

# Estrogen Rapidly Activates the PI3K/AKT Pathway and Hypoxia-Inducible Factor 1 and Induces Vascular Endothelial Growth Factor A Expression in Luminal Epithelial Cells of the Rat Uterus<sup>1</sup>

Armina A. Kazi, Kristin Happ Molitoris, and Robert D. Koos<sup>2</sup>

Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland

## ABSTRACT

We have previously shown that 17 $\beta$ -estradiol ( $E_2$ ) increases vascular endothelial growth factor A (*Vegfa*) gene expression in the rat uterus, resulting in increased microvascular permeability, and that this involves the simultaneous recruitment of hypoxia-inducible factor 1 (HIF1) and estrogen receptor alpha (ESR1) to the *Vegfa* gene promoter. Both events require the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway. However, those studies were carried out using whole uterine tissue, and while most evidence indicates that the likely site of  $E_2$ -induced *Vegfa* expression is luminal epithelial (LE) cells, other studies have identified stromal cells as the site of that expression. To address this question, the pathway regulating *Vegfa* expression was reexamined using LE cells rapidly isolated after  $E_2$  treatment. In addition, we further characterized the nature of the receptor through which  $E_2$  triggers the signaling events that lead to *Vegfa* expression using the specific ESR1 antagonist ICI 182,780. In agreement with previous results in the whole uterus,  $E_2$  stimulated *Vegfa* mRNA expression in LE cells, peaking at 1 h (4- to 14-fold) and returning to basal levels by 4 h. Treatment with  $E_2$  also increased phosphorylation of AKT in LE cells, as well as of the downstream mediators FRAP1 (mTOR), GSK3B, and MDM2. The alpha subunit of HIF1 (HIF1A) was present in LE cells before  $E_2$  treatment, was unchanged 1 h after  $E_2$ , but was >2-fold higher by 4 h. Chromatin immunoprecipitation analysis showed that HIF1A was recruited to the *Vegfa* promoter by 1 h and was absent again by 4 h. The  $E_2$  activation of the PI3K/AKT pathway, HIF1A recruitment to the *Vegfa* promoter, and *Vegfa* expression were all blocked by ICI 182,780. In summary, the rapid  $E_2$ -induced signaling events that lead to the expression of *Vegfa* observed previously using the whole uterus occur in LE cells and appear to be initiated via a membrane form of ESR1.

*AKT, ChIP, chromatin immunoprecipitation, endometrium, ER $\alpha$ , ESR1, estradiol, 17 $\beta$ -estradiol, estrogen, estrogen receptor  $\alpha$ , HIF-1 $\alpha$ , HIF1A, hypoxia-inducible factor 1 alpha, PI3K, PI3K/AKT pathway, rat, signal transduction, uterus, vascular endothelial growth factor, vascular endothelial growth factor A, VEGF, VEGFA, Vegfa*

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<sup>2</sup>Correspondence: Robert D. Koos, Department of Physiology, University of Maryland School of Medicine, 655 West Baltimore St., Baltimore, MD 21201.

FAX: 410 706 8341; e-mail: rkoos@umaryland.edu

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## INTRODUCTION

During the reproductive cycle, the uterine endometrium undergoes a precisely timed complex sequence of physiological and morphological changes in preparation for implantation. These changes are controlled primarily by the ovarian steroid hormones 17 $\beta$ -estradiol ( $E_2$ ) and progesterone. The  $E_2$  regulates the immediate preovulatory phase (proestrus in rodents), which is characterized by endometrial stromal edema, and is followed ~16 h later by a wave of luminal epithelial (LE) cell proliferation. This proliferation is considered one of the critical effects of  $E_2$  on the endometrium, but the mechanisms involved are still incompletely understood. A primary mediator of the effects of  $E_2$  in the endometrium is vascular endothelial growth factor A (VEGFA) [1–4].  $E_2$  rapidly (within ~1 h) and robustly increases *Vegfa* gene expression in the uterus [1, 4]. While this increase is transient, consistent with its being an immediate early gene response [1], it causes the increased stromal microvascular permeability and plasma efflux that are the hallmarks of the initial action of  $E_2$  in the uterus [2]. Evidence indicates that this acute exudation of plasma is essential for subsequent LE cell proliferation, angiogenesis, and other growth and remodeling events [5–8]. We recently showed that  $E_2$  induction of *Vegfa* expression in the uterus requires the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway and involves the recruitment of 1) the heterodimeric transcription factor hypoxia-inducible factor 1 (HIF1), which is made up of HIF1A (also known as HIF-1 $\alpha$ ) and the aryl hydrocarbon receptor nuclear translocator (ARNT [also known as HIF-1 $\beta$ ]) to the hypoxia response element (HRE), and 2) estrogen receptor alpha (ESR1) to proximal transacting transcription factor 1 (SP1)-binding sites on the *Vegfa* gene promoter [4, 9]. This represents the first demonstration of a specific role for HIF1—which is increasingly recognized to be a central regulator of normal development, postnatal physiology, and cancer and other pathologies [10–12]—in a biological action of  $E_2$ .

These findings were made using the whole uterus, which is a complex multilayered organ. Previous *in situ* hybridization (ISH) studies [13–15] in intact rodents indicated that the site of the rapid  $E_2$ -induced *Vegfa* expression in the uterus is the LE cell layer of the endometrium. Other studies [16, 17] performed in ovariectomized animals, however, identified sub-LE stromal cells as the initial site of  $E_2$ -induced *Vegfa* expression. Establishing the exact site of *Vegfa* expression in the endometrium is essential because many effects of  $E_2$  on the uterus, as well as the mammary gland, appear to require interactions between epithelial cells and the stroma [18, 19], although the nature of those interactions is controversial. VEGFA could be the key to that interaction. Identifying the cell type in which *Vegfa* expression initially occurs could help to explain inconsistencies in the literature about both the uterus and mammary gland concerning 1) the relative roles of ESR1 in epithelial cells and stromal cells in the proliferation of the

former, 2) the nature of the stromal contribution to that proliferation, and 3) the apparent additional requirement for systemic factors [7, 18, 20–23]. Therefore, to clarify whether LE cells express the *Vegfa* gene in response to  $E_2$  in the normal uterus and to confirm that the associated signaling events previously identified using the whole uterus occur in this cell type, we reexamined those events in LE cells rapidly isolated after  $E_2$  treatment. We also extended the analysis of the role of the PI3K/AKT pathway in  $E_2$  action by examining  $E_2$ -induced phosphorylation of the downstream PI3K/AKT mediators FRAP1 (also known as molecular target of rapamycin or mTOR), GSK3B (glycogen synthase kinase 3 beta), and MDM2 (mouse double minute protein).

Given that PI3K is a plasma membrane-localized enzyme, its activation by  $E_2$  most likely is initiated through a membrane form of ESR1 [24]. In most cases,  $E_2$  activation of cytoplasmic signaling pathways is blocked by the specific ESR1 antagonist ICI 182,780 [25–29], indicating that a form of ESR1 is involved; however, exceptions to this have been reported [30–33]. The second major objective of these experiments, therefore, was to determine whether the rapid activation of the PI3K pathway by  $E_2$  that leads to HIF1A activation in the uterus is initiated via an ICI 182,780-sensitive receptor.

