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ERBB Receptor Feedback Inhibitor 1 Regulation of Estrogen Receptor Activity Is Critical for Uterine Implantation in Mice[1]

Tae Hoon Kim, Dong-Kee Lee, Heather L. Franco, John P. Lydon, and Jae-Wook Jeong[2]

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas

ABSTRACT

Normal endometrial function requires a balance of progesterone (P4) and estrogen (E2) effects. E2 acts to stimulate the proliferation of uterine epithelial cells, while P4 action inhibits E2-mediated proliferation of the epithelium. P4 through its cognate receptor, the P4 receptor (*Pgr*), has important roles in the establishment and maintenance of pregnancy. In previous studies, we have identified ERBB receptor feedback inhibitor 1 (*Errfi1*) as a downstream target of *Pgr* action in the uterus. Herein, we show that *Errfi1* mRNA expression was significantly increased in the uterus after Day 2.5 of gestation. Its expression is also induced in the uterus by acute E2 treatment, and this induction is synergistically induced by chronic E2 and P4 treatment. Although it is known that conditional ablation of *Errfi1* in the *Pgr*-positive cells (*Errfi1*$^{d/d}$) results in infertility, the function of *Errfi1* in reproductive biology has remained elusive. Using *Errfi1*$^{d/d}$ mice, we have identified *Errfi1* as an important mediator of uterine implantation. Epithelial ESR1 and target genes were significantly increased in the uteri of *Errfi1*$^{d/d}$ mice. Our results identify a new signaling paradigm of steroid hormone regulation in female reproductive biology that adds insight into the underlying dysregulation of hormonal signaling in human reproductive disorders such as endometriosis and endometrial cancer.

Errfi1, *estradiol receptor, estrogen, estrogen receptor, female reproductive tract, implantation, steroid hormones, uterus*

INTRODUCTION

The uterus consists of heterogeneous cell types that undergo dynamic changes to support embryo development and implantation. These phenomena are primarily dependent on coordinated interactions mediated by the ovarian steroid hormones progesterone (P4) and estrogen (E2). E2 stimulates proliferation of both the uterine epithelial and stromal cells in neonatal mice. However, this proliferative action of E2 is restricted to epithelial cells in the adult mouse uterus [1, 2]. In contrast, while P4 is inhibitory to E2-mediated proliferation of the luminal and glandular epithelial cells, P4 (alone or in conjunction with E2) leads to uterine stromal cell proliferation [2–4].

P4 is an essential regulator in the uterus during pregnancy [5, 6]. The physiological effects of P4 are mediated through the P4 receptor (*Pgr*), which exists as two isoforms, PR-A and PR-B, arising from alternate transcriptional start sites on the same gene [7, 8]. *Pgr* is a transcription factor that belongs to the nuclear receptor superfamily [9]. The fertility defects exhibited by the *Pgr* knockout (*Pgr*KO) mice unequivocally demonstrated the critical importance of P4 and its receptor in the establishment and maintenance of pregnancy [6, 10].

Although the effect of the P4-*Pgr* axis on murine uterine function has been extensively investigated, there are several P4-*Pgr*-regulated genes that have been identified. These include genes encoding amphiregulin (*Areg*) [11], histidine decarboxylase (*Hdc*) [12], homeobox A10 (*Hoxa10*) and homeobox A11 (*Hoxa11*) [13], calcitonin (*Calca*) [14, 15], calbindin-D9K (*S100g*) [16], Indian hedgehog (*Ihh*) [17], hypoxia-inducible factor 1 (*Hif1a*) [18], and immune-responsive gene 1 (*Irg1*) [19]. These target genes have been identified by testing candidate genes [11], by differential library screening [14], and by DNA microarray approaches [17, 19]. The advent of the latter high-density DNA microarray technology has immensely improved the ability to identify *Pgr*-regulated genes in the uterus.

