) increased by 4 h after E2-treatment, but basic fibroblast growth factor (*FGF2*), endothelial NO synthase (*NOS3*), angiopoietin (*ANGPT*)*1*, *ANGPT2*, *ANGPT* receptor *Tie2* by 2 h after E2. Expression of mRNA for *FGFR2 IIIc* was increased at 2 h by E2-treatment in ICAR, but not in CAR. By contrast, expression of neuropilin (*NP*)*1* mRNA was increased at 2 h in CAR, but not ICAR. The mRNA expression of *VEGF*, *FGF2*, *HIF1α*, and *PlGF* was positively correlated with mRNA expression of *NOS3*, *VEGFR1*, and *Tie2* suggesting some E2-stimulated interactions between these factors in promoting blood vessel growth. Thus, several major angiogenic factors and their receptors are increased within hours after E2-treatment, which indicates that E2 plays a role in regulation of angiogenesis in the uterus. By using the OVX ewe model, we may begin to understand the molecular basis of E2 effects on angiogenesis in the endometrium and, eventually, how angiogenesis is regulated in normal versus pathological conditions.

Key Words: Uterus; estradiol; gene regulation; nitric oxide; angiogenesis.

Introduction

Estradiol-17β (E2) is a known modulator of the cyclic patterns of increased endometrial angiogenesis, blood flow, and uterine growth that occur during the estrous cycle *(1–3)*. During pregnancy, the uterotropic effects of E2 promote embryonic development and placentation *(1,3–7)*. Estradiol-17β effects on endometrial angiogenesis, which is critical to uterine growth, appear to be mediated through increased expression of at least two key growth factors, vascular endothelial growth factor (*VEGF*) and basic fibroblast growth factor (*FGF2*), and perhaps by others, including endothelial nitric oxide synthase (*NOS3*), hypoxia inducible factor (*HIF*)*1α*, angiopoietin (*ANGPT*)*1* and *2*, placental growth factor (*PlGF*), and their receptors *(3,7–9)*.

VEGF and FGF2 belong to a family of heparin-binding angiogenic factors that are produced by the uterine endometrium (mucosa) of pregnant and nonpregnant cows and ewes *(10–12)*. We have previously shown that E2 replacement in ovariectomized (OVX) ewes stimulated VEGF and *FGF2* mRNA expression by several-fold within a few hours after implanting E2 and that increased uterine growth and microvascular development occurred within 24 h after E2 replacement *(5,6)*. VEGF and FGF2 are thought to stimulate endothelial production of NO by increasing NOS3 expression, whereas NO production causes vasodilation and likely mediates E2-induced increases in uterine blood flow *(13)*. Conversely, NO can regulate expression of *VEGF* and *FGF2 (14)*. Under hypoxic conditions, *VEGF* mRNA expression is stimulated and stabilized by *HIF1α (15,16)*. Hypoxia, VEGF and FGF2 also increase the expression of the potent angiogenic factor, *ANGPT2*, but not *ANGPT1* in microvascular endothelial cells *(17)*.

Additionally, *PlGF* and the receptors neuropilin (*NP*)*1* and *NP2* may play a role in E2-stimulated endometrial angiogenesis through interactions with VEGF and its receptors *(18–20)*.

Based on the above information, we hypothesized that E2 is a major regulator of endometrial expression of several angiogenic factors and receptors. We further suggest that this E2 regulation is tissue-specific and thus would be different in CAR, composed mostly of blood vessels, compared

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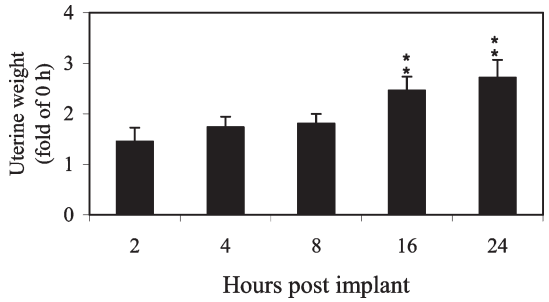

Fig. 1. Weight of the whole uterus after E2-treatment of OVX ewes. Values are presented as the fold-increase of 0 h (control, 17.16 ± 1.41 g) values. Means ± SEM compared with 0 h differ; $^{**}p < 0.01$. The patterns of change in uterine weight, analyzed by regression analysis, were best described by an exponential sigmoidal pattern of change. (See Table 1 for the equation, R^2, and p value.)

with ICAR, which contains the endometrial glands and fewer blood vessels. Therefore, the purpose of this experiment was to evaluate the time-course of expression of mRNA for *VEGF*, *FGF2*, *NOS3*, *HIF1α*, *ANGPT1*, *ANGPT2*, *PlGF*, and their receptors in CAR and ICAR after E2-treatment of OVX ewes.

Results

Uterine Weight

Weight of the intact uterus increased by more than twofold ($p < 0.05$) at 16 h and was nearly threefold ($p < 0.01$) greater at 24 h (Fig. 1) compared with the weight of the uterus at 0 h (17.96 ± 1.41 g).

Expression of VEGF, VEGFR1, and VEGFR2 mRNA

VEGF mRNA expression in CAR increased by >11-fold ($p < 0.01$) at 2 h, >34-fold ($p < 0.01$) at 4 h, and >10-fold ($p < 0.05$) at 8 h, and returned to 0 h values at 16–24 h after E2-treatment (Fig. 2A). In ICAR, *VEGF* mRNA expression increased by >20-fold ($p < 0.01$) at 4 h (Fig. 2A). In CAR and ICAR, expression of mRNA for *VEGFR1* increased by five- to sixfold ($p < 0.01$) at 4–8 h and returned to 0 h values at 16–24 h (Fig. 2B) By contrast, expression of mRNA for *VEGFR2* in CAR and ICAR was not affected by E2-treatment (data not shown).

Expression of FGF2 and FGFR2 IIIc mRNA

Expression of *FGF2* mRNA (Fig. 3A) in CAR and ICAR increased by two- to sixfold ($p < 0.01$) from 2 to 24 h after E2-treament, whereas expression of its receptor, *FGFR2 IIIc* increased twofold ($p < 0.05$) but only in ICAR at 2 h after E2-treatment (Fig. 3B).

