Hypoxia is not required for human endometrial breakdown or repair in a xenograft model of menstruation

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ABSTRACT **Menstrual endometrial breakdown induced by estradiol and progesterone withdrawal is regularly attributed to vasospasm of spiral arteries causing ischemia and hypoxia. We investigated whether hypoxia actually occurred in an *in vivo* model of menstruation. Three complementary approaches were used to look for signs of hypoxia in fragments of human functionalis xenografted to ovariectomized immunodeficient mice bearing pellets-releasing estradiol and progesterone, and then deprived of ovarian steroids. Hormone withdrawal 21 d after grafting induced menstrual breakdown and MMP expression within 4 d. Local partial oxygen pressure (pO_2) was measured by electron paramagnetic resonance using implanted lithium phtalocyanine crystals. In mice with hormone maintenance until sacrifice, pO_2 was low one week after grafting (14.8±3.4 mmHg) but increased twofold from the second week when tissue was largely revascularized. After 3 wk, pO_2 was not modified by hormone withdrawal but was slightly increased on hormone reimpregnation 4 d after removal (34.7±6.1 mmHg) by comparison with hormone maintenance (27.1±8.6 mmHg). These results were confirmed using fluorescence quenching-based OxyLite measurements. In a further search for signs of hypoxia, we did not find significant HIF1-α immunostaining, nor pimonidazole adducts after hormone withdrawal. We conclude that hypoxia is not needed to trigger menstrual-like tissue breakdown or repair in human endometrial xenograft.—Coudyzer, P., Lemoine, P., Jordan, B. F., Gallez, B., Galant, C., Nisolle, M., Courtoy, P. J., Henriet, P., and Marbaix, E.**

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Key Words: mouse model · estradiol and progesterone · matrix metalloproteinases · hypoxia-induced factor 1-α · pimonidazole

THE HUMAN ENDOMETRIUM is a dynamic tissue that undergoes cyclic degradation and regeneration throughout the reproductive life of women. Under the influence of ovarian steroids, the endometrium proliferates and then differentiates to allow blastocyst implantation. If no pregnancy occurs, the fall of progesterone plasma concentration due to corpus luteum involution triggers menstrual endometrium remodeling, leading to shedding of its functional layer associated with bleeding. Besides production and activation of high matrix metalloproteinase (MMP) levels, responsible for the degradation of the endometrial extracellular matrix (reviewed in ref. 1), several other processes are induced in the endometrium in response to progesterone withdrawal and contribute to the spatiotemporal regulation of tissue degradation and/or regeneration (reviewed in refs. 2, 3). These responses include up-regulation of inflammatory mediators [such as interleukin 8 (IL-8) and cyclooxygenase 2 (COX2)] (4) and leukocyte invasion; production of cytokines (IL-1α) (5) and growth factors [transforming growth factor-β (TGF-β)] (6) supporting paracrine interactions between epithelial and stromal cells; and reported vasoconstriction of spiral arterioles, thought to induce hypoxia and followed by up-regulation of vascular endothelial growth

Abbreviations: COX2, cyclooxygenase 2; E, estradiol; EPR, electron paramagnetic resonance; HE, hematoxylin and eosin; HIF1-α, hypoxia inducible factor 1-α; IL, interleukin; LiPc, lithium phtalocyanine; MMP, matrix metalloproteinase; P, progesterone; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; pO_2, partial oxygen pressure; PTGES, prostaglandin E synthase; SCID, severe combined immunodeficiency; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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factor A (VEGFA) and vascular endothelial growth factor receptor 2 (VEGFR2), and angiogenesis (7).

However, the timing and roles of hypoxia in the endometrium are uncertain. In autotransplantation of endometrium into the anterior chamber of the eye of macaques, vasoconstriction of spiral arteries preceded the onset of menstruation (8). Vasoconstriction is thought to be induced by prostaglandins such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) following progesterone withdrawal (9). Episodes of hypoxia might, therefore, occur and participate in triggering menstruation. However, direct measurement of erythrocyte flow by laser-Doppler fluxmetry could not evidence such episodes of endometrial ischemia or ischemia/reperfusion around menstruation (10). Furthermore, immunolabeling of hypoxia-induced factor 1-α (HIF1-α) was reported to be scarce and focal in the cycling endometrium and was intriguingly not confined to the perimenstrual phase (11). Moreover, expression of several MMPs by endometrial stromal cells (11) or explants (12) was decreased under hypoxic conditions.

Alternatively, hypoxia was also suggested to be involved in the transition between tissue degradation and postmenstrual regeneration (13). The current view holds that the early repair starts from the basalis after complete lysis of the functionalis. However, concomitant expression of genes involved in tissue degradation and repair within foci of the superficial layer of menstrual endometria has recently been demonstrated (14). Moreover, signs of regeneration are frequently observed during menstruation (15). Thus, tissue repair could be induced at the same time as tissue degradation and in response to the same global signal.

Despite its recognized importance, menstruation is difficult to appropriately reproduce in animal models and was, therefore, poorly investigated. Indeed, menstruation is normally restricted to human and to a limited number of animal species (*e.g.*, apes, old world monkeys, bats). Moreover, cultures of human endometrial cells or explants lack bloodstream and potential inflammatory cells influx, which seem decisive to induce menstruation. A xenograft model of human menstruation in mice was, therefore, developed to obviate these limitations. Human endometrial explants grafted subcutaneously in immunodeficient mice were shown to reconstitute vascularization (16, 17). In these models, estradiol (E) and progesterone (P) impregnation induced a decidualized stroma and their removal triggered menstrual-like tissue breakdown (see **Figs. 2** and **3**).

