

# Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation

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Many underlying causes of human infertility have been overcome by using *in vitro* fertilization (IVF) and embryo transfer (ET) techniques. Nevertheless, implantation rates in IVF programs remain low despite the transfer of apparently healthy embryos. This suggests that there are problems with the differentiation of the uterus to the receptive state in response to the ovarian hormones estrogen and progesterone. The molecular basis of this receptive state when the uterine environment is conducive to blastocyst acceptance and implantation remains poorly understood. Normally, the “window” of uterine receptivity lasts for a limited time. Using ETs and the progesterone-treated delayed-implantation model in mice, we demonstrate here that levels of estrogen within a very narrow range determine the duration of the window of uterine receptivity. Although estrogen at different physiological concentrations can initiate implantation, we find that the window of uterine receptivity remains open for an extended period at lower estrogen levels but rapidly closes at higher levels. The uterine refractoriness that follows the receptive state at high estrogen levels is accompanied by aberrant uterine expression of implantation-related genes. These results suggest that careful regulation of estrogen levels is one of the important factors for improvement of female fertility in IVF/ET programs.

Synchronized development of the embryo to the blastocyst stage, differentiation of the uterus to the receptive state, and cross-talk between the blastocyst and uterine luminal epithelium are essential to the implantation process (reviewed in refs. 1–3). In mice and rats, estrogen is essential for preparation of the progesterone (P<sub>4</sub>)-primed uterus to the receptive state when the uterine milieu becomes favorable to blastocyst acceptance and implantation (1, 4). Normally, the “window” of uterine receptivity is maintained for a limited period. In mice, the uterus becomes receptive on day 4 of pregnancy or pseudopregnancy and proceeds to the refractory state on day 5 (3). However, the mechanism by which estrogen prepares the P<sub>4</sub>-primed uterus to the receptive state is not clearly defined yet. It is also unknown how the uterus, after achieving the receptive state for a limited period, proceeds to the refractory state. Various factors including cytokines, growth factors, homeobox transcription factors, and cyclooxygenase (COX)-derived prostaglandins participate in these processes through autocrine, paracrine, and/or juxtacrine mechanisms (reviewed in refs. 3, 5, and 6). For example, the gene encoding leukemia inhibitory factor (LIF) is expressed in the mouse uterine glands in response to estrogen stimulation (7, 8), whereas P<sub>4</sub> regulates the gene for amphiregulin in the epithelium and Hoxa-10 in the stroma (9, 10). Furthermore, LIF and Hoxa-10 are considered to be critical to uterine preparation for implantation and decidualization, respectively (11, 12). The genes encoding HB-EGF, COX-2, and LIF are also considered critical to implantation, because these genes are expressed in the uterus at the site of blastocyst apposition before and during the attachment reaction (3). However, their interactions with respect to estrogen and uterine receptivity are not clearly understood.

We hypothesized that a critical level of estrogen is crucial in regulating the window of uterine receptivity for implantation in a P<sub>4</sub>-primed uterus by altering gene expression. We provide evidence from physiological and molecular gene-expression studies for this idea. There is evidence from other studies that ovarian hyperstimulation leads to implantation failure and embryonic resorption in mice (13, 14). Recent evidence also suggests that “on-time” implantation is crucial to successful pregnancy establishment in both humans and mice (15, 16). Thus, uterine receptivity established by coordinated interactions between ovarian P<sub>4</sub> and estrogen is critical to successful implantation and pregnancy establishment. One prediction of our present investigation is that the reduced pregnancy rate in human *in vitro* fertilization (IVF)/embryo-transfer (ET) programs is the result of uterine refractoriness due to higher estrogen levels arising from ovarian hyperstimulation by exogenous gonadotropin administration for retrieving multiple eggs (17–19).

## Methods

**Animal Models, ET, and Treatments.** Adult CD-1 mice were purchased from Charles River Breeding Laboratories. Mice were housed in the Animal Care Facility according to National Institutes of Health and institutional guidelines for laboratory animals. Conditions of delayed implantation were induced by ovariectomizing pseudopregnant (day 1 = vaginal plug) mice on the morning of day 4 (0830–0900 hours) and maintained by daily injections of P<sub>4</sub> (2 mg per mouse) from day 5 until the mice were killed. Day-4 blastocysts recovered from normal pregnant donors were transferred into uteri of these recipients on specific days and under specific treatments as shown in the Fig. 1 schematic diagram. Implantation sites were examined 24 or 48 h after the transfers of blastocysts by the blue-dye method (20). If no implantation sites are noted, uteri were flushed to recover blastocysts, and their gross morphology was examined microscopically. Statistical analysis was performed by ANOVA followed by Fisher’s test.