We recently used oligonucleotide microarrays to identify the genes whose expression is regulated by the P4-*Pgr* axis in the mouse uterus [20, 21]. Using this method, we identified several genes that were regulated by *Pgr* in the uterus in response to P4 [21]. Notably, we found that the expression of ERBB receptor feedback inhibitor 1 (*Errfi1*, *Mig-6*, *RALT*, or *gene 33*) was induced significantly by P4 in a *Pgr*-dependent manner [20, 21]. *Errfi1* is an immediate early response gene that can be induced by various mitogens and commonly occurring chronic stress stimuli [22–25]. It has also been shown to act as a negative feedback inhibitor of epidermal growth factor receptor (EGFR) signaling through direct interaction with the EGFR family [26–29]. *Errfi1* is an adaptor molecule containing a Cdc42/Rac interactive binding (CRIB) [22] domain, Src homology 3 (SH3) binding domain, and 14-3-3 binding domain [22, 30, 31]. Sustained *Errfi1* expression is thought to trigger cells to initiate hypertrophy in chronic pathological conditions such as diabetes and hypertension through stress-activated protein kinase/c-Jun NH_2-terminal kinase activation by binding between its CRIB domain and CDC42 [22, 32]. Ablation of *Errfi1* in mice leads to the development of animals with epithelial hyperplasia, adenoma, and adenocarcinomas in organs such as the lung, gallbladder, and bile duct [33–35]. *ERRFI1* is located on human chromosome 1p36, a locus frequently associated with human cancer [36] and endometriosis. Down-regulated expression of the *ERRFI1* gene is observed in human breast carcinomas, which correlates with reduced overall survival of patients with breast cancer [33, 37]. The *ERRFI1* gene is mutated in human non-small cell lung cancer cell lines NCI-H226 and NCI-H 322M, as well as in one primary human lung cancer [35]. Recently, altered *ERRFI1* expression has been observed in endometrial RNA isolated from women with endometriosis and endometrial cancer [38,

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[2]Correspondence: Jae-Wook Jeong, Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030. FAX: 713 790 1275; e-mail: jjeong@bcm.tmc.edu

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39]. These data point to *ERRFI1* as a tumor suppressor gene in both mice and humans.

Absence of *Errfi1* in mice results in the inability of P4 to inhibit E2-induced uterine weight gain and expression of E2-responsive target genes [20]. *Errfi1*$^{d/d}$ mice develop hyperplasia and endometrial cancer dependent on E2 and P4. In addition, the observation that endometrial carcinomas from women have a significant reduction in *ERRFI1* expression provides compelling support for an important growth regulatory role for *ERRFI1* in the uterus of both humans and mice [20]. This demonstrates that *ERRFI1* is a critical regulator in the tumorigenesis of endometrial cancer. However, the mechanism of *ERRFI1* action in the uterus is uncertain.

Herein, we have used conditional *Errfi1* knockout mice to understand the role of *Errfi1* in uterus. From this study, we have identified *Errfi1* as a critical mediator of embryo implantation. We observed increased E2 signaling in the *Errfi1*$^{d/d}$ uterus that resulted in an implantation defect. Thus, we demonstrate a critical role for *Errfi1* in the preparation of the uterus for the incoming embryo as a mediator of P4 signaling via inhibition of E2 signaling.

MATERIALS AND METHODS

Animals and Tissue Collection

Mice were maintained in the designated animal care facility at Baylor College of Medicine according to the institutional guideline for the care and use of laboratory animals. For the study of steroid hormone regulation, wild-type C57BL/6 mice at age 6 wk were ovariectomized. Two weeks later, ovariectomized wild-type mice were injected with one of the following: vehicle (sesame oil), P4 (1 mg/mouse), E2 (0.1 μg/mouse), or P4 plus E2. Three mice from each group were injected with one of the treatments, and uteri were collected at 4 h, 16 h, or 40 h. The injections were repeated every 12 h for the 16-h and 40-h samples. The mice were anesthetized with Avertin (2,2,-tibromoethyl alcohol; Sigma-Aldrich, St. Louis, MO) and euthanized by cervical dislocation under anesthetic at 4 h, 16 h, or 40 h (4 h after the fourth injection) to collect the uteri. To get uterine samples from early pregnancy mice, wild-type C57BL/6 mice at age 8 wk were mated with wild-type male mice, and different days of pregnant uterine samples were obtained; the morning of vaginal plug was designated as Day 0.5. Uterine tissues were flash frozen at the time of dissection and stored at −80°C for RNA extraction or fixed with 10% (vol/vol) formalin for in situ hybridization. In the implantation study, 8-wk-old female *Errfi1*$^{d/d}$ and *Errfi1*$^{f/f}$ mice were mated with intact wild-type male mice, and 0.1 ml of 1% Chicago Sky Blue 6B (Sigma-Aldrich) was intravenously injected to mice at 5.5 days postcoitus (dpc) of pregnancy before dissection. The number of implantation sites was identified.