In the present study, we used experimental approaches to search for hypoxia in xenografts subjected to continuous EP impregnation, as compared to menstrual remodeling triggered by EP withdrawal. We also looked for hypoxia in xenografts, in which tissue breakdown was arrested and stromal decidualization was reactivated by reimplanting hormonal pellets after menstruation. In this xenograft model, hypoxia is not required to trigger menstrual human endometrium degradation and/or regeneration.

MATERIALS AND METHODS

Animals

Ovariectomized female CB17 mice with severe combined immunodeficiency (SCID) (n=78) were purchased from Charles River Laboratories (L'Arbresle, France). Mice were housed under a barrier husbandry in a pathogen-free environment, fed *ad libitum* with autoclaved laboratory food and water and maintained under a 12:12-h light-dark cycle. All procedures of the animal study were approved by the Ethical Committee for animal welfare at the Université Catholique de Louvain.

Human endometrium collection

Endometrium was collected from 17 hysterectomy specimens of spontaneously cycling women (34 to 57 yr old) operated for benign lesions, such as uterine myomas or adenomyosis. A sample of full-thickness endometrium was fixed in formalin and embedded in paraffin for histological analysis, allowing to confirm the phase of the menstrual cycle (menstrual n=3, proliferative n=7, and secretory n=7), and the absence of endometrial abnormalities. All women gave informed consent and the Ethical Committee of the Université Catholique de Louvain approved the study.

Xenografting of endometrial fragments

The endometrium functionalis was scraped with a sterile surgical blade and cut in fragments of 1–2 mm per side in ice-cold PBS. Mice were subcutaneously grafted with 30 endometrial fragments in each flank. Using a large global volume of tissue ensured that all endometrial cell types were represented in xenografts, *i.e.*, glands surrounded by a stroma containing blood vessels. Pellets of 0.05 or 0.1 mg 17β-estradiol (E) and of 25 mg progesterone (P) (60- or 90-d release; Innovative Research of America, Sarasota, FL, USA) were simultaneously inserted in the neck of the mice and left for 21 d to allow tissue reorganization and differentiation. Since histological appearance of the grafts was similar with both E concentrations (0.05 or 0.1 mg) and both release times (60 or 90 d), data were combined for reporting. After 21 d, EP pellets were removed to induce menstruation or maintained in controls, and mice were sacrificed within 8 d. Four days after pellet removal, EP pellets were inserted again into some mice. The number of mice in each experimental group is indicated in Fig. 1. Pimonidazole analysis (see below) was performed on 28 mice. Xenografts and uterus were collected and either fixed in 10% buffered formalin and embedded in paraffin for histological analysis, embedded in Tissue-Tek OCT compound tissue freezing medium (Sakura, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen-cooled isopentane for frozen sections or frozen at −80°C for RNA extraction. All surgical procedures were conducted under intraperitoneal anesthesia with 12.5 mg/kg of xylazine (Rompun 2%, Bayer HealthCare, Leverkusen, Germany) and 125 mg/kg of ketamin (Ketalar 50 mg/ml; Pfizer, Brussels, Belgium) or under isoflurane (Isoba, isoflurane 100%; Schering-Plough Animal Health, Summit, NJ, USA) anesthesia (3% for induction and 2% for maintenance).

Histological and immunohistochemical analysis

Paraffin-embedded tissue sections (7 μm thick) were prepared from collected grafts and stained with hematoxylin and eosin (HE) or immunolabeled with rabbit anti-human colla-

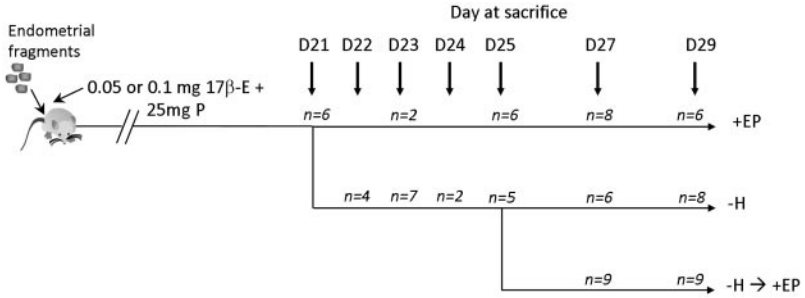

Figure 1. Experimental protocol. Number of sacrificed mice is indicated at each time point.

gen III (PS049, 1:50; Monosan, Sanbio, Uden, The Netherlands) or mouse anti-human HIF1-α (NB100-105, 1:200; Novus Biologicals, Littleton, CO, USA) antibodies, as described previously (18). Before collagen III immunostaining, sections were incubated with Pronase (Pronase E from *Streptomyces griseus*, no. 107433, 1:2,000; Merck, Darmstadt, Germany) at 37°C for 30 min. For HIF1-α immunolabeling, antigen retrieval was achieved by heating sections at 100°C in citrate buffer, pH 5.8, for 75 min.