Expression of NOS3, GUCY1B3, and HIF1α mRNA

Expression of *NOS3* mRNA in CAR and ICAR increased by more than twofold ($p < 0.01$) at 2 h, >13-fold ($p < 0.01$) at 4–8 h, six- to eightfold ($p < 0.01$) at 16 h, and more than threefold ($p < 0.01$) at 24 h after E2-treatment (Fig. 4A). In

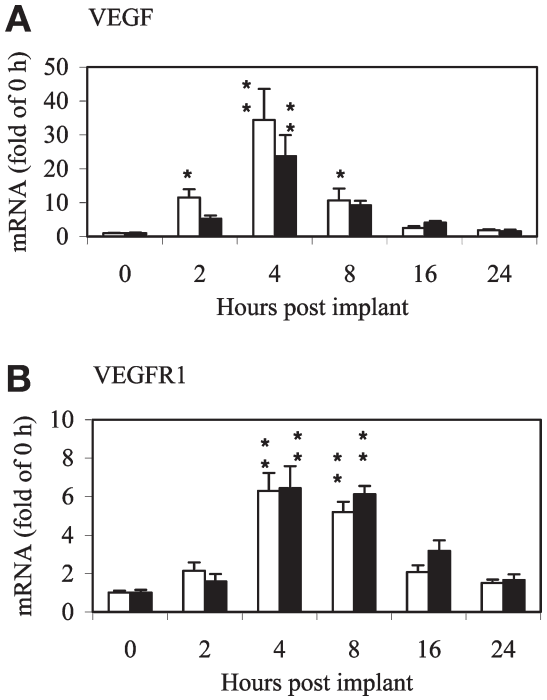

Fig. 2. Endometrial expression of the mRNA for *VEGF* (**A**) and its receptor, *VEGFR1* (**B**), after E2-treatment of OVX ewes. Values are presented as the fold-change of 0 h (control) values. Open bars = CAR, filled bars = ICAR. Means ± SEM compared with 0 h differ; $^{*}p < 0.05$; $^{**}p < 0.01$. The changes in *VEGF* and *VEGFR1* mRNA expression in CAR, analyzed by regression analysis, were best described by a cubic pattern of expression and in ICAR by an exponential sigmoidal pattern of expression. (See Table 1 for the equations, R^2, and p value.)

CAR, expression of mRNA for *GUCY1B3*, the receptor for NO, increased by more than threefold ($p < 0.01$) at 4 h, more than ninefold ($p < 0.01$) at 8 h, and remained at more than twofold ($p < 0.01$) at 16–24 h, whereas in ICAR, *GUCY1B3* mRNA expression was increased by more than sevenfold ($p < 0.01$) at 8 h and more than twofold ($p < 0.01$) at 16 h (Fig. 4B).

Expression of mRNA for *HIF1α* increased in both CAR and ICAR by more than threefold ($p < 0.01$) at 4 h, more than fourfold ($p < 0.01$) at 8–16 h, and more than threefold ($p < 0.01$) at 24 h after E2-treatments (Fig. 4C).

Expression of ANGPT1, ANGPT2, and Receptor Tie2 mRNA

ANGPT1 mRNA expression in CAR and ICAR increased by two- to fourfold ($p < 0.01$), at 2 h returned to approx 0 h values at 4 h, and then decreased ($p < 0.01$) below 0 h values from 8–24 h after E2-treatment (Fig. 5A). In CAR and ICAR, expression of *ANGPT2* mRNA increased by approx twofold ($p < 0.01$) at 2–4 h, by fivefold to eightfold ($p < 0.01$) at 8–16 h, returning to two- to fourfold ($p < 0.01$) above 0 h levels at 24 h (Fig. 5B).

In both CAR and ICAR, expression of mRNA for the *ANGPT* receptor, *Tie2*, increased by approx 2-fold ($p <$

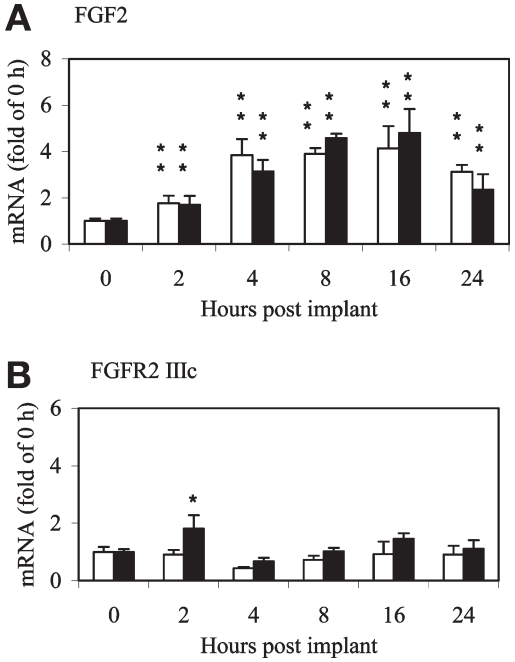

Fig. 3. Endometrial expression of the mRNA for *FGF2* (**A**) and its receptor, *FGFR2 IIIc* (**B**), after E2-treatment of OVX ewes. Values are presented as the fold-change of 0 h (control) values. Open bars = CAR, filled bars = ICAR. Means ± SEM compared with 0 h differ; $*p < 0.05$; $**p < 0.01$. The changes in *FGF2* mRNA expression, analyzed by regression analysis, were best described by an exponential sigmoidal pattern of expression in CAR and ICAR. (See Table 1 for the equations, R^2, and p value.)