**Probes and *in Situ* Hybridization.** Sense or antisense <sup>35</sup>S-labeled cRNA probes were generated by using appropriate polymerases from cDNAs to *Lif*, *Hoxa10*, *Hegf1*, *Areg*, *Ptgs1*, and *Ptgs2* for *in situ* hybridization as described (21). *In situ* hybridization was performed as described (21). Frozen sections (11 μm) were mounted onto poly-L-lysine-coated slides, fixed in cold 4% paraformaldehyde solution in PBS, acetylated, and hybridized at

Abbreviations: P<sub>4</sub>, progesterone; COX, cyclooxygenase; LIF, leukemia inhibitory factor; IVF, *in vitro* fertilization; ET, embryo transfer; E<sub>2</sub>, estradiol-17β; PR, P<sub>4</sub> nuclear receptor.

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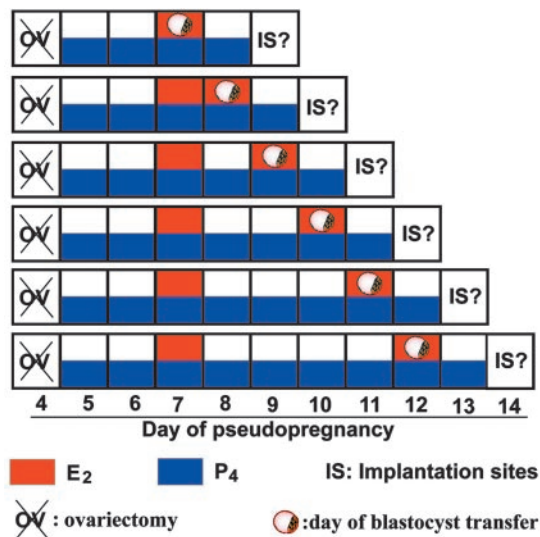


Fig. 1. Schematic outline of experimental designs.

45°C for 4 h in hybridization buffer containing the <sup>35</sup>S-labeled antisense cRNA probes. After hybridization, the sections were treated with RNase A (20 μg/ml) at 37°C for 20 min. RNase A-resistant hybrids were detected by autoradiography. Sections hybridized with the sense probes served as negative controls.

## Results

**Estrogen Modulates the Window of Uterine Receptivity for Implantation.** Uterine preparation to the receptive state by P<sub>4</sub> and estrogen is critical to implantation in mice. Our first objective was to determine the minimum dose of estrogen that is required to initiate implantation in a P<sub>4</sub>-primed uterus. Pseudopregnant recipient mice were ovariectomized on the morning of day 4 to induce the condition of delayed implantation, and this condition was maintained with daily injections of P<sub>4</sub> (2 mg per mouse) from day 5. Day-4 normal blastocysts (hereafter, blastocysts) were transferred into P<sub>4</sub>-treated recipient uterine lumens on day 7 followed immediately by injection of different doses of estradiol-17β (E<sub>2</sub>, 1.5, 3.0, 10.0, or 25.0 ng per mouse). Implantation sites were examined 48 h later by the blue-dye method. We observed that E<sub>2</sub> at 3.0, 10.0, or 25.0 ng was effective in inducing implantation. By contrast, 1.5 ng was suboptimal and ineffective in this response. These results demonstrate that E<sub>2</sub> at all doses between 3.0 and 25.0 ng are equally effective in inducing implantation in P<sub>4</sub>-primed uteri (Table 1).

We next determined the effects of different levels of estrogen on the duration of the window of receptivity. To address this, we again used P<sub>4</sub>-treated delayed recipient mice that were injected with various doses of E<sub>2</sub> (1.5, 3.0, 10.0, or 25.0 ng per mouse) on day 7. On day 8, blastocysts were transferred immediately followed by a second injection of E<sub>2</sub> at 3.0 ng, and implantation sites were examined 48 h later (see Fig. 1). We observed that most of the mice that received 1.5 or 3.0 ng of E<sub>2</sub> showed implantation after receiving the second injection of E<sub>2</sub> at 3.0 ng, whereas those that received 10.0 or 25.0 ng as the first injection had very few or no implantation sites after the second E<sub>2</sub> injection (Table 2). Furthermore, increasing the dose of the second E<sub>2</sub> injection to 10.0 or 25.0 ng did not improve implantation rates. These results demonstrate that lower doses of E<sub>2</sub> maintain the P<sub>4</sub>-primed uterus in a state such that a second estrogen exposure readily induces implantation if blastocysts are present in the uterus. In contrast, the uterus becomes refractory at higher doses of estrogen (10.0 or 25.0 ng) and does not support implantation after a second exposure to estrogen at either a low or high dose (Table 2).