Electron paramagnetic resonance (EPR) oximetry

EPR oximetry enables sensitive, noninvasive and repeated measurements of partial oxygen pressure (pO_2) *in vivo* and proved particularly useful to characterize the tumor environment (19). Small lithium phtalocyanine (LiPc) crystals were used as oxygen reporter probes and were gently inserted, at the time of grafting, into 6 of the 30 endometrial fragments grafted in one flank. The EPR spectra were recorded using an EPR spectrometer (Magnetech, Berlin, Germany) with a low-frequency microwave bridge operating at 1.2 GHz and an extended loop resonator. The EPR line widths (ΔB) were converted to pO_2 by means of a calibration curve determined for LiPc crystals specifically used in this study, with equation: pO_2 (mmHg) = $[\Delta B\ (\mu T) - 3.1]/0.5$.

Oxygen pressure in the xenografts was followed weekly for 3 wk, then daily for up to 8 additional days.

Alternative pO_2 measurement by OxyLite

To confirm the tissue pO_2 measurements obtained by EPR, we used the OxyLite 2000 system (Oxford Optronix, Oxford, UK) in 8 mice. The system consists of a ruthenium probe embedded in silicone at the tip of a 230-μm diameter optic fiber. Oximetry is based on the principle that the lifetime of the fluorescent pulse is inversely proportional to pO_2. OxyLite probe was calibrated by the manufacturer prior to delivery and was inserted with a needle. Results were read for 10 min after signal stabilization. Because insertion of the probe inside the xenograft was likely to alter the tissue, OxyLite measurements were performed only once in a xenograft, immediately before sacrifice of the mouse.

Histological detection of hypoxia by pimonidazole adducts

Mice were intravenously injected with 60 mg/kg pimonidazole (Hypoxyprobe-1 Omni kit, Hypoxyprobe, Burlington, MA, USA), 90 min prior to sacrifice. Pimonidazole forms covalent adducts with various substrates when pO_2 falls <10 mmHg. Frozen sections (7 μm thick) were incubated 40 min with anti-pimonidazole antibody (pAb 2627 rabbit serum, 1:400, Hypoxyprobe, Burlington, MA, USA) and revealed with peroxidase-conjugated dextran molecules carrying anti-rabbit secondary antibodies (Envision, DakoCytomation, Glostrup, Denmark) followed by incubation with H_2O_2 and aminoethyl carbazole as chromogen substrate to stain immune deposits in red and allow easy distinction from brown hemosiderin.

Real-time RT-PCR

After total RNA extraction from xenografts (SV total RNA isolation system; Promega, Madison, WI, USA) and quantification by spectrometry at 260 nm, aliquots of 200 ng RNA were reverse-transcribed using the oligo(dT) protocol of cloned avian myeloma virus RT-PCR system (Invitrogen, Merelbeke, Belgium). Real-time qPCR was performed for β-actin, MMP1, MMP3, TIMP1, TIMP3, VEGFA, VEGFR2, and prostaglandin E synthase (PTGES) on a CFX96 touch real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the Kapa SYBR fast qPCR kit (KapaBiosystems, Woburn, MA, USA). Customized primers **(Table 1**) were obtained from Eurogentec (Seraing, Belgium). After initial denaturation at 95°C for 2 min, the cDNA was amplified by 40 cycles of 3 s at 95°C and 30 s at 60°C. The fluorescence signal was measured after each cycle. A control reaction without cDNA addition was included in each PCR assay, and all PCR were performed in independent duplicates. β-actin mRNA values were used for normalization.

TABLE 1. *Primers used for real-time qPCR*

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-*Actin*	AGCCTCGCCTTTGCCGA	TCACGCCCTGGTGCCTG
MMP1	GCAAACACATCTGACCTACAGG	TCTCAATGGCATGGTCCAC
MMP3	TGTATCTTGCCGGTCATTT	TGTGACAAGGTGCAAGCT
PTGES	TGGGAGGGGCCCACAGGAAG	TCCCGGTCCTCCACCCACTG
TIMP1	GGCTTCACCAAGACCTACA	TTGCAGGGGTGGATAAA
TIMP3	GGTGGGGAAGAAGCTGGT	TTGGTGAAGCCTCGGTAC
VEGFA	TCCACATGCTGCACGCGCAT	TGCCAGTGGTCTCCTGGGCA
VEGFR2	TGGCCAAGCCCCAGGAAGGA	CGGCAGGGAGCCTTGAGCTG

Statistical analysis

For qPCR comparisons, statistical significance was estimated using the two-tailed Wilcoxon two-sample test. For EPR comparisons, Kruskal-Wallis ANOVA and Wilcoxon 2-sample test for *post hoc* testing were used. Differences in immunodetection of pimonidazole adducts were analyzed with the χ^2 test. Differences were considered significant at $P < 0.05$.

RESULTS

Twenty-one days after grafting of human endometrial fragments and implantation of estradiol and progesterone (EP)-delivering pellets, mice were subjected to three different hormonal programs: *1)* pellet maintenance or *2)* pellet removal followed by 1 to 8 additional days; or *3)* pellet removal for 4 d followed by implantation of new pellets for 2 to 4 additional days (Fig. 1). There was no difference between the 17 grafted endometria, according to the phase of menstrual cycle at sampling.

Histology of human endometrium xenografts

In mice kept under EP stimulation for up to 4 wk, xenografts showed large endometrial glands surrounded by a decidualized stroma (Fig. 2*A*, *C*) containing abundant collagen III fibers (Fig. 2*F*, *H*). In contrast, menstrual-like foci of stromal breakdown appeared within 2 d after hormone pellet removal (see asterisk in Fig. 2*B*), extended during the following days and were still present after 6 d, together with focal hemorrhages (see arrowheads at Fig. 2*D*). These foci showed extensive decrease of collagen III fibers at 2 to 6 d after EP withdrawal (Fig. 2*G*, *I*).