## MATERIALS AND METHODS

### *Animals, Treatments, and Tissue Collection*

Animal studies were conducted in accord with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and approved by the Institutional Animal Care and Use Committee, University of Maryland School of Medicine. Immature (21-day-old) female Sprague-Dawley rats (Charles River, Wilmington, MA) were injected (s.c.) with either  $E_2$  (0.05  $\mu\text{g/g}$  body weight) or vehicle (ethanol:PBS ratio, 1:500). In some experiments, ICI 182,780 (3  $\mu\text{g/g}$  body weight [i.p.]; Sigma-Aldrich, St. Louis, MO) or vehicle (dimethylsulfoxide [DMSO]) was administered 1.5 h before  $E_2$  treatment. For the experiment on the role of FRAP1 in  $E_2$ -induced VEGF expression, rapamycin (5  $\mu\text{g/g}$  body weight [i.p.]; LC Laboratories, Woburn, MA) or vehicle (DMSO) was injected 1 h before  $E_2$  treatment.

Animals were killed by cervical dislocation 1 or 4 h after  $E_2$  treatment, the reproductive tract was exposed through a midline incision, and the uterus and ovaries were excised together and placed on a moistened paper towel on top of a frozen gel pack. The ovaries and oviducts, fat, and mesometrial membranes were quickly trimmed away, and the uterus was weighed.

### *Processing of Whole Uterine Samples*

For experiments involving whole uterine tissue, one horn was stored in RNAlater (Qiagen, Valencia, CA) at 4°C. The other horn was flash frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for protein extraction. For chromatin immunoprecipitation (ChIP), uterine horns were slit open longitudinally and fixed in 1.5% formaldehyde/Dulbecco modified Eagle medium-F12 for 15 min at room temperature [9]. Fixation was stopped by adding 1 M glycine to a final concentration of 0.25 M and incubation for 10 min. The fixed horns were rinsed in ice-cold PBS (Quality Biologicals, Gaithersburg, MD) plus 1 $\times$  protease and 1 $\times$  phosphatase inhibitor cocktails (Roche Applied Sciences, Indianapolis, IN), hereafter referred to as PBS+, and stored at  $-80^\circ\text{C}$  for later processing (described herein).

### *LE Cell Isolation*

The LE cells were isolated as previously described by Fagg et al. [34] and by Chen et al. [19]. Briefly, uterine horns were slit open longitudinally and rinsed in 1 ml of ice-cold PBS+. Four to six horns were placed in a round-bottom 14-ml (17  $\times$  100 mm) polypropylene tube containing 1 ml of ice-cold PBS+ and five 3-mm polytetrafluoroethylene solid balls (Fisher Scientific, Fair Lawn, NJ). The horns were then vortexed for six 30-sec cycles with 30 sec on ice between cycles. Residual tissue was removed with forceps, flash frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . The supernatant containing the LE cells was aspirated and divided into aliquots appropriate for RNA (100  $\mu\text{l}$ ) and protein (100  $\mu\text{l}$ ) extraction or ChIP analysis (800  $\mu\text{l}$ ).

For ChIP, an equal volume (800  $\mu\text{l}$ ) of 2% formaldehyde in PBS was added to LE cell aliquots to give a final formaldehyde concentration of 1%, and the

cells were fixed for 10 min at room temperature. Fixed cells were pelleted by centrifugation at  $300 \times g$  for 5 min at 4°C. They were then rinsed by resuspension in 1 ml of ice-cold PBS+ and centrifuged again. The supernatant was removed, and the fixed cell pellet was flash frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

### *RNA Extraction*

The LE cell aliquots for RNA were pelleted by centrifugation ( $300 \times g$  for 5 min) at 4°C, and the supernatant was discarded. The LE cell pellets were resuspended and homogenized in Buffer RLT (Qiagen) by centrifugation through QIAshredder spin columns (Qiagen). Frozen whole uterine tissue or residual tissue was homogenized in ice-cold Buffer RLT using a Mini Beadbeater and 1.0-mm zirconia/silica beads (BioSpec Products, Bartlesville, OK). The beads were removed, and the samples were further homogenized and sheared by centrifugation through QIAshredder spin columns. Total RNA was extracted and purified from homogenates using the RNeasy Mini Kit (Qiagen). The RNA concentration and purity were determined based on 260/280-nm absorbances.

### *RT-PCR Analysis*

*Vegfa* and progesterone receptor (*Pgr*) mRNA expression in LE cells and whole or residual uterine tissue was measured using both conventional and real-time RT-PCR as described previously [4, 9]. The following PCR primers were used in both cases: rat *Vegfa* +12 to +644: forward 5'-GCTCTCTTGGGTGCACTGGA-3' and reverse 5'-CACCGCCTTGGCTTGTCACA-3' (GenBank accession No. NM031836); rat *Pgr* +1910 to +2359: forward 5'-TTATGAGAGCCCTCGATGGT-3' and reverse 5'-GCGAGTGAATGACAATC-3' (GenBank accession No. L16922); and *Rnl8s* (18S rRNA) +364 to +647: forward 5'-CAACTTTCGATGGTAGTCGC-3' and reverse 5'-CGCTATTGGAGCTGGAATTAC-3' (GenBank accession Nos. X01117 and H01593). Rat *Hif1a* primers were +681 to +890: forward 5'-TGCTTGGTGTGATTTGTGA-3' and reverse 5'-GGTCAGATGATCAGATCCA-3' (GenBank accession Nos. AF057308 and NM024359).

### *Protein Extraction*

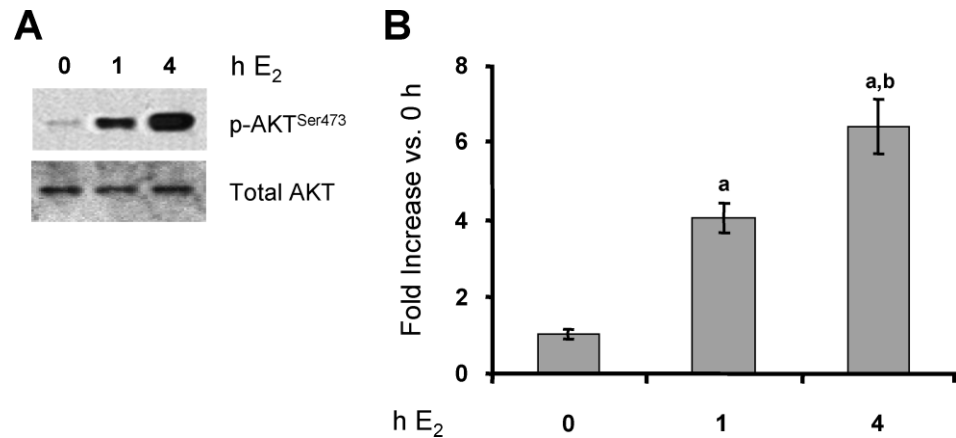
The LE cell aliquots were centrifuged at  $300 \times g$  for 5 min at 4°C, and the supernatant was discarded. The cell pellets were resuspended and lysed in 150  $\mu\text{l}$  of radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% deoxycholic acid, 1 mM edetic acid [EDTA], and protease and phosphatase inhibitors as already described). The samples were then cleared by centrifugation at 3000 rpm for 30 min at 4°C, and the supernatant was collected and stored at  $-80^\circ\text{C}$ .

Whole uterine tissue was homogenized on ice in 200  $\mu\text{l}$  of RIPA buffer using a Tissue-Tearor rotor-stator homogenizer (Biospec Products) at power setting 1.5. The homogenate was then cleared by centrifugation as already described, and the supernatant was collected and stored at  $-80^\circ\text{C}$ .