These results prompted us to examine whether estrogen at a lower dose can prolong the receptive state for an extended period. P<sub>4</sub>-treated ovariectomized pseudopregnant recipients were given an injection of 3.0 ng of E<sub>2</sub> on day 7 followed by blastocyst transfers 24, 48, 72, 96, or 120 h later. Immediately after the ET, the recipients were given a second injection of 3.0 ng of E<sub>2</sub>, and implantation sites were examined 48 h later. The results show that uterine refractoriness is postponed in the majority of the mice at this low dose of 3.0 ng of E<sub>2</sub> for at least 4 days (Table 3). In contrast, when the first injection of E<sub>2</sub> was 25.0 ng the uterus became refractory within 24 h and remained refractory for the next 72 h examined (data not shown).

We next examined whether uterine refractoriness induced at higher doses of E<sub>2</sub> is reversed by increasing the dose of P<sub>4</sub>. Increasing P<sub>4</sub> to 4 mg per mouse did not rescue the implantation failure in recipients that received E<sub>2</sub> at 10.0 or 25.0 ng for the first injection (data not shown). Taken together, these results show that specific levels of estrogen determine the duration of the window of uterine receptivity for implantation in mice. In other words, uterine refractoriness occurs much faster at higher estrogen levels than at lower doses. Furthermore, higher doses of P<sub>4</sub> fail to neutralize estrogen-induced refractoriness.

**Implantation-Specific Gene Expression Is Aberrant at the Blastocyst Site After a High Dose of Estrogen.** The above results suggested that uterine refractoriness induced by higher doses of E<sub>2</sub> is due to aberrant expression of specific genes in the uterus. We therefore asked whether implantation-specific genes are expressed correctly at the implantation sites of P<sub>4</sub>-primed delayed recipients

Table 1. E<sub>2</sub> at 3 ng is the minimal dose for the induction of implantation in P<sub>4</sub>-treated delayed uteri

| Doses of E <sub>2</sub> , ng | No. of recipients | No. of blastocysts transferred | No. of recipients with ISs (%) | No. of ISs (%) | No. of blastocysts recovered from mice without ISs (%) |
|------------------------------|-------------------|--------------------------------|--------------------------------|----------------|--|
| 1.5                          | 5                 | 56                             | 2 (40)                         | 2 (2)          | 14 (40)  |
| 3.0                          | 5                 | 70                             | 5 (100)                        | 30 (43)        |  |
| 10.0                         | 5                 | 70                             | 4 (80)                         | 34 (49)        | 4 (29)   |
| 25.0                         | 5                 | 58                             | 5 (100)                        | 20 (34)        |  |

Recipient mice were ovariectomized on day 4 of pseudopregnancy (0900 hours) and injected daily with P<sub>4</sub> (2 mg per mouse) to induce the condition of delayed implantation. Day-4 normal blastocysts were transferred into uteri of these mice on day 7 at 1000 hours. The recipients received 1.5, 3.0, 10.0, or 25.0 ng of E<sub>2</sub> immediately after blastocyst transfers. Implantation sites (ISs) were examined 48 h after embryo transfer by the blue-dye method. The uteri without implantation sites were flushed to recover unimplanted blastocysts. The rate of ISs in mice treated with 1.5 ng of E<sub>2</sub> was significantly lower ( $P < 0.0001$ ) from that of mice treated with 3.0, 10.0 or 25.0 ng of E<sub>2</sub>.