When mice had been reimplanted with hormone pellets at 4 d after removal and studied after 2 more days, menstrual-like foci vanished and stroma surrounding endometrial glands progressively resumed decidualization (Fig. 2*E*), together with collagen III reappearance or increased immunolabeling (Fig. 2*J*). Since results were indistinguishable between 23 and 25 d and between 27 and 29 d, only one time point is represented.

Effect of ovarian steroids on expression of MMPs and TIMPs in xenografts

In agreement with our previous report documenting physiological menstruation (20), mRNA levels of MMP1 and MMP3 were strongly increased (between 40- and 1000-fold) after hormone withdrawal for 4 and 6 d (**Fig. 3*A*, *B***) but decreased within 2 d after pellet reimplantation (Fig. 3*B*). In contrast, TIMP3 mRNA level was highest when hormones were present then was decreased (between 4- and 16-fold) 2, 4, and 6 d after hormone withdrawal (Fig. 3*C*, *D*) but increased 2 d after pellet reimplantation (Fig. 3*D*). TIMP1 mRNA level was slightly increased 2 d after hormone withdrawal (Fig. 3*C*) and then did not change (Fig. 3*C*, *D*).

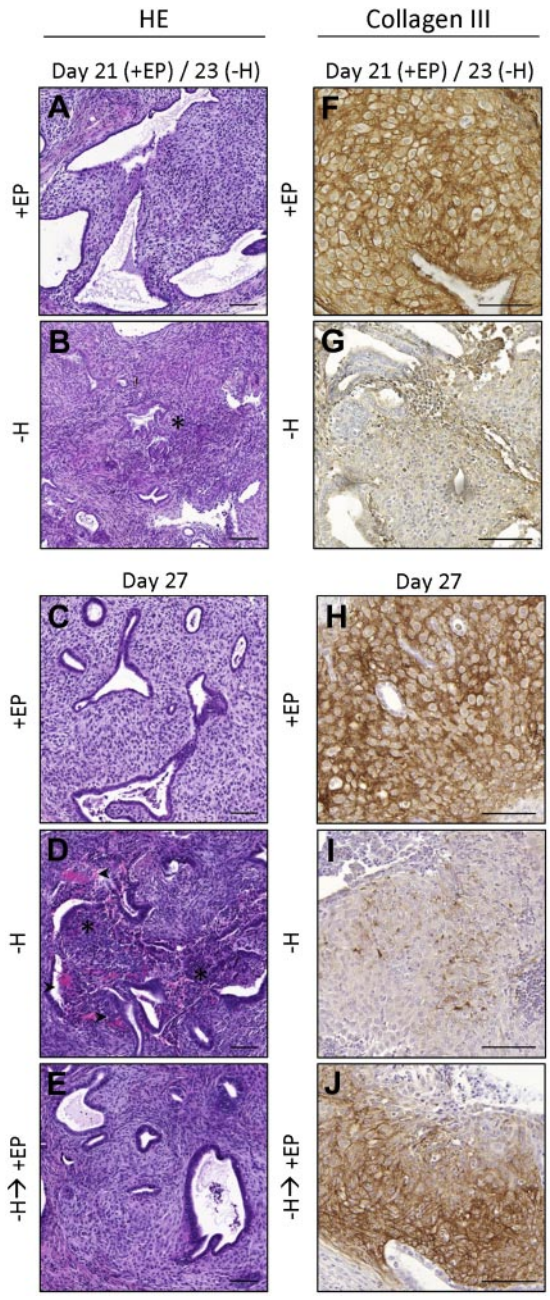

Figure 2. Histology of human endometrial xenografts. Representative micrographs after hematoxylin and eosin (HE) staining (left panels), or collagen III immunostaining (right panels). Mice were sacrificed at 21 d (*A*, *F*) or 27 d (*C*, *H*) after estradiol and progesterone (EP) pellet insertion; at 2 d (*B*, *G*) or 6 d (*D*, *I*) after pellet removal; or at 2 d (*E*, *J*) after pellet reinsertion. Asterisks indicate foci of extracellular matrix breakdown. Arrowheads indicate hemorrhages. Scale bars = 100 μm.

Differences of mRNA levels attenuated or disappeared at d 29 (Fig. 3*B*, *D*).

Monitoring of pO_2 values

As a first experimental approach to test for hypoxia, we measured pO_2 values by EPR in anesthetized mice that had been previously grafted with endometrial frag-