### *Western Blot Analysis*

Protein concentration was determined using the BCA Protein Assay (Pierce Chemical, Rockford, IL). Six to ten micrograms of protein was added to 15  $\mu\text{l}$  of 2 $\times$  SDS loading buffer (0.125 M Tris [pH 6.8], 4% SDS, 20% glycerol, 10%  $\beta$ -mercaptoethanol, and 0.004% bromophenol blue) and incubated at 100°C for 5 min. Protein samples and 10  $\mu\text{l}$  of Precision Plus Protein dual-color standards (Biorad Laboratories, Hercules, CA) were loaded onto 4%–12% gradient Bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) and run at  $\sim 200$  V for 1 h. Proteins were then transferred overnight onto polyvinylidene fluoride membranes (Fisher Scientific). Membranes were blocked with 5% nonfat dry milk (NFD)/1 $\times$  Tris-buffered saline (TBS) with 0.1% Tween-20 (TTBS) for 1 h at 37°C before being incubated with primary antibodies (Supplemental Table 1 available at [www.biolreprod.org](http://www.biolreprod.org)) in 1% NFD/1 $\times$  TBS with 0.05% Tween-20 for either 2 h at room temperature or overnight at 4°C. After four 5-min washes in TTBS, the membranes were incubated for 1 h with either goat anti-mouse horseradish peroxidase (HRP)-conjugated antibody (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA) or goat anti-rabbit HRP-conjugated antibody (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA) in 1% NFD/1 $\times$  TBS with 0.05% Tween-20. The membranes then received four 5-min washes in TTBS. Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA) was used for visualization of protein bands according to the manufacturer's instructions. Whenever blots were reprobed, they were first stripped with Restore Western Blot Stripping Buffer (Pierce Chemical) for 10 min at 37°C.

FIG. 1.  $E_2$  activates the PI3K/AKT pathway in endometrial LE cells. Rats were treated with  $E_2$  for the indicated times or with vehicle (0 h). Total protein was extracted from LE cells, and phosphorylated and total AKT was analyzed by Western blot analysis (representative gels [A] and densitometry [B]). Densitometry results are expressed as the fold increase in phosphorylated (p)-AKT<sup>Ser473</sup> compared with control cells (0-h  $E_2$ ) after normalization to total AKT (mean  $\pm$  SEM,  $n = 3$  independent LE cell samples/group; each sample contained cells pooled from two uteri). a, vs. 0-h  $E_2$  ( $P < 0.01$ ); b, vs. 1-h  $E_2$  ( $P < 0.05$ ).



### ChIP Analysis

ChIP analyses of LE cells and whole uterine tissue were done as previously described [4]. Frozen LE cell pellets were resuspended in nuclear lysis buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 1% SDS, and protease inhibitors as already described) and incubated on ice for 15 min. Frozen fixed whole uterine tissue was homogenized on ice in 200  $\mu$ l of RIPA buffer. The homogenate was then centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was discarded, and the nuclear pellet was resuspended in nuclear lysis buffer (as already described) and incubated on ice for 15 min. Nuclear samples from both LE cells and whole uterine tissue were sonicated on ice for ten 10-sec cycles with 20-sec pauses between each cycle using a Microson Ultrasonic cell disruptor (Misonix, Farmingdale, NY) at power level 2.5. After sonication, the samples were centrifuged at 14000  $\times$  g for 10 min at 4°C. The supernatants were then collected and stored at -80°C.

Primers that encompassed the rat *Vegfa* promoter region -944 to -611 (HRE-containing region) were used for PCR [9]. These were forward 5'-TCTGCCAGACTCCACAGTG-3' and reverse 5'-TGCGTGTTCCTAACACCCAC-3' (Genbank accession No. U22373).

### Statistical Analysis

Statistical analyses were done using factorial analysis of variance and appropriate post hoc tests. An exception was the uterine weight data, which were analyzed using Kruskal-Wallis test (StatView version 4.5; Abacus Concepts, Berkeley, CA).

## RESULTS

### $E_2$ Activates the PI3K/AKT Pathway in LE Cells

The mechanical method used to rapidly isolate LE cells from the uterus has been reported to yield an LE cell fraction of >95% purity [19]. To confirm that cells isolated in this way do not contain a significant level of stromal cell contamination, we examined a functional end point that distinguishes LE cells from stroma, namely,  $E_2$  induction of *Pgr* mRNA expression.  $E_2$  significantly increases *Pgr* expression in the rodent uterus [9, 35], and several studies [36–39] have shown that this occurs exclusively in stromal cells. *Pgr* mRNA levels in the stroma-containing residual tissue were increased significantly (2.6-fold [ $P < 0.05$ ]) at 1 h after  $E_2$  treatment, whereas *Pgr* expression in isolated LE cells went in the opposite direction, with message becoming almost undetectable by 4 h. This confirmed that the LE cell fraction was not contaminated to a significant degree by stromal cells.

The rapid  $E_2$  induction of *Vegfa* expression in the uterus involves activation of the PI3K/AKT pathway and subsequent recruitment of both nuclear ESR1 and HIF1A to the *Vegfa* gene promoter [4, 9]. Therefore, we first determined whether activation of this signaling pathway can be demonstrated in LE cells. As shown in Figure 1, there were ~4-fold and ~6-fold increases in AKT phosphorylation on serine<sup>473</sup> at 1 h and

4 h after  $E_2$  treatment, respectively. The total AKT level was unaffected by  $E_2$  at either time point.

Phosphorylation of AKT leads in turn to activation of a wide range of downstream mediators (including FRAP1 and MDM2) and to inactivation of others (such as GSK3B), but there have been few studies of  $E_2$  regulation of the activity of these proteins in the normal uterus. To determine whether these mediators are phosphorylated in LE cells in response to  $E_2$ , Western blot analyses using phosphospecific antibodies were carried out. Values were normalized to the value of total ESR1 protein, which was unchanged after  $E_2$  treatment at these time points. As shown in Figure 2,  $E_2$  progressively increased phosphorylation of FRAP1, MDM2, and GSK3B after 1 h (2- to 4-fold) and 4 h (5- to 9-fold). In addition, total levels of all three proteins were significantly elevated by 4 h (>2.5-fold in each case). Thus, all of these factors are potential mediators of  $E_2$ -induced effects on uterine LE cells.

### Activation of the PI3K/AKT Pathway in the Uterus Is Blocked by ICI 182,780

The rapid activation of PI3K, an event that occurs at the plasma membrane, by  $E_2$  suggests that it is mediated by a membrane-associated form of ESR1 [24]. Several studies [27, 28, 40–44] have shown that the effects of membrane ESR1, like those of the nuclear form, are blocked by the specific ESR1 antagonist ICI 182,780. Therefore, we determined whether the rapid activation of the PI3K/AKT pathway in the rat uterus is blocked by this antagonist. Having demonstrated that PI3K/AKT activation is similar regardless of whether it is measured in isolated LE cells (Figs. 1 and 2) or in whole uterus [9], this analysis was conducted using the latter. As shown in Figure 3, the  $E_2$ -induced phosphorylation of AKT, as well as that of FRAP1 and GSK3B, is completely blocked by pretreatment of rats with ICI 182,780, indicating that activation of this pathway originates with a membrane form of ESR1.