Quantitative Real-Time PCR

RNA was extracted from the uterine tissues using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA). Expression levels of *Errfi1*, E2 receptor α (*Esr1*), its target genes, and *Pgr* mRNA were measured by real-time RT-PCR TaqMan analysis using the ABI Prism 7700 Sequence Detector System according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Prevalidated probes and primers for *Errfi1* (*Mm00505292_m1*), *Esr1* (*Mm00433149_m1*), *C3* (*Mm01232779_m1*), *Ltf* (*Mm00434787_m1*), *Muc1* (*Mm00449604_m1*), *Clca3* (*Mm00489959_m1*), *Pgr* (*Mm00435628_m1*), and 18S rRNA (4319413E) were purchased from Applied Biosystems. The cDNA was produced from 1 μg of total RNA using random hexamers and MMLV RT (Invitrogen Corp., Carlsbad, CA). The RT-PCR was performed using RT-PCR Universal Master Mix reagent (Applied Biosystems) according to the manufacturer's instructions. All real-time PCR was performed using six independent RNA sets. The mRNA quantities were normalized against 18S rRNA using ABI rRNA control reagents. Statistical analyses were performed using one-way ANOVA analysis, followed by Tukey post hoc multiple range test or Student *t*-test using the Instat package from GraphPad (San Diego, CA).

In Situ Hybridization

The spatial expression of *Errfi1* mRNA in uterine tissue sections was determined by in situ hybridization as described previously by Simmons et al. [40]. Briefly, uterine tissues were fixed in 10% (vol/vol) formalin. After overnight fixation at room temperature, tissues were dehydrated through a series of ethanol and then processed for paraffin embedding. Paraffin sections were mounted onto poly-L-lysine-coated slides (VWR Scientific Products, West Chester, PA) and then used for in situ hybridization. The *Errfi1* templates for antisense and sense cRNA probes were amplified using PCR primer containing the T7 polymerase promoter sequence flanking the antisense and sense nucleotide primer sequence, respectively. The riboprobes were generated by in vitro transcription (Promega, Madison, WI) of 0.1 μg of amplified DNA products using [^{35}S] uridine triphosphate (Amersham Biosciences, Piscataway, NJ). Slides were incubated for 7 min at room temperature in proteinase K (20 μg/ml) in a buffer containing 50 mM Tris and 5 mM edetic acid (EDTA) (pH 8.0). Slides were then acetylated with acetic anhydride, dehydrated, and exposed to either denatured antisense or sense probes (5×10^6 cpm/slide) in hybridization buffer (50% [vol/vol] formamide, 10% [wt/vol] dextran sulfate, 5× Denhardt solution, 300 mM NaCl, 5 mM EDTA [pH 8], 20 mM Tris [pH 8.0], and 0.05 mg/ml of yeast tRNA). Hybridization was performed at 55°C overnight in a humidity chamber containing 5× saline and sodium citrate (SSC) and 50% (vol/vol) formamide. Coverslips were floated off the slides by placement in 5× SSC and 10 mM β-mercaptoethanol (βME) for 30 min at 55°C, and remaining coverslips were gently removed with a pair of forceps. Sections were then washed as follows: 50% formamide, double-strength SSC, and 50 mM βME for 20 min at 65°C; single-strength TEN (0.05 M NaCl, 10 mM Tris [pH 8], and 5 M EDTA) for 10 min at room temperature; and then three times in single-strength TEN for 10 min at 37°C. Hybridized slides were exposed to 20 μg/ml of RNase A for 30 min at 37°C. Slides were washed in 50% (vol/vol) formamide, 2× SSC, and 100 mM 2-mercaptoethanol, followed by 2× SSC at 55°C for 30 min; dehydrated in a graded series of ethanol in 0.3 M ammonium acetate; and exposed to Biomax MR film overnight (Kodak, Rochester, NY). The following morning, slides were dipped in autoradiography emulsion (GE Healthcare, Piscataway, NJ) and placed at 4°C in a lightproof box for several days. Following development, slides were counterstained with hematoxylin.