**Table 2. Effects of E<sub>2</sub> on the duration of the window of uterine receptivity for implantation**

| Treatment (E <sub>2</sub> , ng per mouse)    |  | No. of recipients | No. of blastocysts transferred | No. of mice with ISs (%) | No. of ISs (%) | No. of blastocysts recovered from mice without ISs (%) |
|--|--|-------------------|--------------------------------|--------------------------|----------------|--|
| Day 7 (1st injection of E <sub>2</sub> , ng) | Day 8 (2nd injection of E <sub>2</sub> , ng) |                   |                                |                          |                |  |
| 1.5  | 0  | 5                 | 74                             | 0                        | 0              | 19 (26)  |
|  | 1.5  | 4                 | 49                             | 3 (75)                   | 19 (39)        | 0  |
|  | 3.0  | 7                 | 92                             | 7 (100)                  | 28 (30)        | 0  |
| 3.0  | 0  | 4                 | 46                             | 1 (25)                   | 8 (17)         | 0  |
|  | 3.0  | 10                | 146                            | 9 (90)                   | 49 (34)        | 0  |
|  | 10.0   | 4                 | 56                             | 4 (100)                  | 15 (27)        | 0  |
| 10.0   | 0  | 3                 | 37                             | 1 (33)                   | 1 (3)          | 1 (7)  |
|  | 3.0  | 6                 | 84                             | 0                        | 0              | 0  |
|  | 10.0   | 8                 | 94                             | 2 (25)                   | 6 (6)          | 3 (19)   |
| 25.0   | 3.0  | 6                 | 84                             | 0                        | 0              | 14 (17)  |
|  | 25.0   | 4                 | 48                             | 0                        | 0              | 5 (10)   |

The recipient mice were ovariectomized on day 4 of pseudopregnancy (0900 hours) and injected daily with P<sub>4</sub> (2 mg per mouse) to induce the condition of delayed implantation. On day 7, the recipients received the first injection of E<sub>2</sub> at 1.5, 3.0, 10.0, or 25.0 ng. On day 8, day-4 normal blastocysts were transferred into these recipients immediately followed by a second injection of the vehicle (oil) or E<sub>2</sub> at 1.5, 3.0, 10.0, or 25.0 ng. Implantation sites (ISs) were examined 48 later. The uteri without implantation sites were flushed to recover unimplanted blastocysts. The IS rate in mice receiving the first E<sub>2</sub> injection at 1.5 or 3.0 ng followed by the second injection of 1.5, 3.0, or 10.0 ng of E<sub>2</sub> was significantly higher ( $P < 0.001$ ) than those receiving the first E<sub>2</sub> injection at 10.0 or 25.0 ng.

given E<sub>2</sub> at 3.0 or 25.0 ng immediately after blastocyst transfer on day 7. After 24 h, we examined the expression of *Lif*, *Hegfl* (HB-EGF), and *Ptgs2* (COX-2), because of their known roles in implantation in mice (reviewed in ref. 3), and found that expression was normal (data not shown). We next asked whether these genes are expressed correctly when uterine receptivity is sustained by a lower dose of E<sub>2</sub> or when uterine refractoriness is induced by a higher dose. To address this issue, P<sub>4</sub>-treated recipients with conditions of delayed implantation received an injection of either 3.0 or 25.0 ng of E<sub>2</sub> on day 7. Blastocysts were transferred on day 8 immediately followed by a second injection of 3.0 ng of E<sub>2</sub>. After 24 h, sections of uteri with or without implantation sites but containing blastocysts were processed for *in situ* hybridization. We observed that *Lif*, *Ptgs2*, and *Hegfl* genes were expressed correctly at the implantation sites when the mice received the first and second injections of E<sub>2</sub> at 3.0 ng. In contrast, mice receiving 25.0 ng of E<sub>2</sub> as a first injection completely lacked any sign of implantation or the expression of these genes adjacent to blastocysts after the second injection of E<sub>2</sub> at 3.0 ng (Fig. 2a). These results suggest that uteri maintained in a receptive state at the lower E<sub>2</sub> dose behaved normally with respect to implantation and gene expression, whereas implantation fails and gene expression becomes aberrant in the uterus proceeding to refractoriness in response to E<sub>2</sub> at 25.0 ng.

**Genes Associated with Uterine Preparation to the Receptive State Are Aberrantly Expressed After a High Dose of Estrogen.** Expression of *Ptgs1* (COX-1), *Lif*, *Hoxa10*, and *Areg* (amphiregulin) is normally associated with uterine preparation to the receptive phase (3). We observed that 24 h after an injection of E<sub>2</sub> at 3.0 or 25.0 ng, the expression pattern of *Hoxa10* was normal. However, the expression of *Ptgs1* became aberrant, whereas the expression of *Areg* was undetectable in the uterus after an injection of 25.0 ng of E<sub>2</sub>. The uterine expression of *Ptgs1* became mostly confined to glands at 25.0 ng of E<sub>2</sub> (Fig. 2b). These results suggest that the aberrant expression of *Ptgs1* and down-regulation of *Areg* at higher E<sub>2</sub> levels are indicative of uterine refractoriness. All these genes are expressed in a cell-specific manner throughout the mouse uterus in the morning and afternoon on day 4 of pregnancy before or at the time of the attachment reaction (8, 9, 12, 22). Overall, these results suggest that a rapid onset of uterine refractoriness at a higher estrogen level is due to a failure in maintaining the correct expression of genes associated with uterine receptivity and attachment reaction. A similar observation was noted when a receptive uterus on day 4 proceeds to a refractory state in the afternoon of day 5 in intact pseudopregnant mice (data not shown).