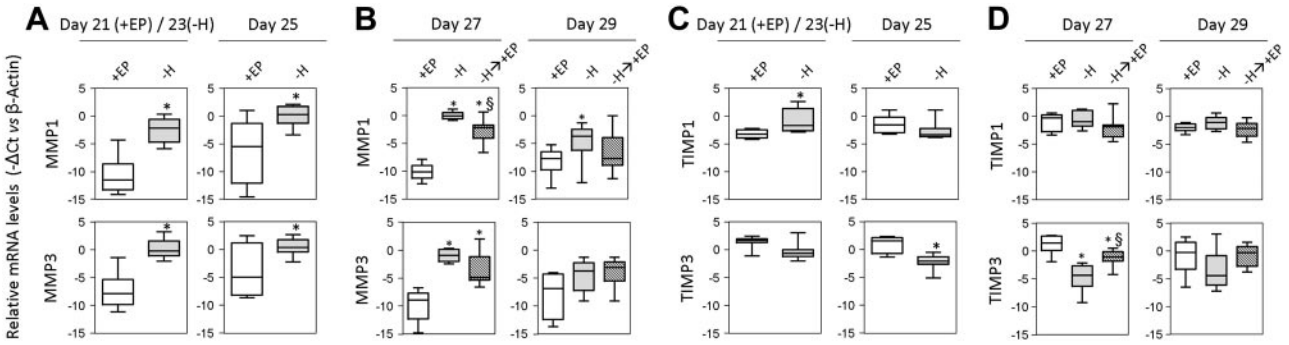

Figure 3. Response of human MMP and TIMP mRNA levels in xenografts to EP withdrawal or reexposure. mRNA levels of MMP1, MMP3 (*A*, *B*), and TIMP1, TIMP3 (*C*, *D*), were measured by real-time quantitative PCR in xenografted tissue from mice treated with pellets (white boxes), after pellet removal (gray boxes) or after pellet reimplantation (hatched boxes). PCR data were normalized to β-actin mRNA and are presented as box plots showing 25th and 75th percentiles and medians with whiskers showing minimal and maximal values. Number of mice for each condition is shown in Fig. 1. $^{*}P < 0.05$ *vs.* +EP. $^{\S}P < 0.05$ *vs.* –H.

ments containing LiPc crystals (**Fig. 4*A***). The technique is based on the properties of molecular oxygen, which alters the EPR spectrum of paramagnetic species, such as LiPc. LiPc crystals were, therefore, used as oxygen-sensitive probes (see inset in Fig. 4*B*).

As control for hypoxia detection, pO_2 measured in xenografts of 6 mice (2 from each group at d 27–29) dropped from 31.3±6.9 mmHg under anesthesia down to 10.8±2.4 mmHg ($P<0.002$) 5 min after sacrifice. This value was considered as full hypoxia. Out of 267 pO_2 values measured in living mice for the whole study, all but 3 were above this level (Fig. 4*A*). Overall, the values ranged between 7.4 and 45.8 mmHg and varied from day to day, even in daily measures from individual mice.

In mice in which hormone pellets were maintained until sacrifice, pO_2 in grafts was lower 7 d after grafting (mean ± SD, 14.8±3.4 mmHg) than at any other time point tested (23.7±6.7 mmHg by pooling grafts from d 14–29), indicating that revascularization was not yet completed. Removal of hormone pellets did not significantly modify pO_2 neither during the first 4 d (23.7± 7.0 when withdrawn *vs.* 23.6±6.3 mmHg when maintained) nor during the next 4 d (30.7±8.0 *vs.* 27.2±8.6 mmHg). Reinsertion of new EP-delivering pellets after 4 d of deprivation resulted in a further slight increase of pO_2 values (34.7±6.1 mmHg) that was only significant by comparison with pellet maintenance.

By histological examination, we ensured that LiPc crystals did not interfere with overall endometrial structure nor generate fibrotic areas (Fig. 4*B*). In a limited number of mice ($n=8$), pO_2 values measured on the other flank by the OxyLite method (insertion of a 230-μm diameter probe) were found to be similar to those measured by EPR (Fig. 4*C*).

Lack of detection of histological hypoxic molecular markers

To further search at higher histological resolution for local markers of hypoxia in our xenografts, we immunolabeled HIF1-α and pimonidazole adducts. The transcription factor HIF1-α is rapidly degraded in a normoxic