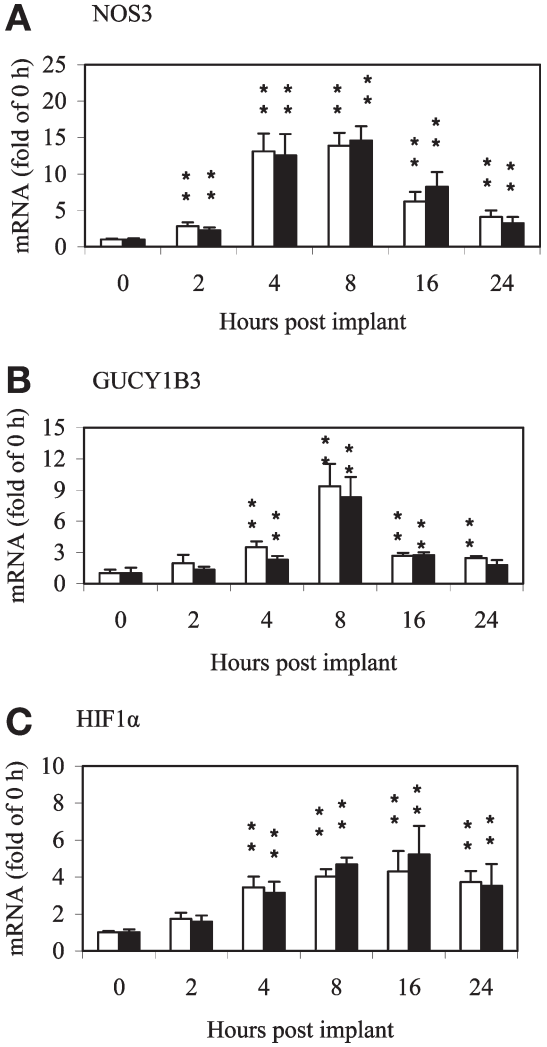

Fig. 4. Endometrial expression of the mRNA for *NOS3* (**A**), its receptor, *GUCY1B3* (**B**), and *HIF1α* after E2-treatment of OVX ewes. Values are presented as the fold-change of 0 h (control) values. Means ± SEM compared with 0 h differ; $**p < 0.01$. Open bars = CAR, filled bars = ICAR. The changes in *NOS3* and *GUCY1B3* mRNA expression, analyzed by regression analysis, were best described by a cubic pattern of expression in CAR and an exponential sigmoidal pattern of expression in ICAR, whereas those of *HIF1α* were best described by a exponential sigmoidal pattern of expression in both CAR and ICAR. (See Table 1 for the equations, R^2, and p value.)

0.01) at 2–8 h; in CAR Tie2 mRNA returned to 0 h values by 16–24 h after E2-treatment, whereas in ICAR, the two-fold increase in expression was maintained at 16 h and returned to 0 h values at 24 h after E2-treatment (Fig. 5C).

Expression of PlGF, NP1, and NP2 mRNA

Expression of *PlGF* mRNA in CAR increased by >10-fold ($p < 0.01$) at 4 h and returned to 0 h values by 16 h after E2-treatment (Fig. 6A). Similarly, in ICAR, expression of *PlGF* increased by >17-fold ($p < 0.01$) at 4 h and returned to 0 h values by 24 h (Fig. 6A). In CAR, expression of *NP1* mRNA was increased by >2.5-fold ($p < 0.01$) at 2 h and then decreased to 0 h values at 4–24 h (Fig. 6B). In ICAR, although the pattern of expression of *NP1* mRNA paralleled that of CAR, the changes were not significant except by regression analysis (see below). Expression of mRNA for *NP2* was unchanged by E2-treatment in CAR and ICAR (data not shown).

Data for the concentration of 18S rRNA in each sample was similar across all treatments ($p > 0.05$) indicating that similar concentrations of tcRNA were present in each sample.

Regression Analyses

All significant regression analyses equations, along with their R^2, and p values are shown in Table 1.

Uterine weight changes were best described by an exponential sigmoidal regression pattern.

VEGF and *VEGFR1* mRNA expression were best described by cubic regression curves in CAR and by exponential sigmoidal regression curves in ICAR. *FGF2* mRNA expression in CAR and ICAR were best described by exponential sigmoidal regression curves. *NOS3* and *GUCY1B3* mRNA expression were best described by cubic regression curves in CAR and by exponential sigmoidal regression curves in ICAR. *HIF1α* mRNA expression in CAR and ICAR were best described by exponential sigmoidal regression curves. *ANGPT1* and *ANGPT2* mRNA expression in CAR

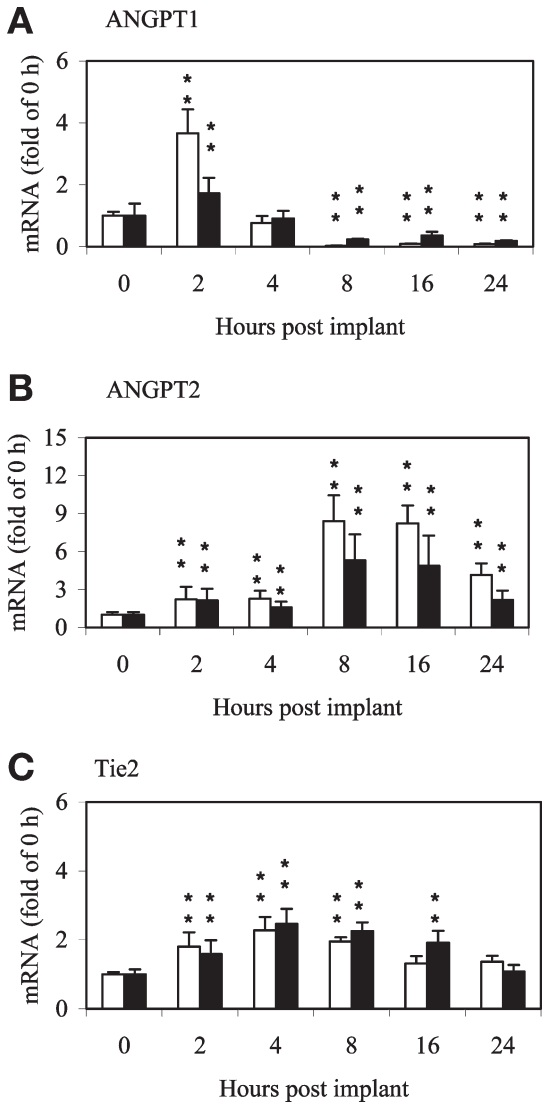

Fig. 5. Endometrial expression of the mRNA for *ANGPT1* (**A**), *ANGPT2* (**B**), and *Tie2* (C) after E2-treatment of OVX ewes. Values are presented as the fold-change of 0 h (control) values. Means ± SEM compared with 0 h differ; $^{**}p < 0.01$. Open bars = CAR, filled bars = ICAR. The changes in *ANGPT1* and *ANGPT2* mRNA expression, analyzed by regression analyses, were best described by an exponential sigmoidal pattern of expression in CAR and ICAR, whereas those of *Tie2* were best described by a cubic pattern of expression. (See Table 1 for the equations, R^2, and p value.)