### HIF1A Is Present in LE Cells and Further Induced by $E_2$

We have previously shown that HIF1A is present in the immature rat uterus before  $E_2$  treatment and that  $E_2$  further induces its expression, but not that of ARNT or ESR1, by 4 h [4]. Isolated LE cells also expressed detectable HIF1A, ARNT, and ESR1 protein under basal conditions (Fig. 4A). As in the whole uterus, both ARNT and ESR1 levels were unchanged after  $E_2$  treatment, as was the level of HIF1A at 1 h. By 4 h after  $E_2$ , however, the level of HIF1A protein had doubled (Fig. 4, A and B). *Hif1a* mRNA was also increased ~2.5-fold by 4 h ( $P < 0.05$  [data not shown]). This increase in HIF1A expression suggests



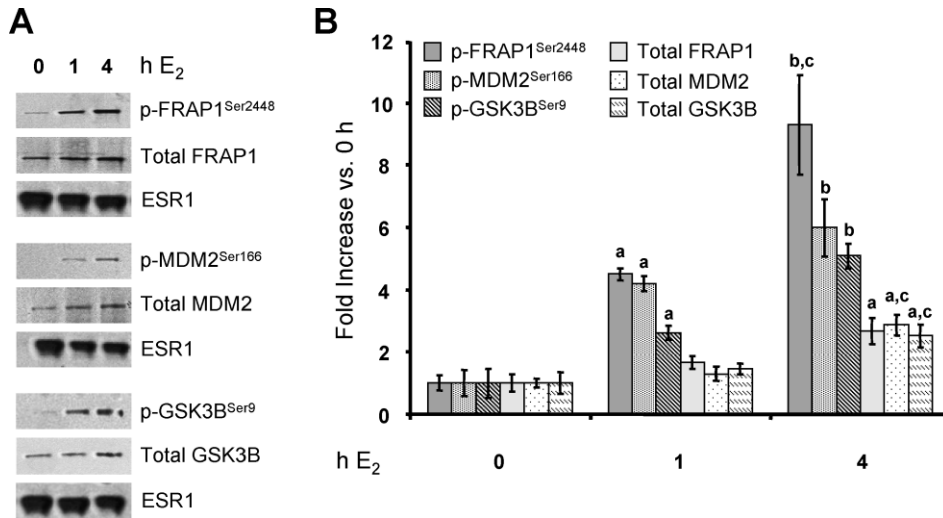


FIG. 2. E<sub>2</sub> induces phosphorylation of FRAP1, MDM2, and GSK3B in LE cells. Rats were treated as described in Figure 1. Phosphorylated (p) and total FRAP1, MDM2, and GSK3B and total ESR1 were analyzed by Western blot analysis (representative blots [A] and densitometry [B]). Densitometry results are expressed as the fold increase in phosphorylated protein compared with that in control cells (0-h E<sub>2</sub>) after normalization to total ESR1 protein (mean ± SEM, n = 3 LE cell samples/group). a, vs. 0-h E<sub>2</sub> (P < 0.01); b, vs. 0-h E<sub>2</sub> (P < 0.05); c, vs. 1-h E<sub>2</sub> (P < 0.05).

that it has a sustained role in E<sub>2</sub> action on LE cells. As shown in Figure 4, C and D, the stimulation of HIF1A expression by E<sub>2</sub> at 4 h is also blocked by ICI 182,780. To our knowledge, this is the first time that expression of the HIF1A gene has been demonstrated to be mediated by ESR1; whether this involves both membrane and nuclear forms of ESR1 is still unknown.

*E<sub>2</sub> Stimulates Vegfa Expression in LE Cells*

In the whole uterus, E<sub>2</sub> induces a large increase in Vegfa mRNA levels by 1–2 h, with a return to basal levels by 4 h [1, 4, 9]. As shown in Figure 5, Vegfa mRNA expression in isolated LE cells increased strongly by 1 h after E<sub>2</sub> treatment, before declining to basal levels by 4 h. In the animals from which the cells were isolated 4 h after E<sub>2</sub> treatment, there was a >50% increase in uterine wet weight (Fig. 5C), which is dependent on VEGFA [2]. Thus, isolated epithelial cells show exactly the same pattern of Vegfa expression that is observed when RNA is prepared from the whole uterus, consistent with their being the primary site of that increase. The E<sub>2</sub> induction of Vegfa expression in the endometrium and the increase in uterine weight at 4 h were both blocked by ICI 182,780 (Fig. 6, A and B). Because our work indicates that both membrane and

nuclear ESR1 mediate E<sub>2</sub>-induced Vegfa expression, the effect of ICI 182,780 is likely due to inhibition of both.

*E<sub>2</sub>-Induced Vegfa Gene Expression in LE Cells Does Not Involve FRAP1*

FRAP1 can be effectively inhibited in vivo by rapamycin. To determine whether FRAP1 has a role in E<sub>2</sub> activation of the Vegfa gene in the uterus, the effect of this inhibitor was examined. To confirm the effectiveness of rapamycin in blocking E<sub>2</sub>-induced FRAP1 activity, the phosphorylation state of RPS6KB1 (ribosomal protein S6 kinase, 70 kDa, polypeptide 1), a downstream target of FRAP1, was examined. The E<sub>2</sub> caused a marked increase in its phosphorylation by 4 h, without affecting the total RPS6KB1 levels (Fig. 7A). Pretreatment of rats with rapamycin effectively blocked this effect, indicating that the dose of rapamycin was sufficient to inhibit FRAP1 activity in vivo. Blocking FRAP1 activity, however, had no effect on E<sub>2</sub>-induced increases in Vegfa expression or uterine weight (Fig. 7, B and C). Thus, FRAP1 is not required for E<sub>2</sub> induction of Vegfa expression. This lack of connection of FRAP1 to Vegfa expression is also suggested by the absence of

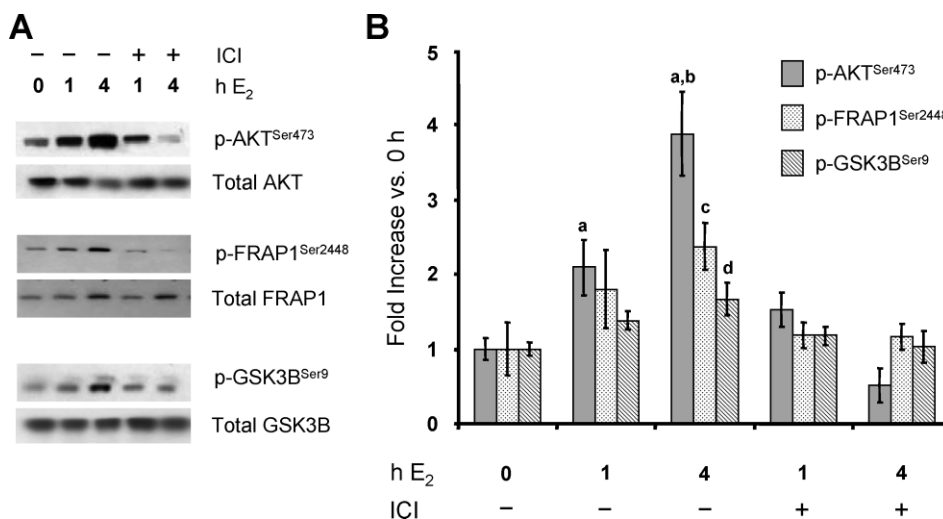
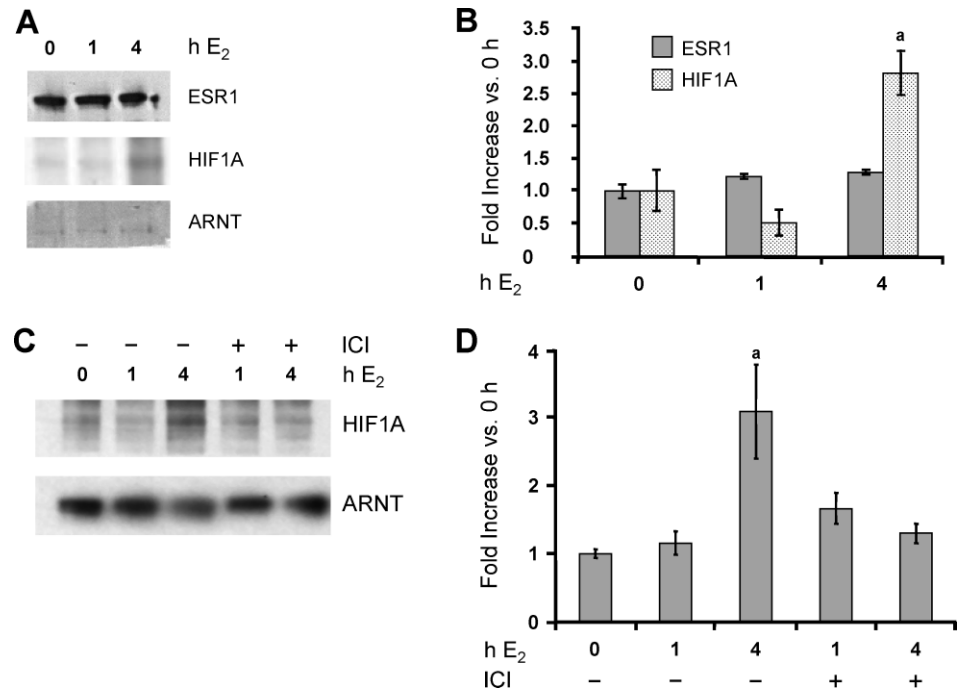