Immunohistochemistry

Uterine sections from paraffin-embedded tissue were cut at 5 μm and mounted on silane-coated slides, deparaffinized, and rehydrated in a graded alcohol series. Sections were preincubated with 10% normal serum in PBS (pH 7.5) and then incubated with primary antibody diluted in 10% normal serum in PBS (pH 7.5) overnight at 4°C at the following dilutions: 1:500 for PGR (rabbit polyclonal anti-human PGR; DAKO Corp., Carpinteria, CA), 1:500 for ESR1 (mouse monoclonal anti-human ESR1; DAKO Corp.), 1:500 for phospho-ESR1 (rabbit monoclonal anti-human phospho-ESR1; Abcam, Cambridge, MA), 1:800 for MUC1 (rabbit polyclonal anti-human MUC1; Abcam), and 1:800 for LTF (rabbit polyclonal anti-human LTF; Millipore, Bedford, MA). Rabbit IgG isotype control was used for a negative control. On the following day, sections were washed in PBS and incubated with secondary antibody diluted 1:200 (biotinylated anti-rabbit IgG anti-rabbit IgG for anti-PR, anti-phospho-ESR1, MUC1, and LTF and biotinylated anti-mouse IgG for anti-ESR1; Vector Laboratories, Burlingame, CA) for 1 h at room temperature and subsequently washed three times with PBS. Horseradish peroxidase-conjugated streptavidin (Vector Laboratories) at a dilution of 1:1000 was added to the slides and incubated for 30 min. Immunoreactivity was detected using the Vectastain Elite ABC kit (Vector Laboratories). After this, the nuclei were stained with hematoxylin for 30 sec. The slides were subsequently washed in water and increasing gradients of ethanol, then placed in xylene before mounting in a xylene-based mounting solution, and observed under a microscope.

RESULTS

Expression of Errfi1 During Early Pregnancy

We examined the expression profile of *Errfi1* mRNA in mouse uteri during the preimplantation, periimplantation, and postimplantation periods by performing real-time RT-PCR to measure levels of *Errfi1*. The initiation of pregnancy was marked by the presence of the postcoital vaginal plug (0.5 dpc). As shown in Figure 1A, the expression of *Errfi1* was detected on 0.5 dpc, which gradually increased until 5.5 dpc, reaching statistical significance after 2.5 dpc in the uterus. Next, the spatial expression profile of *Errfi1* mRNA was assayed during early pregnancy using in situ hybridization. The uterine sections were cut from the tissues, fixed, and embedded as described in the *In Situ Hybridization* subsection of *Materials and Methods*. In situ hybridization analysis of uterine cross