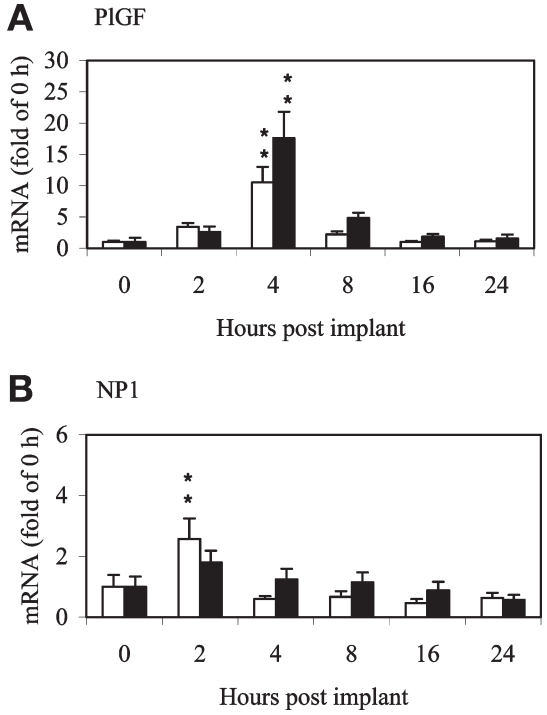

Fig. 6. Endometrial expression of the mRNA for *PlGF* (**A**), and *NP1* (**B**) after E2-treatment of OVX ewes. Values are presented as the fold-change of 0 h (control) values. Means ± SEM compared with 0 h differ; $^{**}p < 0.01$. Open bars = CAR, filled bars = ICAR. The changes in *PlGF* mRNA expression in CAR and ICAR, analyzed by regression analyses, were best described by a cubic pattern of expression, whereas those of *NP1* in ICAR were best described by a linear pattern of expression. (See Table 1 for the equations, R^2, and p value.)

and ICAR were best described by exponential sigmoidal regression curves.

However, *Tie2* mRNA expression in CAR and ICAR were best described by cubic regression curves. *PlGF* mRNA expression in CAR and ICAR were best described by cubic regression curves. *NP1* mRNA expression in ICAR was best described by a linear regression curve. The regression analysis of the E2-response curves was non-significant for the receptors *VEGFR2*, *FGFR2 IIIc*, and *NP2* in both CAR and ICAR and for *NP1* in CAR (data not shown).

Evaluation of Correlations

The correlation coefficients and p values for significant correlations are either summarized in the text below or shown in Table 2.

Uterine Weight

Uterine weight was negatively correlated with *ANGPT1* mRNA expression in both CAR ($R^2 = -0.397$; $p = 0.0330$) and ICAR ($R^2 = -0.480$; $p = 0.0085$), but was not correlated with other angiogenic factors in ICAR. However, in CAR, uterine weight tended to be positively correlated with mRNA expression of *FGF2* ($R^2 = 0.363$; $p = 0.0527$) and *HIF1α* ($R^2 = 0.322$; $p = 0.0889$) and negatively correlated with *VEGF* ($R^2 = -0.322$; $p = 0.0883$) and *NP1* ($R^2 = -0.336$; $p = 0.0745$).

VEGF, VEGFR1, and VEGFR2

In CAR and ICAR, expression of *VEGF* mRNA was positively correlated with the mRNA expression of *VEGFR1*, but not with *VEGFR2*, with *Tie2*, but not *ANGPT1* or *ANGPT2*, with *NOS3*, but not *GUCY1B3*, and with *PlGF*, but not *NP1* or *NP2* (Table 2) Additionally, in CAR and ICAR, *PlGF* mRNA expression was positively correlated with *VEGFR1* and the *ANGPT* receptor *Tie2* (Table 2).

Table 1
Regression Equations, R^2, and p Values for Significant Changes in Uterine Weight and Expression of mRNA for Angiogenic Factors

Factor	Tissue	Regression equation	R^2	p value
Total Weight	Uterus	$Y = 20.3298e^{0.07769x - 0.00182x^2}$	0.5530	$p < 0.0001$
VEGF	CAR	$Y = 0.94501 + 2.13722x - 0.23614x^2 + 0.00611x^3$	0.3929	$p = 0.0033$
	ICAR	$Y = 0.474e^{0.275x - 0.0131x^2}$	0.4470	$p = 0.0002$
VEGFR1	CAR	$Y = 0.01454 + 0.03275x - 0.00320x^2 + 0.000077x^3$	0.6656	$p < 0.0001$
	ICAR	$Y = 0.0169e^{0.251x - 0.0105x^2}$	0.4836	$p = 0.0001$
FGF2	CAR	$Y = 0.564e^{0.191x - 0.00657x^2}$	0.5337	$p < 0.0001$
	ICAR	$Y = 0.328e^{0.244x - 0.00923x^2}$	0.5636	$p < 0.0001$
NOS3	CAR	$Y = -0.07879 + 1.34903x - 0.12077x^2 + 0.00279x^3$	0.6294	$p < 0.0001$
	ICAR	$Y = 0.354e^{0.380x - 0.0150x^2}$	0.6903	$p < 0.0001$
GUCY1B3	CAR	$Y = -0.18331 + 1.53646x - 0.13251x^2 + 0.00299x^3$	0.4524	$p = 0.0009$
	ICAR	$Y = 0.513e^{0.286x - 0.0111x^2}$	0.4802	$p = 0.0001$
HIF1α	CAR	$Y = 0.099e^{0.186x - 0.0061x^2}$	0.5530	$p < 0.0001$
	ICAR	$Y = 0.064e^{0.237x - 0.0083x^2}$	0.4797	$p = 0.0002$
ANGPT1	CAR	$Y = 0.0039e^{-0.480x + 0.0146x^2}$	0.5462	$p < 0.0001$
	ICAR	$Y = 0.00027e^{-0.190x + 0.0497x^2}$	0.4246	$p = 0.0006$
ANGPT2	CAR	$Y = 0.0138e^{0.315x - 0.0106x^2}$	05662	$p < 0.0001$
	ICAR	$Y = 0.0100e^{0.225x - 0.0084x^2}$	0.2304	$p = 0.0291$
Tie2	CAR	$Y = 0.19057 + 0.06804x - 0.00698x^2 + 0.000176x^3$	0.3689	$p = 0.0055$
	ICAR	$Y = 0.135401 + 0.05121x - 0.00418x^2 + 0.000085x^3$	0.3831	$p = 0.0051$
PlGF	CAR	$Y = 0.01622 + 0.01740x - 0.00201x^2 + 0.00005308x^3$	0.3416	$p = 0.0094$
	ICAR	$Y = 0.00919 + 0.02257x - 0.00245x^2 + 0.00006325x^3$	0.3348	$p = 0.0129$
NP1	ICAR	$Y = 0.19159 - 0.00455x$	0.1665	$p = 0.0252$

Table 2
Correlations Between Expression of mRNA for Angiogenic Factors after E2 Treatment of OVX Ewes