FIG. 3. The E<sub>2</sub> activation of the PI3K/AKT pathway in the uterus is blocked by ICI 182,780. Rats were treated with either vehicle or the ESR1 antagonist ICI 182,780 (ICI) 1.5 h before treatment with either vehicle or E<sub>2</sub> for the indicated times. Total protein was extracted from whole uteri, and phosphorylated (p) and total AKT, FRAP1, and GSK3B were analyzed by Western blot analysis (representative blots [A] and densitometry [B]). Densitometry results are expressed as the fold increase in phosphorylated protein expression compared with that of uteri not exposed to either ICI or E<sub>2</sub> (0 h) after normalization to the total level of each protein (mean ± SEM, n = 3–4 uteri/group). a, vs. 0 h and ICI 4-h E<sub>2</sub> (P < 0.01); b, vs. 1-h E<sub>2</sub> and ICI 1-h E<sub>2</sub> (P < 0.01); c, vs. 0 h, ICI 1-h E<sub>2</sub>, and ICI 4-h E<sub>2</sub> (P < 0.05); d, vs. 0 h, ICI 1-h E<sub>2</sub>, and ICI 4-h E<sub>2</sub> (P < 0.05).

FIG. 4. **A** and **B**)  $E_2$  induces HIF1A expression in LE cells. Rats were treated as described in Figure 1. Total protein was extracted from LE cells, and ESR1, HIF1A, and ARNT proteins were analyzed by Western blot analysis (representative gels [A] and densitometry [B]). Densitometry results are expressed as the fold increase in each protein compared with cells not exposed to  $E_2$  (0-h  $E_2$ ) after normalization to ARNT (mean  $\pm$  SEM,  $n = 3$  LE cell samples/group). a, vs. 0 h and 1-h  $E_2$  ( $P < 0.01$ ). **C** and **D**) The  $E_2$  induction of HIF1A expression in the uterus is blocked by ICI 182,780. Rats were treated as described in Figure 3. Total protein was extracted from whole uteri, and HIF1A and ARNT proteins were analyzed by Western blot analysis (representative gels [C] and densitometry [D]). Densitometry results are expressed as the fold increase in HIF1A compared with uteri not exposed to either ICI or  $E_2$  (0 h) after normalization to ARNT (mean  $\pm$  SEM,  $n = 3$ –4 uteri/group). a, vs. 0 h, 1-h  $E_2$ , ICI 1-h  $E_2$ , and ICI 4-h  $E_2$  ( $P < 0.01$ ).



phosphorylation of RPS6KB1 at 1 h, when *Vegfa* mRNA expression is already maximal.

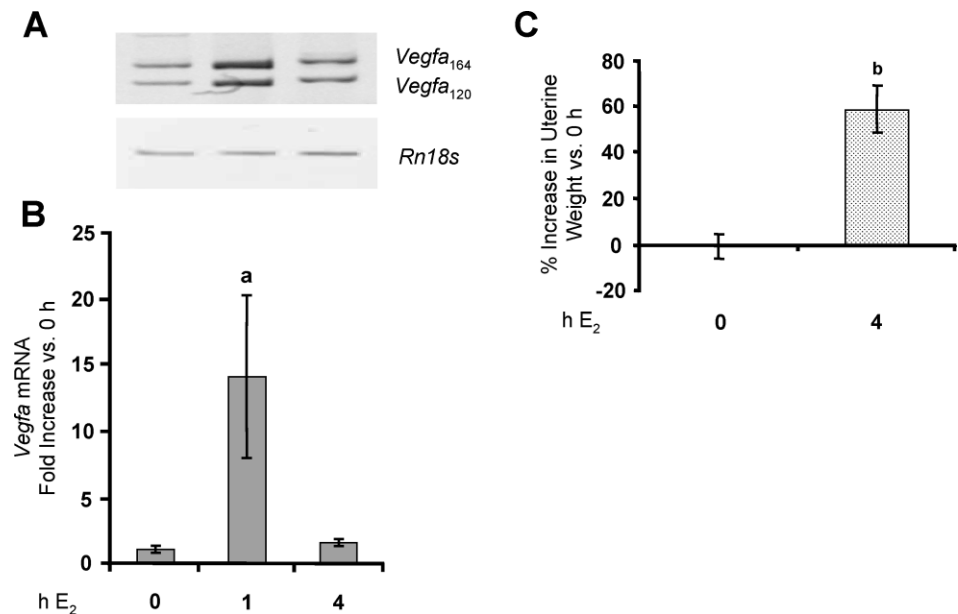
#### *E<sub>2</sub> Rapidly Induces Recruitment of HIF1A to the Vegfa Gene Promoter in LE Cells*

The  $E_2$ -induced PI3K/AKT activation leads to the rapid recruitment of HIF1A to the HRE of the *Vegfa* promoter [9]. ChIP analyses to detect  $E_2$ -induced transcription factor binding to the *Vegfa* promoter showed that  $E_2$  increased HIF1A binding to the *Vegfa* promoter in rat LE cells at 1 h, which was lost again by 4 h (Fig. 8, B and C). This pattern correlated with  $E_2$ -induced *Vegfa* mRNA expression (Figs. 5 and 6). This recruitment of HIF1A to the *Vegfa* gene promoter was blocked by ICI 182,780 (Fig. 8D), again indicating the involvement of membrane ESR1.

#### DISCUSSION

The rapid but transient increase in *Vegfa* expression in LE cells after  $E_2$  treatment is identical to that previously observed using the whole uterus [1, 4, 9]. This is consistent with several ISH studies [13–15] that identified the LE cells as the only site of  $E_2$ -induced *Vegfa* expression in the rodent endometrium. In the mouse, *Vegfa* expression was also restricted to LE cells on Day 1 of pregnancy, when (according to the authors) the uterus is primarily under the influence of preovulatory  $E_2$  [45–47]. However, two other studies [16, 17] reported that  $E_2$  induction of *Vegfa* mRNA occurs in stromal cells. In both cases, the animals used were ovariectomized, in contrast to the previously mentioned studies, which were all done using intact animals. This suggests that removal of the ovaries may cause a shift in  $E_2$ -induced *Vegfa* expression from LE cells to sub-LE stromal

FIG. 5.  $E_2$  induces *Vegfa* expression in endometrial LE cells. Rats were treated as described in Figure 1. Total RNA was extracted from LE cells, and *Vegfa* mRNA was analyzed by conventional (A) and real-time (B) RT-PCR. Real-time results are expressed as the fold increase in *Vegfa* mRNA levels compared with untreated controls (0 h) after normalization to *Rn18s* (mean  $\pm$  SEM,  $n = 3$  LE cell samples/group). a, vs. 0 h and 4 h  $E_2$  ( $P < 0.05$ ). **C**) Uteri were weighed before LE cell isolation, and the percentage increase in wet weight (normalized to body weight) vs. 0 h is given (mean  $\pm$  SEM,  $n = 6$  uteri/group). b, vs. 0 h ( $P < 0.001$ ).