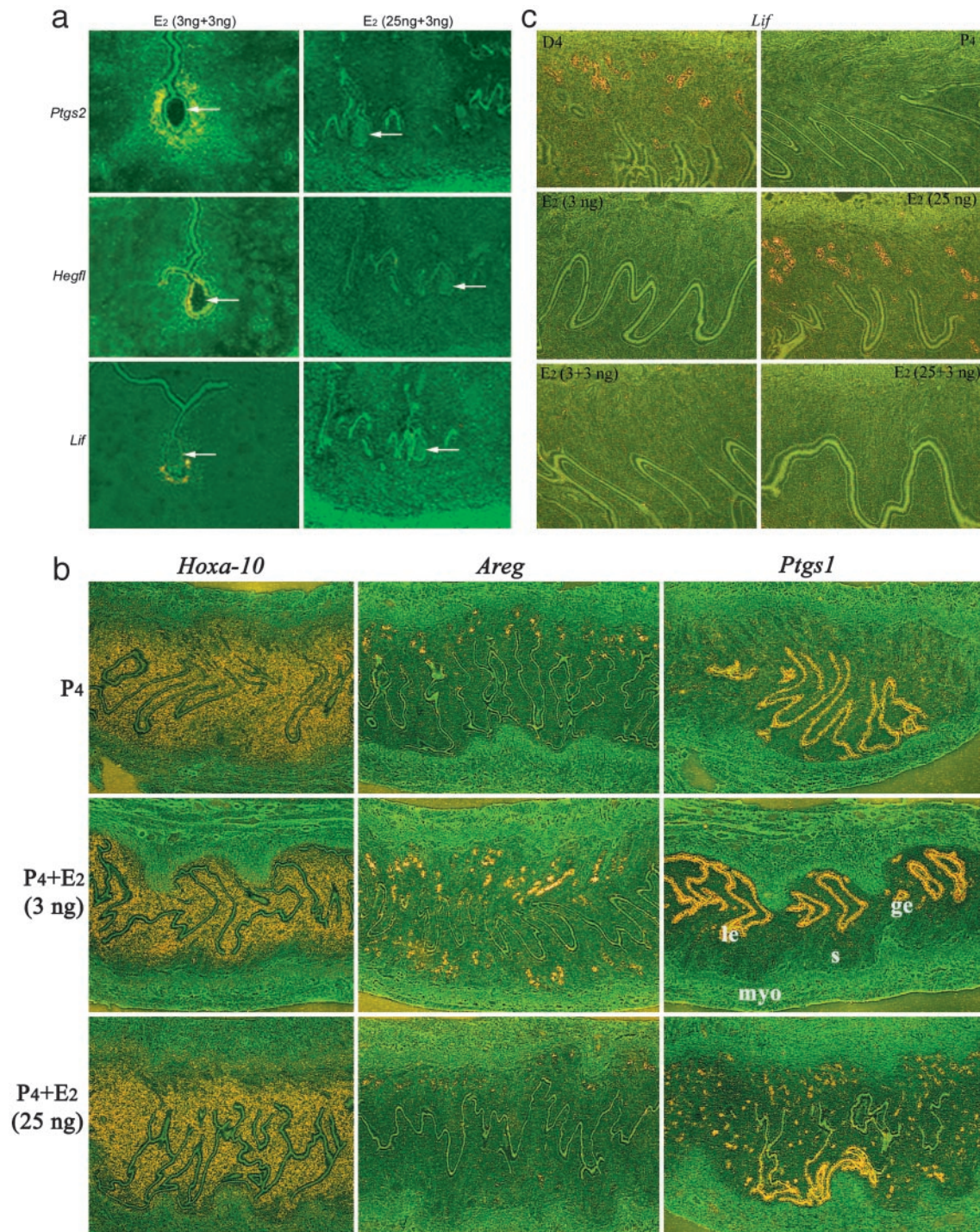
An interesting observation was noted with respect to *Lif* expression. *Lif* is normally expressed in the mouse uterus in a