Factor	VEGFR1	VEGFR2	FGFR2 III c	NOS3	GUCY1B3	HIF1α	ANGPT2	Tie2	PlGF
VEGF	$R^2 = 0.688$	NS	NS	$R^2 = 0.607$	NS	NS	NS	$R^2 = 0.687$	$R^2 = 0.727$
CAR	$p < 0.0001$			$p = 0.0003$				$p < 0.0001$	$p < 0.0001$
	$R^2 = 0.757$	NS	NS	$R^2 = 0.595$	NS	NS	NS	$R^2 = 0.647$	$R^2 = 0.906$
ICAR	$p < 0.0001$			$p = 0.0005$				$p < 0.0001$	$p < 0.0001$
FGF2	$R^2 = 0.607$	NS	NS	$R^2 = 0.651$	$R^2 = 0.410$	$R^2 = 0.874$	$R^2 = 0.590$	$R^2 = 0.549$	
CAR	$p = 0.0003$			$p < 0.0001$	$p = 0.0219$	$p < 0.0001$	$p = 0.0005$	$p = 0.0014$	NS
	$R^2 = 0.656$	$R^2 = 0.590$	$R^2 = 0.374$	$R^2 = 0.678$	$R^2 = 0.567$	$R^2 = 0.908$	$R^2 = 0.724$	$R^2 = 0.679$	
ICAR	$p < 0.0001$	$p = 0.0006$	$p = 0.0418$	$p < 0.0001$	$p = 0.0011$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	NS
NOS3	$R^2 = 0.895$	NS	NS	—	$R^2 = 0.448$	$R^2 = 0.619$	$R^2 = 0.445$	$R^2 = 0.733$	$R^2 = 0.614$
CAR	$p < 0.0001$				$p = 0.0115$	$p = 0.0002$	$p = 0.0122$	$p < 0.0001$	$p = 0.0002$
	$R^2 = 0.887$	NS	NS	—	$R^2 = 0.423$	$R^2 = 0.618$	$R^2 = 0.527$	$R^2 = 0.849$	$R^2 = 0.501$
ICAR	$p < 0.0001$				$p = 0.0199$	$p = 0.0003$	$p = 0.0028$	$p < 0.0001$	$p = 0.0048$
HIF1α	$R^2 = 0.483$	$R^2 = 0.398$	$R^2 = 0.390$	$R^2 = 0.619$	$R^2 = 0.324$	—	$R^2 = 0.723$	$R^2 = 0.544$	NS
CAR	$p = 0.0059$	$p = 0.0266$	$p = 0.0303$	$p = 0.0002$	$p = 0.0754$*		$p < 0.0001$	$p = 0.0016$	
	$R^2 = 0.512$	$R^2 = 0.686$	$R^2 = 0.406$	$R^2 = 0.618$	$R^2 = 0.416$	—	$R^2 = 0.795$	$R^2 = 0.601$	NS
ICAR	$p = 0.0038$	$p < 0.0001$	$p = 00261$	$p = 0.0003$	$p = 0.0223$		$p < 0.0001$	$p = 0.0005$	
ANGPT2	NS	$R^2 = 0.443$	$R^2 = 0.381$	$R^2 = 0.445$	NS	$R^2 = 0.723$	—	NS	NS
CAR		$p = 0.0126$	$p = 0.0345$	$p = 0.0122$		$p < 0.0001$			
	$R^2 = 0.389$	$R^2 = 0.731$	$R^2 = 0.447$	$R^2 = 0.527$	NS	$R^2 = 0.795$	—	$R^2 = 0.493$	NS
ICAR	$p = 0.0338$	$p < 0.0001$	$p = 0.0132$	$p = 0.0028$		$p < 0.0001$		$p = 0.0057$	
PlGF	$R^2 = 0.657$	NS	NS	$R^2 = 0.614$	NS	NS	NS	$R^2 = 0.598$	—
CAR	$p < 0.0001$			$p = 0.0002$				$p = 0.0005$	
	$R^2 = 0.745$	NS	NS	$R^2 = 0.501$	NS	NS	NS	$R^2 = 0.598$	—
ICAR	$p < 0.0001$			$p = 0.0048$				$p = 0.0005$	

NS = non significant.
*$p = 0.0754$, above the level of significance at $p < 0.05$, but less than $p < 0.1$, indicating a tendency.

VEGFR1 mRNA expression was also positively correlated with *GUCY1B3* mRNA expression in CAR (R^2 = 0.426; p = 0.0170) and ICAR (R^2 = 0.505; p = 0.0043), whereas *VEGFR2* mRNA expression was positively correlated with *FGFR2 IIIc* mRNA expression in CAR (R^2 = 0.675; $p <$ 0.0001) and ICAR (R^2 = 0.784; $p < 0.0001$).

FGF2 and FGFR2 IIIc

Expression of *FGF2* mRNA was positively correlated with mRNA expression of its receptor, *FGFR2 IIIc*, and with *VEGFR2* in ICAR, but not in CAR. In CAR and ICAR, *FGF2* mRNA expression was positively correlated with *VEGFR1*, *NOS3* and *GUCY1B3*, *HIF1α*, and with *ANGPT2* and *Tie2* mRNA expression (Table 2).

NOS3, GUCY1B3, and HIF1α

In addition to positive correlation with *VEGF* and *FGF2* mRNA expression, expression of *NOS3* mRNA in CAR and ICAR was positively correlated with mRNA expression of its receptor *GUCY1B3* and *VEGFR1*, *HIF1α*, *ANGPT2*, *Tie2*, and *PlGF* (Table 2).

ANGPT1, ANGPT2, and Tie2

ANGPT1 mRNA expression tended to be negatively correlated with *ANGPT2* mRNA expression (R^2 = –0.342; p = 0.0601). Expression of mRNA for *ANGPT2* was positively correlated with mRNA expression of its receptor, *Tie2*, and with *VEGFR1* in ICAR, but not in CAR (Table 2). Additionally, in CAR and ICAR, *ANGPT2* mRNA expression was positively correlated with *VEGFR2*, *FGFR2 IIIc*, *NOS3*, and *HIF1α* mRNA expression (Table 2). *Tie2* mRNA expression was positively correlated with *VEGFR1* mRNA expression in CAR (R^2 = 0.797; $p < 0.0001$) and ICAR (R^2 = 0.839; $p < 0.0001$) and with *VEGFR2* mRNA expression in CAR (R^2 = 0.354; p = 0.0266) and ICAR (R^2 = 0.475; p = 0.0081).

PlGF, NP1, and NP2

As stated above, *PlGF* mRNA expression was positively correlated with *VEGF* and *NOS3* in CAR and ICAR. In CAR, *NP1* mRNA expression was positively correlated with *ANGPT1* mRNA expression (R^2 = 0.737; $p < 0.0001$) and in ICAR, *NP1* mRNA expression was positively correlated with *NP2* mRNA expression (R^2 = 0.489; p = 0.0149). *NP2* mRNA expression was positively correlated with *FGFR2 IIIc* mRNA expression (R^2 = 0.440; p = 0.0066) in ICAR.