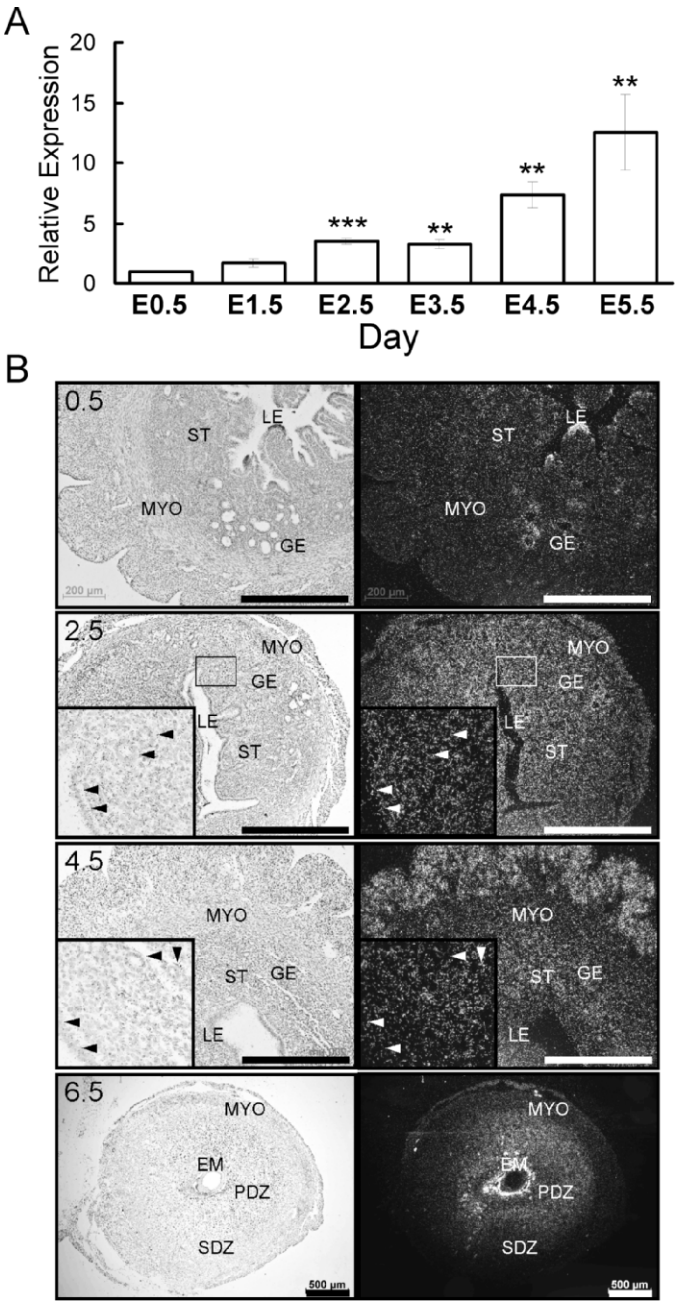

FIG. 1. The expression of *Errfi1* by real-time RT-PCR and in situ hybridization in pseudopregnancy. **A**) The expression level of *Errfi1* was measured in uteri of pseudopregnancy. Total RNA used for the RT-PCR assays was prepared from pseudopregnant uteri. The results represent the mean ± SEM of three independent RNA sets. $^{**}P < 0.01$ and $^{***}P < 0.001$. **B**) The localization pattern of *Errfi1* mRNA by in situ hybridization during early pregnancy. Nuclei are counterstained with hematoxylin. E, embryonic day; EM, embryo; LE, luminal epithelium; GE, glandular epithelium; PDZ, primary decidual zone; SDZ, secondary decidual zone; ST, stroma cells; MYO, myometrium; bar = 500 μm. Arrowheads indicate luminal and glandular epithelium.

sections revealed increased expression of *Errfi1* at 2.5 dpc and 4.5 dpc compared with 0.5 dpc in the stroma, luminal epithelium, and glandular epithelium. The expression of *Errfi1* mRNA was also evident in the decidua on 6.5 dpc.

Steroid Hormone Regulation of Errfi1

We previously identified *Errfi1* as a P4- and *Pgr*-regulated gene in the murine uterus using DNA microarray analysis [21, 41]. In this study, the effect of E2 and E2 plus P4 on *Errfi1* expression was examined. Ovariectomized wild-type female mice were daily injected with one of the following: vehicle (sesame oil), P4 (1 mg/mouse), E2 (0.1 μg/mouse), or P4 plus E2 (1 mg of P4 and 0.1 μg of E2 per mouse). Mice were euthanized at 4 h, 16 h, and 40 h after the initial hormone treatment (n = 5 mice per treatment) and subjected to real-time PCR to characterize the effect of steroid hormone treatment on the relative expression of *Errfi1*. As shown in Figure 2, P4 induced *Errfi1* mRNA expression at 4 h but not 16 h and 40 h after treatment, as in our previous microarray results [20, 21]. The expression of *Errfi1* mRNA was significantly increased at 4 h, 16 h, and 40 h after E2 treatment. The results suggest that E2 induces the expression of *Errfi1* in the uterus. Figure 2 also shows that treatment of mice with E2 plus P4 significantly increased the induction of *Errfi1* at 16 h and 40 h. These results indicate that the expression of *Errfi1* was induced by acute E2 or P4 treatment and that *Errfi1* was synergistically induced by chronic E2 plus P4 treatment in the uterus.

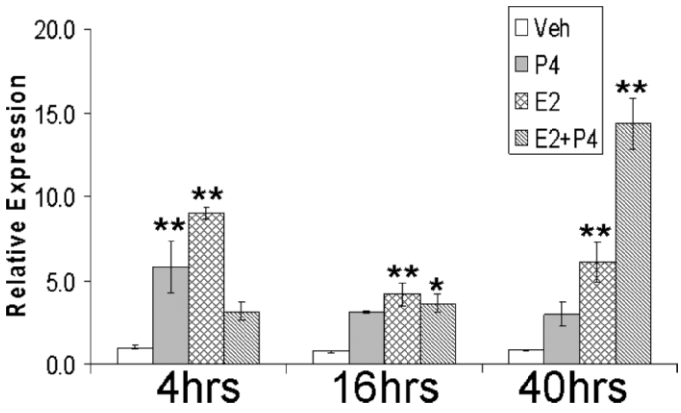

FIG. 2. The expression pattern of *Errfi1* by E2 and P4 in the uterus. Total RNA used for the RT-PCR assays was prepared from wild-type mice that were treated with P4, E2, E2 plus P4, or vehicle (sesame oil [Veh]) for 4 h, 16 h, and 40 h. The results represent the mean ± SEM of three independent RNA sets. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Effect of Errfi1 *Ablation on Implantation*