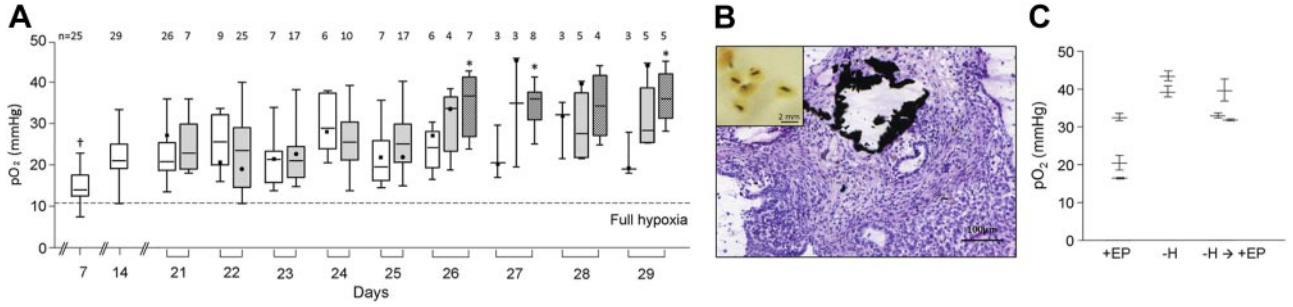

Figure 4. Monitoring of pO_2 values by EPR or OxyLite. *A)* pO_2 measurements by EPR at the indicated days in anesthetized animals with EP pellets (white boxes); after pellet withdrawal (gray boxes); and after pellet reinsertion (hatched boxes). The gray box at d 21 corresponds to pO_2 measured 1–5 h after pellet removal. Full hypoxia value (dotted line at 10.8 mmHg) was determined as the average pO_2 measured in 6 dead mice 5 min after sacrifice. pO_2 values are presented as box plots showing 25th and 75th percentiles, median as horizontal line and whiskers showing minimal and maximal values. Superimposed dots and squares indicate serial pO_2 measurements in 2 representative mice. Number of mice is indicated above each box. $^{*}P < 0.05$ *vs.* pellet maintenance at the same day. $^{\dagger}P < 0.05$ *vs.* any other group. *B)* Representative micrograph of a hematoxylin-and-eosin-stained histological section of a xenograft containing a LiPc crystal collected 3 wk after grafting. The crystal fragmented during microtome sectioning. Inset shows a low-power view of LiPc crystals inserted in endometrial fragments before grafting. Scale bars=100 μm in histological microphotograph and 2 mm in the inset. *C)* pO_2 measurements by OxyLite in animals bearing EP pellets for 29 d ($n=3$); 8 d after pellet withdrawal ($n=2$); and 4 d after pellet reinsertion ($n=3$). Each symbol represents mean value ± SD of 8 measurements over 10 min.

environment but stabilized under hypoxia (21). HIF1-α detection was validated in histological sections from endometrial explants cultured for 24 h under hypoxia (12), in which HIF1-α was strongly detected in the nucleus of epithelial and endothelial cells (**Fig. 5*B***) whereas no staining was observed in explants from the same endometrium cultured under normoxia (Fig. 5*A*). In the human endometrium xenografts, exceptionally a few glandular cells showed nuclear staining (arrowheads on Fig. 5*D*), and this rare finding showed no relation with estradiol and progesterone impregnation (Fig. 5*C–L*).

Alternatively, we exploited pimonidazole, which forms immunodetectable adducts in cells under pO_2 <10 mmHg (22). Pimonidazole detection was validated in frozen sections from endometrial explants cultured for 2 h under hypoxia (3% O_2), in which pimonidazole adducts were identified in the stromal cells (Fig. 5*N*) in contrast to explants from the same endometrium cultured under normoxia (21% O_2) (Fig. 5*M*). No such adducts were immunodetected in xenografts subjected to continuous EP impregnation (Fig. 5*O, Q, U, X*). There was no significant difference in adduct formation after hormone withdrawal (Fig. 5*P, R, V, Y*) since most xenografts did not show any staining except for 3 out of 16 mice (mice sacrificed at d 22–24). In these animals, adducts (Fig. 5*S–T*) were detected in small foci close to menstrual-like areas or in the lumen of endometrial glands. Foci deprived of NADH dehydrogenase activity, indicating necrosis, were found in ~10% of 60 tested grafted mice (Supplemental Fig. S1) and, interestingly, were localized in close vicinity of adducts in one of the 3 mice showing pimonidazole adducts.

Quantification of VEGFA, VEGFR2, and PTGES mRNA in xenografts

Angiogenesis is essential for tissue growth or regeneration, as well as wound healing, and is driven by VEGF, itself induced by hypoxia (23). To test whether a putative hypoxia would also modify VEGF expression in the xenografts, we measured the expression of VEGFA and VEGFR2 by quantitative RT-PCR. The mRNA levels of both targets did not