Discussion

In this experiment, we have re-examined the effects of E2 on uterine weight and endometrial expression of *VEGF* and *FGF2* mRNA and further evaluated E2 effects on the mRNA expression of several additional angiogenic factors, including *NOS3*, *ANGPT1*, *ANGPT2*, *PlGF*, and *HIF1α* and their receptors, which have been demonstrated to have major roles in mediating and regulating vascular function in many organs, including the uterus *(3,28)*. As expected, uterine weight was similar to that reported previously using our established OVX ewe model *(5,21)*. Uterine weight increased several hours after E2 replacement, reaching two- to threefold levels above controls by 16–24 h after E2 *(6,21)*.

In intact ewes, the effects of E2 on endometrial growth and angiogenesis cannot be separated from other factors affecting uterine function, whether examined during the estrous cycle or pregnancy. However, in this experiment, by using the OVX ewe model, the effects of E2 removal and replacement on angiogenesis and growth of the uterus, an extremely E2-sensitive tissue, could be quantified. Moreover, we have shown that the E2 concentrations, achieved at 24 h after E2 replacement in the OVX ewe, were similar to E2 concentrations at estrus *(29)*. Uterine fresh and dry weights, based on tissue hypertrophy and water content, were the greatest immediately after estrus *(29)*. During early pregnancy in ewes, uterine wet and dry weights increased immediately after a dramatic increase in cell proliferation *(10,30)*. Similarly, E2 replacement in OVX ewes caused profound increases in uterine tissue mass by 24 h, due to cellular hypertrophy rather than to hyperplasia *(5,21)*. However, uterine tissue mass at estrus was never achieved in OVX ewes by replacing E2 alone or both E2 and progesterone, confirming that E2 interacts with other factors or has additional effects on the uterus of intact ewes that were not completely mimicked by steroid replacement *(5,21,29)*. The E2-stimulated increases in tissue mass were accompanied by dramatic increases in endometrial microvascular volume to support tissue growth *(5)*. Thus, we hypothesized that E2 would also cause an orchestrated increase in expression of angiogenic factors within 24 h after E2 replacement in OVX ewes.

Previously, using ribonuclease protection assays (RPA), we have reported that E2-treatment of OVX ewes increased *VEGF* and *FGF2* mRNA expression several-fold within a few hours after implanting E2 *(5)*. Additionally, we and others have shown that these E2-induced changes were accompanied by increased *VEGF* and *FGF2* protein and by 5- to 10-fold increases in uterine angiogenesis and blood flow *(1,2,5,6)*. In the present study, we used qRT-PCR to re-examine E2-induced effects on mRNA for VEGF (based on detection with a probe-primer set that detected all isoforms of *VEGF* mRNA) *(22)* and *FGF2* in OVX ewes and found even more striking increases in the expression of *VEGF* and *FGF2* mRNA (>20-fold for *VEGF* and >4-fold for *FGF2* by 4 h after E2). Thus, the patterns of increase in uterine weight and expression of *VEGF* and *FGF2* mRNA were similar to those observed in our previous studies. However, qRT PCR was more sensitive and perhaps more quantitative than the RPA (shown by the increased magnitude of response to E2 for *VEGF* and *FGF2* mRNA).

Additionally, others have reported similar effects of E2 on endometrial expression of *VEGF* and/or *FGF2* mRNA and protein in sheep, rats, and humans *(1,31–33)*. By contrast, Welter et al. *(33)* reported that in OVX pigs, E2 de-

creased and P4 increased endometrial expression of *VEGF* mRNA. However, the fact that VEGF is readily induced by estrogens has been well established *(9)*. The *VEGF* gene contains an estrogen-response element (ERE), which may help explain the relatively rapid response to E2 by the OVX sheep endometrium *(34)*. Co-cultures of human endometrial glandular epithelial and endothelial cells showed that an estrogen-dependent expression of *VEGF* by glandular epithelial cells may have a major role in regulating angiogenesis *(35)*. In addition, estrogen-dependent synthesis of *VEGF* or other factors locally within the stroma may also influence angiogenesis *(35)*. This study and our previous studies have shown that E2 stimulates an immediate and dramatic up-regulation of *VEGF* mRNA that precedes the growth and microvascular development of the endometrium *(5,6,21)*

Our data showed increased expression of *VEGFR1* mRNA in CAR and ICAR after E2 replacement, which correlated with the mRNA expression of *VEGF*, while *VEGFR2* mRNA expression remained unchanged. These receptors, two of the four high-affinity VEGF receptors now identified, are members of subclass II receptor tyrosine kinases and have similar structural organizations *(36,37)*. VEGFR1 has greater affinity for VEGF than VEGFR2 does, but EGFR2 has been reported to be phosphorylated more efficiently after ligand binding and has been reported to be a more potent stimulator of endothelial cell mitogenesis, chemotaxis, and changes in morphology *(38,39)*. It has been suggested that VEGFR1 maintains vascular integrity and regulates the actions of VEGF by keeping angiogenesis in check, while VEGFR2 is thought to stimulate angiogenesis *(40,41)*.

The VEGF receptor data from this study conflict with those from an earlier OVX pig study demonstrating that VEGFR1 was unchanged by steroids and VEGFR2 was increased by E2 plus progesterone treatment, but not by E2 or progesterone alone *(33)*. The most current dogma is that the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF is VEGFR2 *(42)*. Our data suggest that in OVX ewes, the immediate effects of E2 on *VEGF* mRNA expression and subsequent endometrial angiogenesis may be mediated through VEGFR1 rather than through VEGFR2. Moreover, there are several other receptors that apparently interact with VEGF, including the neuropilins, NP1 and NP2, and it has been shown that VEGFR1 will also bind PlGF *(42)*. Thus, these complex interactions warrant further investigation.

We have shown in this study that the increase in *VEGF* and *FGF2* mRNA expression in CAR and ICAR occurred within 2 h after E2, suggesting a direct response to E2 by both factors. FGF2 is a pleiotropic growth factor that not only promotes angiogenesis in tissues, but has major roles in regulation of cellular proliferation and anti-apoptotic effects *(43)*. We have previously suggested that VEGF and FGF2 act synergistically to promote blood vessel growth *(5,6)*. However, in the present study, the *FGF2* mRNA expression was maintained at a threefold increase even at 24 h, whereas *VEGF* mRNA decreased to nearly 0 h values by 16 h after E2, which implies only a brief period of synergistic effects. Others have shown that E2 treatment of OVX rats increased *FGF2* mRNA expression in the uterus and that progesterone augmented this response *(44)*. Thus, in intact ewes, the E2-stimulated increase in expression *FGF2* mRNA may need to be maintained longer than *VEGF* mRNA expression in order to support FGF2-stimulated cell proliferation and tissue growth, which occurs somewhat later and may be sustained by progesterone.