**Table 3. E<sub>2</sub> at 3 ng prolongs uterine receptivity beyond 24 h**

| Time of blastocyst transfer after 1st E <sub>2</sub> (3 ng) injection, h | No. of recipients | No. of blastocysts transferred | No. of mice with ISs (%) | No. of ISs (%) | No. of blastocysts recovered from mice without ISs (%) |
|--|-------------------|--------------------------------|--------------------------|----------------|--|
| 24   | 10                | 146                            | 9 (90)                   | 49 (34)        | 0  |
| 48   | 4                 | 51                             | 4 (100)                  | 10 (20)        | 0  |
| 72   | 5                 | 67                             | 4 (80)                   | 26 (40)        | 3 (21)   |
| 96   | 4                 | 49                             | 3 (75)                   | 16 (33)        | 0  |
| 120  | 5                 | 70                             | 2 (40)                   | 7 (10)         | 0  |

The recipients were ovariectomized on day 4 of pseudopregnancy at 0900 hours and injected daily with P<sub>4</sub> (2 mg/ml) to induce the condition of delayed implantation. On day 8, the recipients received the first injection of E<sub>2</sub> at 3 ng. Day-4 normal blastocysts were transferred into these recipients 24, 48, 72, 96, or 120 h after the first injection of E<sub>2</sub> followed by a second injection of E<sub>2</sub> at 3 ng immediately after blastocyst transfers. Implantation sites (ISs) were examined 48 later. The uteri without ISs were flushed to recover unimplanted blastocysts. The IS rate was statistically insignificant ( $P > 0.05$ ) among mice receiving blastocyst transfers 24, 48, 72, or 96 h after the first 3-ng E<sub>2</sub> injection; the IS rate, however, was lower ( $P < 0.05$ ) in mice receiving blastocyst transfers at 120 h than in those receiving transfers 24 or 72 h after the first 3-ng E<sub>2</sub> injection.





**Fig. 2.** Uterine gene expression in receptive and refractory uteri. (a) *Lif*, *Ptgs2*, and *Hegfl* expression is aberrant at the site of a blastocyst in a refractory uterus induced by 25 ng of E<sub>2</sub>. Recipient mice were ovariectomized on day 4 of pseudopregnancy and injected daily with 2 mg of P<sub>4</sub> to induce the condition of delayed implantation. On day 7, the recipients received the first injection of E<sub>2</sub> at 3 or 25 ng. On day 8, blastocysts were transferred into these recipients immediately followed by a second injection of 3 ng of E<sub>2</sub>. Uterine sections containing blastocysts were processed for *in situ* hybridization 24 h later. (Magnification,  $\times 100$ .) Arrows indicate the location of blastocysts. (b) Uterine expression of *Areg* and *Ptgs1* becomes aberrant at a higher E<sub>2</sub> level. Pseudopregnant mice ovariectomized on day 4 were injected daily with P<sub>4</sub> to induce the condition of delayed implantation. On day 7, they received an injection of 3 or 25 ng of E<sub>2</sub>. Uteri were processed for *in situ* hybridization 24 h later. (Magnification,  $\times 40$ .) Note that although *Hoxa-10* expression is similar at 3 or 25 ng of E<sub>2</sub>, the expression of *amphiregulin* and *COX-1* is aberrant at 25 ng of E<sub>2</sub>. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium. (c) Uterine *Lif* expression is different at higher and lower doses of E<sub>2</sub>. As stated above, pseudopregnant mice ovariectomized on day 4 were treated with P<sub>4</sub> daily to induce the condition of delayed implantation. On day 7, they received 3 or 25 ng of E<sub>2</sub> or received the first injection of E<sub>2</sub> at 3 or 25 ng followed by a second injection of 3 ng of E<sub>2</sub> 24 h later. Uteri were processed for *in situ* hybridization at different times. Results of *Lif* expression at 24 h are shown. (Magnification,  $\times 100$ .) E<sub>2</sub> at 3 ng as one or two injections failed to detect glandular *Lif* expression, whereas 25 ng of E<sub>2</sub> as a single injection induced this expression. The expression was undetectable when the first E<sub>2</sub> injection was at 25 ng followed by a second injection at 3 ng. Representative sections of day 4 (D4) pregnant uterus showing glandular *Lif* expression and of P<sub>4</sub>-treated uteri showing the absence of *Lif* expression were used as positive and negative controls, respectively.