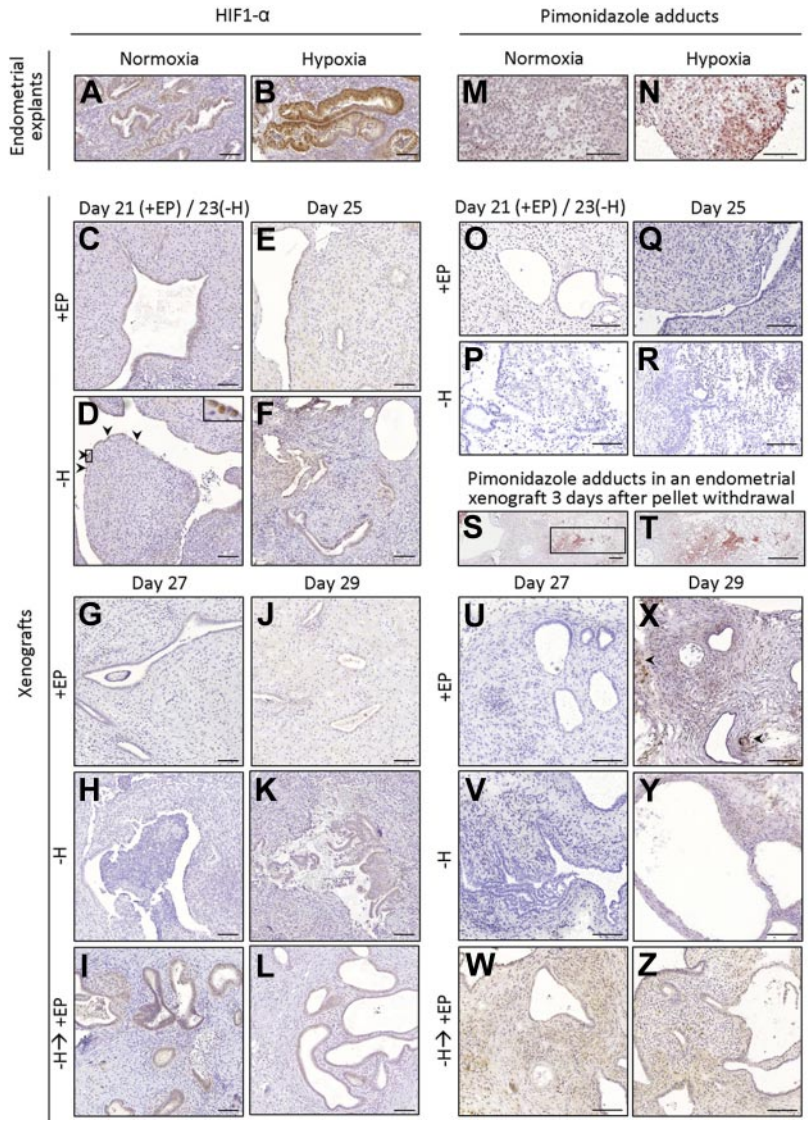

Figure 5. Histological testing of hypoxia in xenografts. Representative micrographs of HIF1-α immunostaining (*A–L*) and pimonidazole adducts detection (*M–Z*). Mice were sacrificed at 21 d (*C, O*), 25 (*E, Q*), 27 (*G, U*) or 29 d (*J, X*) after EP pellet insertion; at 2 d (*D, P*), 4 d (*F, R*), 6 d (*H, V*), or 8 d (*K, Y*) after pellet removal; or at 2 d (I, W) or 4 d (L, Z) after pellet reinsertion. HIF1-α immunostaining was validated using sections from endometrial explants cultured for 24 h under normoxic (*A*) or hypoxic (*B*) conditions. Rare epithelial cells showing HIF1-α immunostaining are indicated by arrowheads in *D* and shown at higher magnification in the inset. Pimonidazole adducts immunostaining was validated using sections from endometrial explants cultured for 2 h under normoxic (*M*) or hypoxic (*N*) conditions, with 200 μM pimonidazole added to culture medium. Rare foci in 3 xenografted mice showed pimonidazole adducts (*S*, enlarged in *T*). Brown signal (arrowheads on *X*) indicates hemosiderin deposit. Scale bars=100 μm.

change after removal of EP pellets but transiently decreased almost 4-fold on reexposure to EP for 2 d.

Prostaglandins are thought to be involved in triggering menstruation (24), and PGE2 was shown to participate in HIF1-α stabilization in human endometrium (13). Therefore, we also looked for the expression level of PTGES. PTGES mRNA level transiently increased by 10-fold in endometrial xenografts at 6 d after EP withdrawal (**Fig. 6**).

DISCUSSION

In this study, we used an *in vivo* model of human endometrium xenografts into SCID mice to determine whether hypoxia is required for the induction of menstrual-like endometrium degradation after EP withdrawal and/or for its regeneration when resuming hormone treatment. Although ovarian steroid withdrawal obviously induces menstruation, the detailed mechanisms leading to extracellular matrix breakdown and bleeding are still insufficiently documented, in particular, the long-lasting hypothesis of vasospasm-induced hypoxia proposed more than 70 yr ago (8). Using 3 different approaches, *i.e.*, direct measurement of local pO_2 with millimetric crystal probes and EPR; formation of hypoxia-induced adducts by the pimonidazole assay; and hypoxia-induced HIF1-α stabilization, we found no consistent evidence for hypoxia under conditions where EP removal induced menstrual remodeling and local hemorrhages.

We selected the xenograft model because our time course study demonstrated that functional chimeric

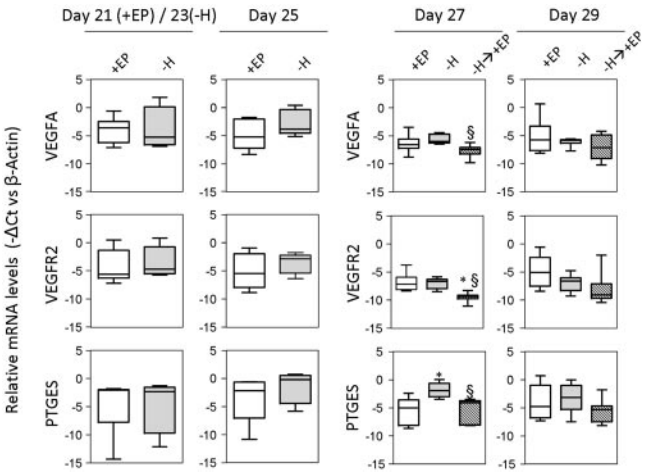

Figure 6. Response of human VEGFA, VEGFR2, and PTGES mRNA to EP withdrawal or reexposure in xenografts. mRNA levels of VEGFA, VEGFR2, and PTGES were measured by real-time PCR in xenografted tissue from mice bearing EP pellets (white boxes), after pellet removal (gray boxes) or after pellet reimplantation (hatched boxes). PCR data were normalized to β-actin mRNA and are presented as box plots showing 25th and 75th percentiles and medians with whiskers showing minimal and maximal values. Number of mice at each time point is shown in Fig. 1. $^{*}P < 0.05$ *vs.* +EP. $^{\S}P < 0.05$ *vs.* –H.

vasculature, connecting human and SCID mouse vessels, was settled after 3 wk (16). Other investigations have previously evaluated angiogenesis in endometrial tissue transplanted into nude mice (25) and demonstrated that revascularization occurred between d 5 and 8 after implantation (26). In the present study, the chimeric vasculature delivered appropriate oxygen supply throughout the grafts, as demonstrated by high pO_2 values in grafts under EP, from 2 wk after grafting, whereas lower pO_2 values measured at 1 wk after grafting indicated that vascularization was not yet effective at that time. Admittedly, xenografts were fragments of endometrial functionalis in absence of basalis, therefore, devoid of spiral arterioles. Absence of hypoxia in grafts could, thus, result from differences in vessel architecture (absence of spiral arteries) and, consequently, lack of vasoconstriction induced on EP withdrawal. Nevertheless, menstrual-like tissue breakdown clearly occurred in grafts despite absence of spiral arterioles. It is also well known that atrophic endometrium on progesterone treatment may bleed, although it is devoid of spiral arterioles (27). Similarly, endometriotic lesions may show menstrual-like breakdown and bleeding, although lacking spiral arterioles (28).