Because *Errfi1* ablation resulted in numerous pathologies and decreased longevity [34, 42–44], we generated conditional *Errfi1* ablation ($PR^{cre/+}Errfi1^{f/f}$ and $Errfi1^{d/d}$) in the reproductive tract by breeding $Errfi1^{f/f}$ mice and PR^{Cre} (official allele symbol $Pgr^{tm2(cre)Lyd}$) mice to effectively investigate the role of *Errfi1* in the regulation of uterine function [20, 45, 46]. Female $Errfi1^{d/d}$ mice were infertile [22]. This defect was not due to an ovarian phenotype, as these mice exhibited normal ovarian morphology, steroidogenesis, and function [20]. Uterine function was first assessed by examining the ability of $Errfi1^{d/d}$ mice to undergo a decidual response. Female $Errfi1^{d/d}$ mice exhibited a normal decidual response. Thus, our previous results suggest that the fertility defect of the $Errfi1^{d/d}$ mice occurred either before implantation or after decidualization.

To dissect the cause of infertility, 8-wk-old female $Errfi1^{d/d}$ and $Errfi1^{f/f}$ control mice were placed with intact wild-type male mice. Mice were euthanized at 5.5 dpc of pregnancy, and the number of implantation sites was identified. We dissected mice on the morning of 5.5 dpc and counted the number of implantation sites by injecting Chicago Sky Blue dye. Implantation sites were not detected in the mutant uterine horns (n = 7), while normal implantation sites (mean ± SEM,

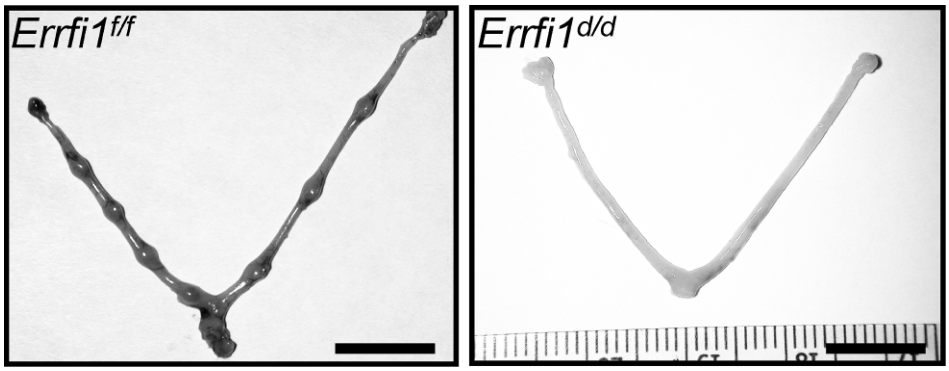

FIG. 3. Implantation defect in *Errfi1*$^{d/d}$ mice. Mice were euthanized at 5.5 dpc of pregnancy, and the number of implantation sites was counted by injecting Chicago Sky Blue dye. Implantation sites were not detected in the mutant uterine horns (n = 7), while normal implantation sites (mean ± SEM, 7.25 ± 0.48) were scored in the controls (n = 4). Bar = 1 cm.

7.25 ± 0.48) were scored in the controls (n = 4) (Fig. 3). This result demonstrates that the fertility defect of the *Errfi1*$^{d/d}$ mice occurs in the preimplantation period.

E2 Receptor Activity Is Enhanced in the Uteri of Errfi1$^{d/d}$ *Mice*

One of the major roles of P4 is to down-regulate *Esr1* activity in the uterine luminal epithelium, which consequently opens the uterine receptivity window [47, 48]. Because *Errfi1*$^{d/d}$ mice have an implantation defect, we investigated whether *Errfi1* is a mediator of P4′s suppression of ESR1 activity during the preimplantation period. To address this, the expression level of E2-responsive genes, chloride channel calcium-activated 3 (*Clca3*), mucin 1 transmembrane (*Muc1*), complement component 3 (*C3*), and lactotransferrin (*Ltf*) was examined by quantitative real-time RT-PCR analysis. The expression of *Clca3*, *Muc-1*, *C3*, and *Ltf* was significantly increased in the *Errfi1*$^{d/d}$ mice compared with the *Errfi1*$^{f/f}$ mice (Fig. 4A). Immunohistological staining detected high LTF and MUC1 protein expression in the epithelium of the *Errfi1*$^{d/d}$ mice compared with the *Errfi1*$^{f/f}$ mice (Fig. 4B). These results demonstrate that E2 activity is indeed enhanced in the uterine epithelial compartment of the *Errfi1*$^{d/d}$ mice.