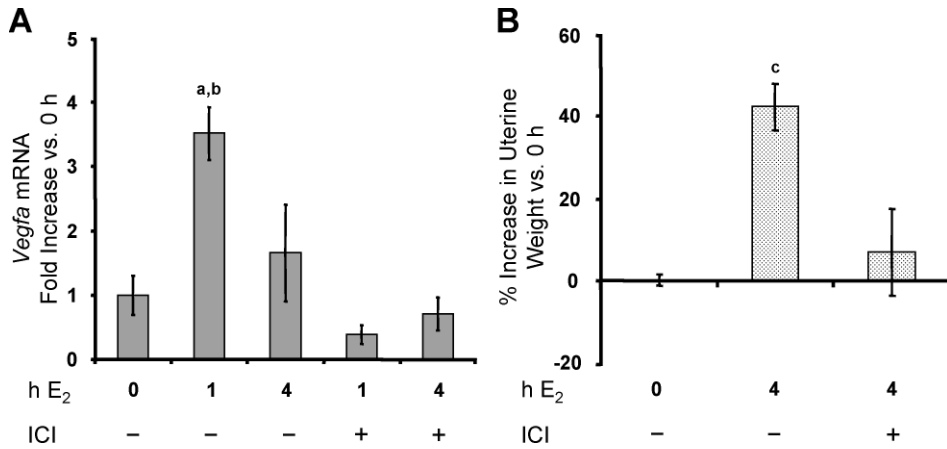


FIG. 6. The E<sub>2</sub> induction of *Vegfa* expression in the uterus is blocked by ICI 182,780. Rats were treated as described in Figure 3. **A**) Total RNA was extracted from whole uteri, and *Vegfa* mRNA was analyzed using quantitative real-time RT-PCR (mean ± SEM, n = 3–4 uteri/group). a, vs. 0 h, ICI 1-h E<sub>2</sub>, ICI 4-h E<sub>2</sub> (P < 0.001); b, vs. 4-h E<sub>2</sub> (P < 0.01). **B**) Uteri were weighed, and the percentage increase in wet weight (normalized to body weight) is compared vs. uteri not exposed to either ICI or E<sub>2</sub> (0 h; mean ± SEM, n = 3–4 uteri/group). c, vs. 0 h and ICI 4-h E<sub>2</sub> (P < 0.01).

cells. In one of the two studies in ovariectomized animals [17], *Vegfa* expression reverted to LE cells by 24 h after E<sub>2</sub> treatment, by which time stromal expression was very low, confirming that shifts in the cellular site of *Vegfa* expression do occur. The mechanical approach we used for the isolation of LE cells does not yield a pure population of stromal cells; after vortexing, the horns still contain residual LE cells and glandular epithelial cells. Thus, we cannot rule out the possibility that E<sub>2</sub> also affects *Vegfa* expression by stromal cells. As discussed previously, however, all ISH studies conducted to date in intact rodents have shown expression only in LE cells [13–15, 46–48], while stromal expression has been reported only in ovariectomized animals [16, 17]. Thus, regardless of the model used, E<sub>2</sub>-induced expression has been found to occur either in one compartment or the other. The fact that the pattern of E<sub>2</sub>-induced *Vegfa* expression in LE cells, as well as the signaling events previously linked to that expression, is identical to that seen when the whole uterus was used is further evidence that the LE cells are the site of that expression.

Establishing the exact spatial and temporal patterns of *Vegfa* expression in the uterus is important because of the critical role VEGFA may have in the initiation of E<sub>2</sub>-induced endometrial growth. VEGFA mediates the early effects of E<sub>2</sub> on the

endometrial microvasculature [2], namely, markedly increased permeability and plasma exudation, which are thought to be essential for normal LE cell proliferation [5–7]. Most important, VEGFA from the mammary epithelium has also recently been shown to be essential for normal alveolar expansion and microvascular function in the mouse mammary gland [48, 49]. It follows that VEGFA produced in epithelial cells must be secreted and diffused to nearby subepithelial capillaries. The primary isoforms of VEGFA expressed in the uterus are the more soluble forms [1]. Thus, synthesis in LE cells is compatible with a paracrine action on the sub-LE capillary bed. We propose, therefore, that the key events in LE cell proliferation are 1) E<sub>2</sub>-induced LE cell *Vegfa* expression, 2) VEGFA-induced increased microvascular permeability, and 3) increased delivery of growth factors to LE cells from plasma (Fig. 9). This model is entirely consistent with a recent study [22] that attributes the growth of ESR1– mammary epithelial cells to a direct effect of E<sub>2</sub> on a subpopulation of ESR1+ epithelial cells. Zhu and Pollard [50] have proposed that the E<sub>2</sub>-induced factor that causes endometrial LE cell proliferation is insulin-like growth factor 1 (IGF1), as IGF1 receptors are required for that effect. Sato et al. [7] reached a similar conclusion in investigations using IGF1-null mice but found that the involved IGF1 originated from the circulation. These

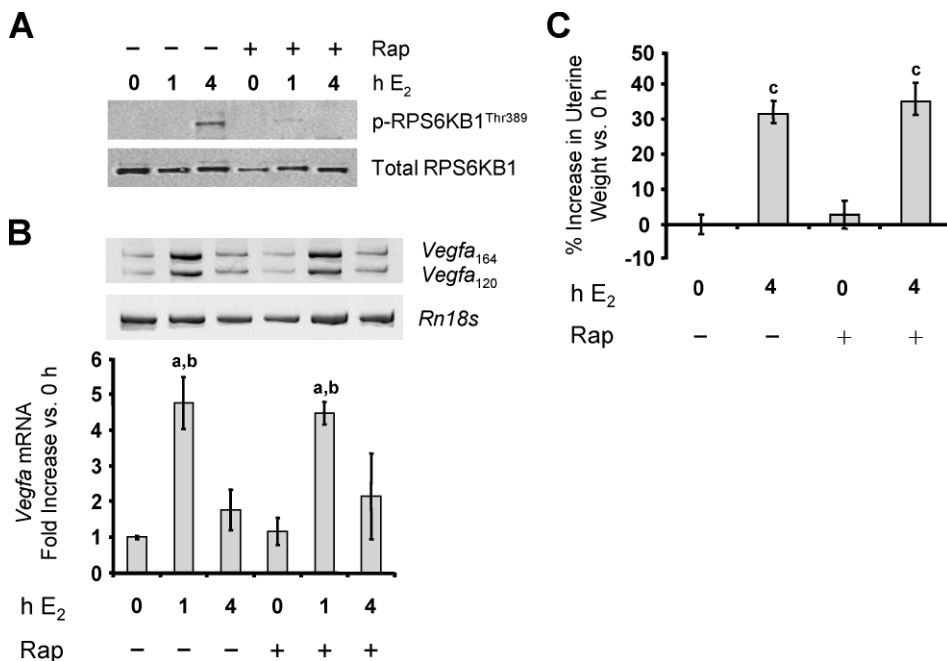
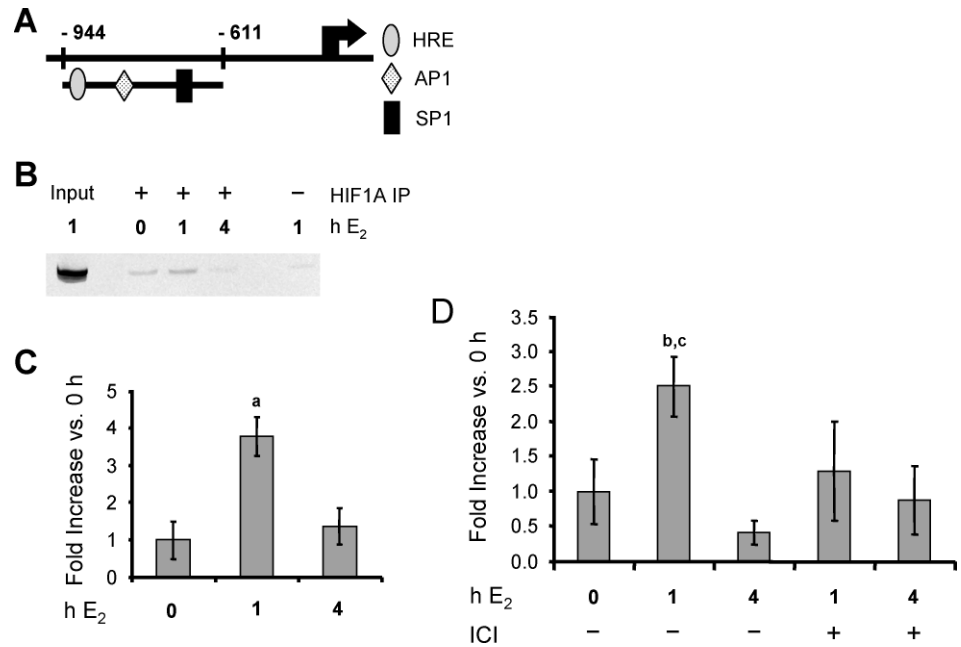