We found that the mRNA for the *FGF2* receptor (*FGFR2 IIIc*) only increased at 2 h in ICAR, but not in CAR. In sheep, the maternal CAR tissues are the sites for attachment of the placenta and contain numerous blood vessels for uteroplacental exchange, whereas the ICAR tissues are composed of many glands, which are also well vascularized *(5,6)*. We have previously shown that FGF2 protein is found within the glandular and luminal endothelium and that FGF2 protein was persistently present, although at lower levels, even in long-term OVX ewes *(6)*. Thus, the E2-stimulated increase in the FGF2 protein within the numerous glands present in ICAR may result in increased mRNA for the *FGFR2 IIIc*, as a localized response from that tissue compartment.

In this study, *NOS3* mRNA increased several-fold in CAR and ICAR from 2 until 24 h after E2-treatment. Interactions among *VEGF*, *FGF2*, and *NOS3* expression are believed to stimulate increased production of NO by vascular pericytes *(14)*. NO production causes vasodilation and likely mediates E2-induced increases in uteroplacental blood flow *(1,14)*. The promoter region of the *VEGF* gene contains a hypoxia response element (HRE) that is required for transcriptional activation by NO and/or hypoxia, with a binding site for *HIF1α* within the HRE *(34)*. Under hypoxic conditions, *VEGF* mRNA is upregulated and stabilized by *HIF1α (15-17)*. Additionally, *ANGPT2* is upregulated by hypoxia, but no HRE has been identified in the *ANGPT2* gene *(17)*. We did find significant correlations between NOS3 mRNA expression and the mRNA for *VEGF*, *VEGFR1*, *FGF2*, *HIF1α*, *ANGPT2*, *Tie2*, and *PlGF*. This indicates that these angiogenic factors and/or their receptors are orchestrating a coordinated response to E2-treatment of OVX ewes, which implies that these factors interact to maintain uterine function under normal physiological conditions.

The expression of the NOS3 receptor, *GUCY1B3*, was also increased in both CAR and ICAR. However, in the OVX rat uterus, an inhibition of *GUCY1B3* mRNA and protein expression was detected by 3 h after E2-treatment *(45)*. This indicates a species-specific difference in E2 effects on *GUCY1B3* mRNA expression. We also found that *GUCY1B3* mRNA expression was positively correlated with NOS3 and several other factors, such as *FGF2*, *HIF1α*, and *VEGFR1*, but not with *VEGF* mRNA expression. Thus, these interactions should be evaluated in future studies.

HIF1α increased several-fold by 4 h after E2 and the enhanced expression was maintained through 24 h. In OVX

mice, E2-treatment also increased *HIF1α* mRNA expression, but the expression decreased by 24 h *(46)*. Additionally, increased expression of *HIF1α* was demonstrated in an E2 receptor knockout mouse model *(46)*. During hypoxic conditions, NO acts as a transcriptional activator of VEGF and the binding of HIF1α within the HRE of the *VEGF* gene upregulates and stabilizes *VEGF* mRNA expression *(15,16)*. Interestingly, *HIF1α* mRNA expression was not correlated with *VEGF* mRNA expression in this study but was correlated with both VEGF receptors, with *FGF2* and *FGFR2 IIIc*, with *NOS3* and *GUCY1B3*, and with *ANGPT2* and *Tie2*.

ANGPT1 mRNA expression increased by 2 h after E2 and then its mRNA expression decreased in CAR and ICAR. In contrast, *ANGPT2* mRNA expression not only increased by 2 h after E2-treatment, but more than doubled the 2 h increase at 8–16 h. Thus, when *ANGPT1* mRNA decreased, *ANGPT2* increased. Both *VEGF* and *ANGPT1* are produced by pericytes *(47)*. In contrast, endothelial cells selectively express *ANGPT2* mRNA in adult tissues that undergo periodic vascular remodeling, such as the uterus, ovary, and placenta *(17, 48)*. Additionally, *VEGF* and *FGF2* expression and hypoxia increase expression of *ANGPT2*, but not *ANGPT1 (17,49)*. This could explain why *ANGPT2* expression increased after the initial increase of *VEGF* and *FGF2* in our study. In fact, *FGF2* mRNA expression correlated with expression of *ANGPT2*, but not with *ANGPT1*.

Tie2 mRNA initially increased and then decreased to 0 h levels by 24 h. Tie2 is the common receptor for ANGPT1 and ANGPT2 and likely mediates their effects on angiogenesis of endometrial tissues *(48)*. *Tie2* is found in endothelial cells, which are the source of *ANGPT2* expression as well *(48,50)*. The current hypothesis is that *ANGPT2* has destabilizing, anti-angiogenic effects on the vasculature, downregulates *Tie2*, and thus has a role in vascular regression *(27,48,49,51)*. However, mounting evidence has shown that the combination of *ANGPT2* and *VEGF* is required for the destabilization of existing vessels as they prepare to remodel during an angiogenic event. Furthermore, it has been suggested that the upregulation of *ANGPT2* expression may be a common pathway for mediating the effects of VEGF and other angiogenic regulators, such as hypoxia and FGF2 *(17,28)*. On the other hand, ANGPT1 expression is believed to counteract the actions of VEGF, by stabilizing the blood vessels, making them less permeable and leaky *(28)*. ANGPT1 also enhances Tie2 expression and is critical for embryonic vascular development and survival. Our data showing an initial increase in *ANGPT1* and *Tie2* mRNA expression followed by a decrease in *ANGPT1* mRNA expression and simultaneous increase in *VEGF* and *ANGPT2* mRNA expression after E2-treatment of OVX ewes support the recent suggestion that a combination of *ANGPT2* and *VEGF* is required for the destabilization of existing vessels as they prepare to remodel during an angiogenic event.

We have shown that E2-treatment not only increased *PlGF* mRNA expression in endometrial tissues of OVX ewes but was positively correlated with *VEGF* mRNA expression, suggesting that both are mediators of the effect of E2 on angiogenesis of endometrial tissues. Additionally, we report that *PlGF* mRNA expression is correlated with expression of *VEGFR1* but not *VEGFR2*, as others have also found *(53–55)*. Interestingly, although the heparin-binding form of PlGF has been reported to bind both NP1 and NP2 *(19)*, we found no correlations between the E2-stimulated expression of *PlGF* mRNA and either NP.