The chimeric vasculature also ensured hormone delivery within the grafts, as illustrated by the various responses to EP addition or withdrawal, mimicking key features of the physiological menstrual cycle. In particular, stromal cells underwent decidualization on prolonged EP exposure; however, tissue shrinkage, degradation of collagen fibers, and expression of MMPs were induced when hormones were withdrawn. Infiltration by blood leukocytes of the areas of stromal breakdown has also been observed after steroid removal in similar models (29, 30). In our xenografts, steroid withdrawal did not significantly reduce the pO_2 values measured using either LiPc crystals combined with EPR oximetry or OxyLite as *in situ* sensors. Only three values were slightly under the level set for full hypoxia (10 mmHg). Arguably, EPR only provides average pO_2 values in a millimetric range, but pimonidazole adducts were microscopically immunodetected in only 3 out of 16 graft samples collected after hormone withdrawal, whereas staining was totally absent in mice under sustained hormone treatment. Such adducts were barely detectable and restricted to small foci clearly below spatial resolution level of EPR, indicating that hypoxia would be very limited in space. Necrosis was also found in similar limited areas of menstrual stromal breakdown, as well as in the cellular debris found inside the lumen of reconstructed glands, likely as a consequence of the blunt configuration of endometrial glands in the subcutaneous grafts in contrast to the eutopic endometrium, where cellular debris can be expelled in the menstrual fluid.

HIF1-α is a heterodimeric nuclear transcription factor that mediates the effects of hypoxia (21). Stabilization of HIF1-α in endometrium during menstrual cycle is debated. In one study (11), very limited cytoplasmic staining was detected without correlation with the

timing along the menstrual cycle. In subsequent reports (31, 32), staining was intense in the nucleus of stromal and epithelial cells in the functional layer during the late secretory and menstrual phases. In our xenografts, HIF1-α was not detected under hormonal impregnation nor on induction of menstrual-like tissue breakdown. The lack of HIF1-α immunostaining in grafts likely indicates an absence of sustained hypoxia but cannot exclude the existence of transient hypoxic episodes.

Pimonidazole adducts are considered to be stable once formed. In our conditions, they can only form during the 90-min interval between injection and sacrifice. It is possible that transient episodes of hypoxia lasting for a few minutes allow the formation of adducts that can be detected at sacrifice. This could explain why no extensive necrosis was found at the vicinity of pimonidazole adducts. In contrast, HIF1-α is known to be rapidly stabilized on hypoxia but to rapidly disappear on return to normoxia. This could be responsible for the lack of detection of HIF1-α if hypoxic episodes are only transient. However, transient hypoxia likely implies ischemic episodes induced by arteriolar vasospasm, a mechanism that cannot be produced by blood capillaries, which are the only functional vessels found in the xenografts.

VEGF is a key factor in physiological and pathological angiogenesis. Increased VEGF expression has been shown in menstrual human endometrium (33) and could participate in early vascular repair (34). The effect of VEGF on angiogenesis during repair was demonstrated by using VEGF-Trap, a VEGF blocker, in Rhesus monkey and murine models (7). Indeed, complete inhibition by VEGF trap of neovascularization during endometrial repair suggests that VEGF is essential. Because hypoxia has been shown to stimulate VEGF mRNA expression in decidualized stromal cells (35), we also evaluated expression of VEGFA and VEGFR2 in xenografts. EP impregnation did not change the mRNA levels of both targets, in good agreement with previous reports, indicating that VEGF secretion by human endometrial stromal and epithelial cells increases in response to hypoxia but is unresponsive to estradiol and progesterone (36, 37). However, absence of increased mRNA levels on hormone withdrawal does not support the occurrence of hypoxia in xenografts.

Recently, it has been proposed that hypoxic events in the perimenstrual human endometrium could trigger expression of tissue repair mediators, such as IL-8 (4), *i.e.*, that hypoxia would rather play a role during postmenstrual regeneration. In our model, reintroduction of ovarian steroids after induction of menstruation led to the loss of stromal menstrual-like foci and to progressive differentiation of stromal cells that mimics tissue repair. However, we found no sign of hypoxia in xenografts on hormone reinsertion. Since a recent study highlighted the importance of ischemia/reperfusion in recruiting bone marrow-derived stem cells to endometrium (38), the potential to reconstitute endometrium without significant decrease in volume after several rounds of consecutive hormonal withdrawal and replacement remains to be investigated in this xenograft model.

In summary, although our results show that removal of hormone pellets induces MMP expression and tissue breakdown, hypoxia was not detected by EPR and minimal biological signs for transient episodes of hypoxia were rarely detected in a small proportion of xenografts, indicating that this event is not required to trigger menstruation or for tissue repair at least in the model of xenografted human endometrium. In this regard, designing drugs against pathologies related to menstruation (such as dysfunctional uterine bleeding or development of endometriosis) based on hypoxic pathways would prove useless. FJ

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