FIG. 7. The E<sub>2</sub>-induced *Vegfa* expression in LE cells is not mediated by FRAP1. Rats were treated with either vehicle or rapamycin (Rap) 1 h before treatment with either vehicle or E<sub>2</sub> as described previously, and LE cells were isolated. **A**) Expression of the FRAP1 target ribosomal protein S6 kinase, polypeptide 1 (RPS6KB1, also known as p70S6 kinase) and phosphorylated (p) RPS6KB1<sup>Thr389</sup> was analyzed by Western blot analysis (representative gels; n = 3 cell samples/group). **B**) *Vegfa* mRNA was analyzed as described previously (mean ± SEM, n = 3 cell samples/group). a, vs. 0 h and Rap 0 h (P < 0.01); b, vs. 4-h E<sub>2</sub> and Rap 4-h E<sub>2</sub> (P < 0.05). **C**) Uteri were weighed at 4 h, and the percentage increase in wet weight (normalized to body weight) is compared vs. uteri not exposed to either E<sub>2</sub> or rapamycin (0 h; mean ± SEM, n = 6 uteri/group). c, vs. 0 h and Rap 0 h (P < 0.0001).

FIG. 8.  $E_2$  induces recruitment of HIF1A to the *Vegfa* gene promoter in LE cells. Rats were treated as described in Figure 1.

Protein-chromatin complexes were extracted from isolated LE cells, and HIF1A binding to the HRE on the rat *Vegfa* gene promoter was analyzed by ChIP as described in *Materials and Methods*. **A)** Schematic of *Vegfa* promoter region. AP1, activator protein 1. **B)** Representative PCR gel. IP, immunoprecipitation. **C)** Densitometry results expressed as fold increase compared with control cells (0 h; mean  $\pm$  SEM,  $n = 3$  pools/group). a, vs. 0 h and 4-h  $E_2$  ( $P < 0.05$ ). **D)**  $E_2$ -induced recruitment of HIF1A is blocked by ICI 182,780. Rats were treated as described in Figure 3. HIF1A binding to *Vegfa* promoter in the whole uterus was analyzed by ChIP. Densitometry results are expressed as fold increase vs. uteri not exposed to either ICI or  $E_2$  (0 h; mean  $\pm$  SEM,  $n = 3$  uteri/group). b, vs. 0 h, ICI 1-h  $E_2$ , and ICI 4-h  $E_2$  ( $P < 0.05$ ); c, vs. 4-h  $E_2$  ( $P < 0.01$ ).



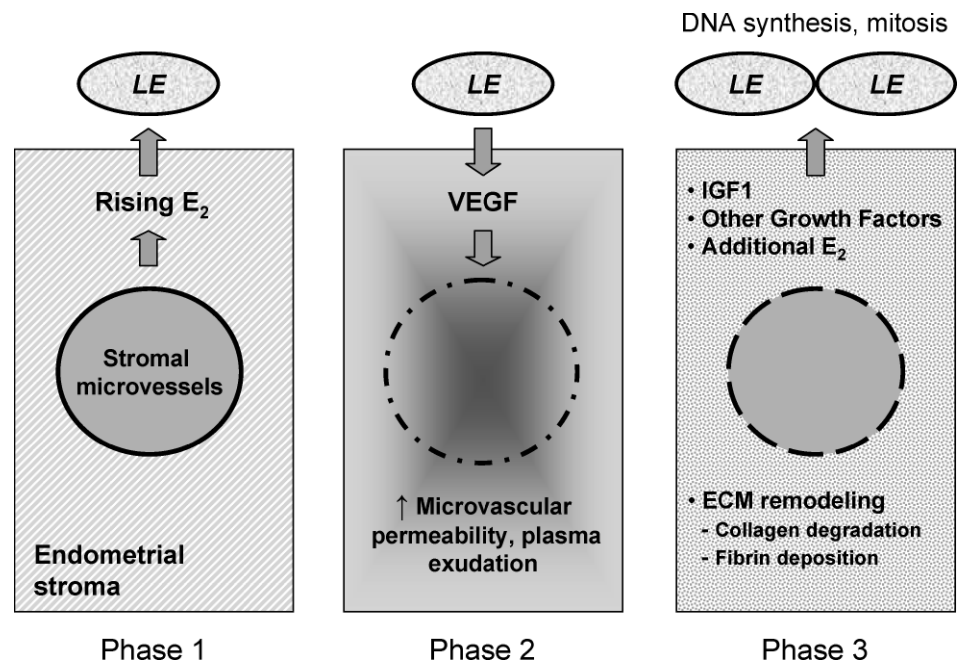
findings are compatible with the model proposed herein (Fig. 9) in which  $E_2$  acts on LE cells to induce *Vegfa* expression, which in turn enhances delivery of circulating IGF1 to LE cells via induction of interendothelial cell gaps. VEGFA is a potent inducer of such gaps [51], which appear in endometrial microvessels following  $E_2$  treatment [52, 53]. Furthermore, circulating IGF1 enters tissues from the blood primarily via the interendothelial cell route [54, 55].

We also show that key signaling events that we have previously linked to  $E_2$ -induced *Vegfa* expression using the whole uterus, namely, activation of the PI3K/AKT pathway and recruitment of HIF1A to the *Vegfa* promoter within 1 h, occur in LE cells. Thus, LE cells possess all of the signaling machinery necessary for activating *Vegfa* expression in response to  $E_2$ , and, conversely, stromal cell-derived factors are not involved in *Vegfa* expression. This is consistent with the rapid nature of the induction and with previous findings

that  $E_2$  regulation of the *Vegfa* gene occurs at the transcriptional level and does not require the synthesis of new proteins [1, 56]. As when the whole uterus was used [4], we also found that  $E_2$  induces a further increase in HIF1A levels in LE cells by 4 h. We previously showed that this increase in HIF1A protein is matched by an increase in *Hif1a* mRNA [4], and this was again confirmed in LE cells. Thus,  $E_2$  induces activation of HIF1A from the basal pool by 1 h and a further increase in the level of HIF1A by 4 h. The role of the latter increase in the effects of  $E_2$  on the uterus remains to be determined.