Treatment with E2 changed *NP1* mRNA expression in CAR only at 2 h, but there was a linear regression pattern for *NP1* mRNA expression in ICAR. Neuropilins regulate signal transduction through formation of complexes with the tyrosine-kinase receptors of VEGF (including VEGFR1 and VEGFR2). Neuropilins appear to have a VEGF isoform-specific activity or interaction with the VEGFs. For example, NP1 forms a complex with VEGFR2 that enhances VEGF 165 activity *(19,56)*. NP1 does not have a tyrosine kinase domain and is a membrane-bound protein *(57)*. NP2 is an isoform-specific receptor for VEGF that interacts with both VEGF165 and VEGR145, but not with VEGF121 *(58)*. Both neuropilins also interact with other members of the VEGF family *(19)*. However, our data indicate that E2 is not a major regulator of *NP* mRNA expression in the ovine endometrium.

In summary, we have found that in ovine uterine tissues, E2-treatment of OVX ewes stimulated an orchestrated regulation of the mRNA for angiogenic genes and some, but not all, of their receptors within a few hours after E2. The factors responding to E2-treatment include *VEGF*, *VEGFR1*, *FGF2*, *NOS3*, *GUCY1B3*, *HIF1α*, *PlGF*, *ANGPT1*, *ANGPT2*, and *Tie2*. This new information supports our hypothesis that E2 is a major regulator of endometrial expression of angiogenic factors in sheep. Furthermore, our data provide a foundation for further cellular and molecular investigations of how steroids and growth factors interact to regulate uterine function. Understanding the effects of E2 on growth factors, their receptors and other factors that coordinate and control angiogenesis in uterine tissues may help establish strategies to stimulate blood vessel growth that promotes placental development or to block angiogenesis that promotes tumors or other pathological processes.

Materials and Methods

Animals and Treatments

The protocols and animal care for this study were approved by the Institutional Animal Care and Use Committee at NDSU. We used an established OVX ewe model that we have validated and described in several studies *(5,6,21)*. Briefly, on d 10–12 after estrus, ewes (n = 32) of mixed breed were OVX and allowed to recover for at least 30 d before steroid treatments were begun. Silicone elastomer implants containing 100 mg of E2 (Sigma, St. Louis, MO) were inserted subcutaneously into each ewe, and the uterus was collected from ewes (n = 4–6 per group) at 0 h (con-

Table 3
Sequence of TaqMan Primers and Probes for Ovine HIF1a, PlGF, and NP2

Oligonucleotide[a]	Nucleotide Sequence	GenBank accession number[b]
HIF1α FP	5'-CGC ATC TTG ATA AGG CTT CTG TT-3'	AF233078
HIF1α RP	5'-CAC CAG CAT CCA GAA GTT TCC T-3'	
HIF1α Probe	5'(6FAM)-TGA GGC TCA CCA TCA GCT ATT TGC GTG-(MGBNFQ)3'	
PlGF FP	5'-CCC TGG AGA CAG CCA ACG T-3'	AY157708
PlGF RP	5'-GGC TGG TCC AGA GAG TGG TAC T-3'	
PlGF Probe	5'(6FAM)-CCA TGC AGC TCA TG-(MGBNFQ)3'	
NP2 FP	5'-CAT CAA GAG CCA CTG GAG AAC TT-3'	AF534636
NP2 RP	5'-CCG GCC AAA CTC CAT TCC-3'	
NP2 Probe	5'(6FAM)-CAG TGC AAT GTC CCT C-(MGBNFQ)3'	

[a]FP, forward primer; RP, reverse primer.
[b]Nucleotide sequences for ovine-specific genes were obtained from the National Center for Biotechnology Information (2004) database.

trols), or at 2, 4, 8, 16, or 24 h after receiving the E2 implant *(5,6,21)*. After weighing the intact uterus, endometrial caruncular (CAR) and intercaruncular (ICAR) tissues were dissected from the endometrium, snap-frozen, and stored at –70°C for RNA isolation and quantitative real-time RT-PCR (qRT-PCR) analysis of gene expression.

Quantitative Real-time RT-PCR

All procedures for determining the expression of mRNA for angiogenic factors in ovine tissues by qRT-PCR have been reported previously *(22–24)*. Briefly, snap-frozen tissues were homogenized in Tri-Reagent (Molecular Research Inc, Cincinnati, OH) according to the manufacturer's specifications. The quality and quantity of total RNA were determined via capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE). Real-time RT-PCR reagents, probes, and primers were purchased from and used as recommended by Applied Biosystems (Foster City, CA). For each sample, 30 ng total RNA was reverse transcribed in triplicate 20-μL reactions using random hexamers. Taqman probe and primer sets for *VEGF*, *VEGFR1*, *VEGFR2*, *FGF2*, *NOS3*, *GUCY1B3*, *HIF1α*, *ANGPT1*, *ANGPT2*, and *Tie2 (22)* and for *FGFR2 IIIc* and *NP1 (24)* were used as described. Additionally, sequence-specific Taqman probe and primer sets were designed for sequences of *HIF1α*, *PlGF*, and *NP2* using the Primer Express Software from Applied Biosystems (Table 3). The ABI PRISM 7000 was used for detection of sequences amplified at 60°C typically for 40 or 45 cycles (Applied Biosystems). Quantification was determined from a relative standard curve of dilutions of the cDNA generated from tcRNA pooled from placentomes collected on d 130 of pregnancy. This standard was selected because the relative expression of each angiogenic gene analyzed was robust at this stage of pregnancy and could be diluted to give a good representative range of expressions that covered the mRNA expression of the samples in this study. To control for variations in the amount of RNA used, individual samples were also analyzed for concentrations of 18S RNA using the 18S PDAR kit from Applied Biosystems.

Statistical Analysis

Data were analyzed by using the General Linear Models (GLM) procedure of the Statistical Analysis System, with time after E2-treatment as the main effect *(25)*. Uterine weight values and the mRNA values were evaluated as a fold-increase relative to the control (0 h), which was assigned a value of 1. Differences between specific means were determined using LSMeans test or Bonferroni's *t*-test *(26,27)*. Uterine weights and the E2-response curves of all angiogenic factors and receptors were also analyzed by linear and nonlinear regression using PROC REG of SAS *(25)*, and the best-fit curves (based on the lowest *p* value and greatest R^2) were selected. For the regression analysis, uterine weight and E2-response curves of all angiogenic factors and receptors were evaluated without converting the data to fold-increase values. Additionally, correlations between uterine weights and expression of the angiogenic factor genes and between expression of the angiogenic factors themselves were determined using the PROC CORR of SAS *(25)*.

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