biphasic manner: first in uterine glands on day 4 of pregnancy followed by transient expression in stromal cells surrounding the blastocyst at the time of attachment reaction at midnight of day 4 that persists through the morning of day 5 (8). However, in the ovariectomized or P<sub>4</sub>-primed ovariectomized mice, estrogen at higher doses rapidly induces *Lif* expression in uterine glands (7, 8). We sought to examine by *in situ* hybridization whether *Lif* expression is altered in the P<sub>4</sub>-primed uterus in response to a low or high dose of E<sub>2</sub>. We observed that E<sub>2</sub> at 3.0 ng as a single injection or two injections at 24-h intervals failed to induce *Lif* expression in uterine glands at 1, 6, or 24 h after the last E<sub>2</sub> injection. In contrast, E<sub>2</sub> at 25.0 ng as a single injection, as expected, induced *Lif* expression in P<sub>4</sub>-primed uterine glands at these times. However, *Lif* expression was undetectable in uterine glands when the first injection of E<sub>2</sub> was given at 25.0 ng followed 24 h later by a second injection of E<sub>2</sub> at 3.0 ng (Fig. 2c). Recall that 3.0 ng of E<sub>2</sub> as single or two injections can induce implantation with correct expression of *Lif* in stromal cells surrounding the implanting blastocyst (see Fig. 2a). These results suggest that stromal cell *Lif* expression at the site of blastocyst during the attachment reaction is more important than the glandular *Lif* expression.

### Discussion

Preimplantation embryo development and uterine preparation for implantation are two major determinants of female fertility. Despite significant developments in IVF/ET technology in humans, the pregnancy success rates remain disappointingly low. The question of uterine receptivity for implantation is an important issue, because a low pregnancy success rate in IVF/ET programs is considered to be due to a higher incidence of implantation failure. This low rate of success is perceived as the result of transfer of IVF-derived embryos into the nonreceptive uterus. One cause of this low rate could be the high levels of estrogen resulting from hyperstimulation of the ovary by gonadotropin administration to retrieve multiple eggs (17–19, 23), thereby rendering the uterus refractory. Thus, uterine receptivity established by coordinated interactions of ovarian P<sub>4</sub> with estrogen is critical to successful implantation and pregnancy outcome. However, critical levels of P<sub>4</sub> and estrogen, their relative importance, and mechanism of actions in regulating uterine receptivity and refractoriness have not been examined in a physiologically defined system. Our results using a delayed-implantation model in mice provide evidence that the levels of estrogen within a very narrow range are critical determinants for transforming uterine receptivity to a refractory state, suggesting that the uterus is extremely sensitive to estrogen levels with respect to implantation (see Fig. 3). This remarkable sensitivity of the uterus to estrogen perhaps plays a significant role in ensuring on-time implantation, which is critical to successful pregnancy establishment and outcome.

Our results in mice raise the interesting possibility that the uterus becomes refractory after a considerably prolonged period of receptive state at a low dose of E<sub>2</sub> (3.0 ng). These results also show that sharp changes occur with respect to uterine receptivity within a narrow range of E<sub>2</sub> levels (3.0–25.0 ng). However, the mechanism by which E<sub>2</sub> at 3.0 ng, but not at 10.0 or 25.0 ng, can prolong the receptive phase is not clearly understood. Doses of 10.0 and 25.0 ng of E<sub>2</sub> as a first injection should not be considered nonphysiological, because both can induce implantation in a P<sub>4</sub>-primed uterus similar to 3.0 ng of E<sub>2</sub>. Therefore, we suggest that implantation-inducing changes in a P<sub>4</sub>-primed uterus produced by different doses of E<sub>2</sub> during the initial phase, i.e., before 24 h, are similar, if not identical, to those changes occurring in the normal pregnant uterus with active blastocysts. This is consistent with our observation of normal expression of genes encoding LIF, COX-2, and HB-EGF at the site of implantation induced by either a low or high dose of estrogen.

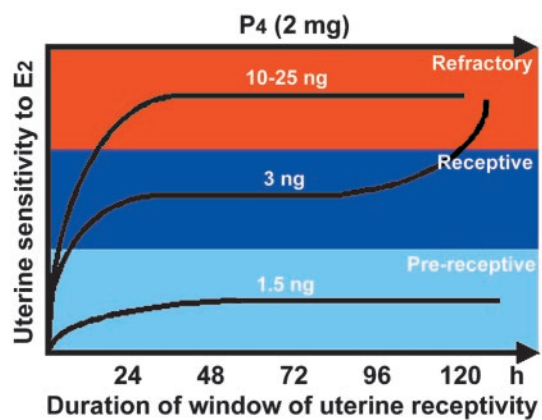


Fig. 3. A scheme depicting modulation of the window of receptivity in the P<sub>4</sub>-primed uterus in response to changing estrogen levels. This scheme shows that estrogen at a low threshold level extends the window of uterine receptivity for implantation, but higher levels rapidly close this window, transforming the uterus into a refractory state.