Although we postulate that the activation of PI3K/AKT is mediated by a membrane-associated form of ESR1 (described herein), others have attributed it to IGF1 secreted by stromal cells in response to  $E_2$  [50]. As already discussed, however, systemic rather than locally produced IGF1 appears to mediate  $E_2$ -induced LE cell proliferation [7]. We propose that VEGFA

FIG. 9. The proposed role of VEGFA in endometrial epithelial cell proliferation and stromal remodeling. As  $E_2$  levels rise during proestrus (or in response to exogenous  $E_2$  in immature or ovariectomized animals), *Vegfa* is expressed by LE cells (Phase 1). VEGFA induces a large increase in the permeability of stromal microvessels, flooding the stroma with plasma, the edema that is the sine qua non of the early effects of  $E_2$  on the uterus (Phase 2). The plasma is rich in growth factors such as IGF1 and carries additional  $E_2$  bound to serum proteins. Growth factors and  $E_2$  act in concert to induce maximal LE cell proliferation (Phase 3). The plasma also contains plasminogen that triggers the collagenolytic cascade and the degradation of the stromal extracellular matrix (ECM), as well as fibrinogen, which is converted to fibrin, creating a temporary matrix that promotes cell proliferation, cell migration, and angiogenesis [8]. The  $E_2$  also primes stromal fibroblasts for later proliferation in response to progesterone.





is the primary  $E_2$ -induced paracrine factor, which in turn causes the delivery of IGF1 via the endocrine route.

The  $E_2$  activation of PI3K/AKT led to phosphorylation of the downstream effectors FRAP1, GSK3B, and MDM2 in LE cells. This indicates that they could be involved in the uterotrophic effects of  $E_2$ . All three have been linked to  $E_2$ -induced cell proliferation [19, 50, 57, 58]. They have also been linked to HIF1A activation and/or *Vegfa* expression in other systems [59–67]. We did not find any linkage, however, between FRAP1 activation and *Vegfa* expression. There are other reports of increased HIF1A activity and/or *Vegfa* induction independent of FRAP1 [68, 69]. FRAP1 may be involved in other later  $E_2$  effects in the uterus, as has been shown for GSK3B in  $E_2$ -induced cell proliferation [19]. Indeed, phosphorylation and total expression of FRAP1 and GSK3B in LE cells were highest after 4 h of  $E_2$  treatment, when  $E_2$ -induced *Vegfa* expression has already returned to basal levels. Additional work is needed to determine the specific roles of these downstream effectors in mediating the effects of  $E_2$  in the uterus. Although GSK3B can be inhibited by LiCl, the design of a study on its role in  $E_2$  action is not straightforward because its activity is inhibited by  $E_2$  itself [50]. MDM2 can be inhibited by Nutlin3 [65], but this compound has not yet been shown to be effective in vivo.

PI3K/AKT could also activate HIF1A independent of any of these factors. First, AKT can be translocated to the nucleus, where it could directly phosphorylate the coactivator EP300 (E1A-binding protein p300 [commonly known as just p300]) [70], which is a key regulator of HIF1A activity [71–76]. A recent study [77] found that  $E_2$  induces nuclear localization of activated AKT in myocardial cells, and in preliminary studies we have confirmed that this is also true for LE cells (Kazi and Koos, unpublished results), although we have not yet connected this to phosphorylation of EP300. Another recent study [78] found that PI3K/AKT stimulation of HIF1A transcriptional activity on the promoter for the gene for glucose transporter, type 1 (which, like *Vegfa*, is induced by  $E_2$  in the endometrium) involved dissociation of the transcriptional inhibitor FOXO3 from a complex with HIF1A and EP300 and its translocation to the cytoplasm.

We have now linked activation of PI3K/AKT to  $E_2$ -induced *Vegfa* expression using both the whole uterus and isolated LE cells. PI3K activation of AKT occurs at the plasma membrane. Thus, a membrane  $E_2$  receptor is likely involved, and there is now strong evidence that a subpopulation of ESR1 resides at the plasma membrane [28] and can activate PI3K [79]. Most recently, Pedram and colleagues [80] showed that the E domain of ESR1 localized to the membrane was all that was needed for  $E_2$  activation of PI3K. Numerous studies [27, 28, 40–44, 81] indicate that ICI 182,780 blocks both nuclear and membrane forms of ESR1, including ESR1 targeted specifically to the plasma membrane [82]. Effects of ICI 182,780 specifically on PI3K/AKT activation by  $E_2$ , however, vary among studies. In some cases, ICI 182,780 did not block  $E_2$ -stimulated AKT activation [30–33]. On the other hand, it did inhibit  $E_2$ -induced AKT phosphorylation in endothelial cells [25, 26, 28], MCF-7 cells [28], cerebral blood vessels [27], and rat uterus in vivo [29]. Our finding that it inhibits  $E_2$ -induced activation of PI3K/AKT and HIF1A in the uterus provides further evidence that those effects are mediated by a form of ESR1 localized at the cell membrane. Finally, the phytoestrogen diosgenin has been reported to induce *Vegfa* expression in osteoblasts via the PI3K/AKT pathway and HIF1A [83], and, as herein, these effects were completely blocked by ICI 182,780. The lack of effect of ICI 182,780 in some studies may reflect differences in methods such as time of treatment. In one study [84], for

example, ICI 182,780 inhibited  $E_2$ -induced activation of AKT at 30 min but not at 3 min.

As proposed previously [4, 9],  $E_2$  induction of *Vegfa* expression appears to involve both membrane and nuclear forms of ESR1, with the former mediating activation of the PI3K/AKT signaling pathway and the latter being recruited to proximal SP1 sites on the *Vegfa* promoter. Because the amount of material obtained from LE cells was limited, we were unable to repeat the ChIP studies of ESR1 recruitment carried out previously in the whole uterus. As we clearly show, however, LE cells contain abundant ESR1 (Figs. 2 and 4); in other studies, we have found that there is a marked translocation of ESR1 from the cytoplasm to the nuclei of LE cells after  $E_2$  treatment (Kazi et al., unpublished results). We are examining the relationships between PI3K/AKT activation, HIF1 binding, and nuclear ESR1 recruitment to the *Vegfa* promoter, events that appear to be closely linked [9] (Kazi et al., unpublished results). We have not yet fully examined possible roles of the  $\beta$  form of  $E_2$  receptor (ESR2) in any of the effects of  $E_2$  or ICI 182,780 on *Vegfa* expression or related signaling events. Studies [85–87] in ESR1- and ESR2-null animals or using ESR1- or ESR2-specific ligands, however, have clearly shown that ESR1 has the major role in the effects of  $E_2$  on the endometrium, although ESR2 may modulate its activity [88].

In conclusion, linking the PI3K/AKT pathway and HIF1 to  $E_2$  induction of *Vegfa* in LE cells advances our understanding of the molecular mechanisms that underlie the effects of  $E_2$  in the uterus and probably other tissues as well. It also suggests novel targets and strategies for the treatment of uterine disorders that are exacerbated by  $E_2$ . Excessive HIF1 and VEGFA are associated with both endometriosis [89–91] and endometrial cancer [92, 93], which is also strongly linked to high PI3K/AKT activity [94].

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