However, the duration of the window of uterine receptivity is drastically curtailed at higher estrogen levels. This is coincident with the absence of uterine expression of *Lif*, *Ptgs2*, and *Hegfl* at the site of the blastocyst when the mice received a first injection of E<sub>2</sub> at 25.0 ng followed by a second injection of 3.0 ng of E<sub>2</sub> immediately following blastocyst transfer. In contrast, these genes were expressed correctly at the implantation sites when the first and second injections were restricted to 3.0 ng. It could be postulated that the maintenance of the receptive state was altered at the molecular level in the presence of higher estrogen levels, leading to implantation failure. This postulation is supported by aberrant expression of genes encoding COX-1 and amphiregulin but not *Hoxa-10* in the uterus 24 h after an injection of E<sub>2</sub> at a higher dose. These changes could be due to alteration of nuclear receptors for estrogen (ER) and P<sub>4</sub> (PR). Indeed, we observed that PR expression in the luminal epithelium but not in stromal cells was down-regulated 24 h after an injection of 25.0 ng of E<sub>2</sub>; little alteration in PR expression was noted at 3.0 ng of E<sub>2</sub>. In contrast, the ER $\alpha$  expression pattern in luminal and stromal cells was similar at both E<sub>2</sub> concentrations (data not shown). Collectively, these results provide evidence that uterine gene expression conducive to blastocyst implantation is maintained at a lower E<sub>2</sub> dose, whereas gene expression promptly becomes aberrant in the uterus, becoming refractory at higher E<sub>2</sub> levels. These results also suggest that the molecular programming of the uterus with respect to specific gene expression is altered depending on the E<sub>2</sub> levels within a very narrow range, regulating uterine receptivity and refractoriness.

An alternative possibility for prolonging the state of uterine receptivity could be due to differential responses of different regions of the uterus to a low dose of E<sub>2</sub>. For example, at 3.0 ng of E<sub>2</sub> specific areas of the uterus may become receptive, whereas the remaining areas may still be in the neutral state. These remaining neutral areas then respond to a second injection of E<sub>2</sub>. In contrast, at 25.0 ng of E<sub>2</sub> the entire uterus may respond rapidly to reach a maximally receptive state followed by a refractory state by 24 h. This is a possibility if the uterus is comprised of sensitive and less-sensitive areas that respond differentially to hormones and local factors. The validity of this possibility will require a closer examination of gene expression along the entire uterus under defined experimental conditions.

It is well documented that the effects of P<sub>4</sub> and E<sub>2</sub> are either synergistic or antagonistic with respect to various uterine functions and gene expression (5, 24). Therefore, it was obligatory to determine whether increasing the P<sub>4</sub> doses would counteract the



effects of higher doses of E<sub>2</sub> in extending the uterine receptivity. Our observation of failure of P<sub>4</sub> at an elevated level to reverse the adverse effects of higher doses of estrogen on uterine receptivity indicates that specific uterine functions are more sensitive to estrogen and independent of P<sub>4</sub> levels. For example, the down-regulation of PR expression in the luminal epithelium at a higher E<sub>2</sub> level suggests that the ineffectiveness of a higher dose of P<sub>4</sub> apparently includes a change in the luminal epithelium. Because our results provide strong evidence that higher estrogen levels promptly transform the uterus to the refractory state, it may be possible to extend the state of uterine receptivity by neutralizing excess estrogen by the use of an aromatase inhibitor at the time of gonadotropin stimulation in human IVF/ET programs for correcting the cause of uterine refractoriness at higher estrogen levels. Indeed, decreasing estradiol levels during the preimplantation period by using a follicle-stimulating hormone/step-down regimen has been claimed to increase uterine receptivity in humans (18). However, there is

also evidence that ovarian hyperstimulation does not adversely affect uterine receptivity for implantation in IVF/ET programs (25), suggesting that the range of estrogen levels is less restrictive in humans than in mice. Nonetheless, the information obtained from the present investigation in mice should provide valuable information for improving the implantation rate of the IVF-derived embryos in women. Complications associated with multiple embryo implantations also raise serious clinical and social concerns. Thus, prolonging the uterine receptive state may circumvent the necessity of multiple ET to improve pregnancy rates in humans.

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