In an attempt to further dissect the molecular mechanism of enhanced E2 activity in the mutant uterine epithelium, we

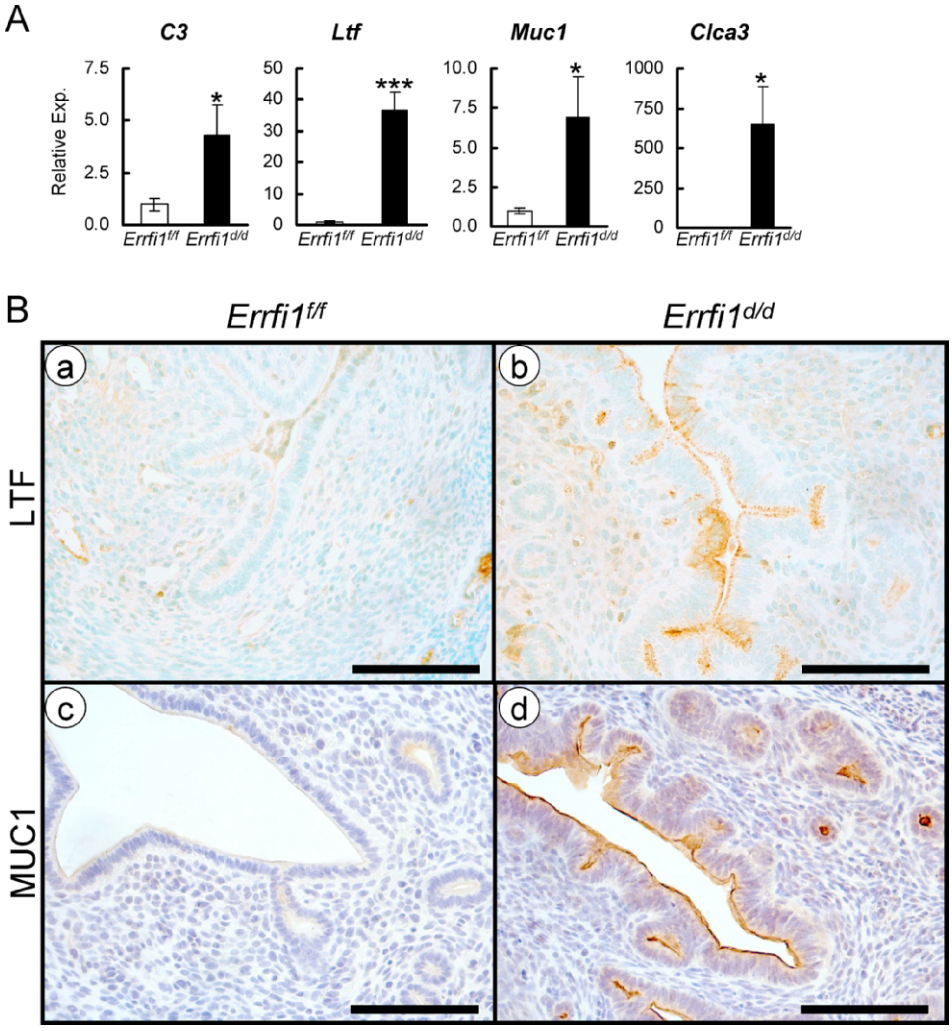

FIG. 4. An increase in expression of *Esr1*-regulated genes in *Errfi1*$^{d/d}$ mice. **A**) Real-time RT-PCR analysis of *C3*, *Ltf*, *Muc1*, and *Clca3* was performed on uteri of *Errfi1*$^{f/f}$ and *Errfi1*$^{d/d}$ mice at 3.5 dpc. The results represent the mean ± SEM of six independent mouse sets. *$P < 0.05$ and ***$P < 0.001$. **B**) Immunohistochemical analysis of LTF (a and b) and MUC1 (c and d) in uteri of *Errfi1*$^{f/f}$ (a and c) and *Errfi1*$^{d/d}$ (b and d) mice at 3.5 dpc. Bar = 200 μm.

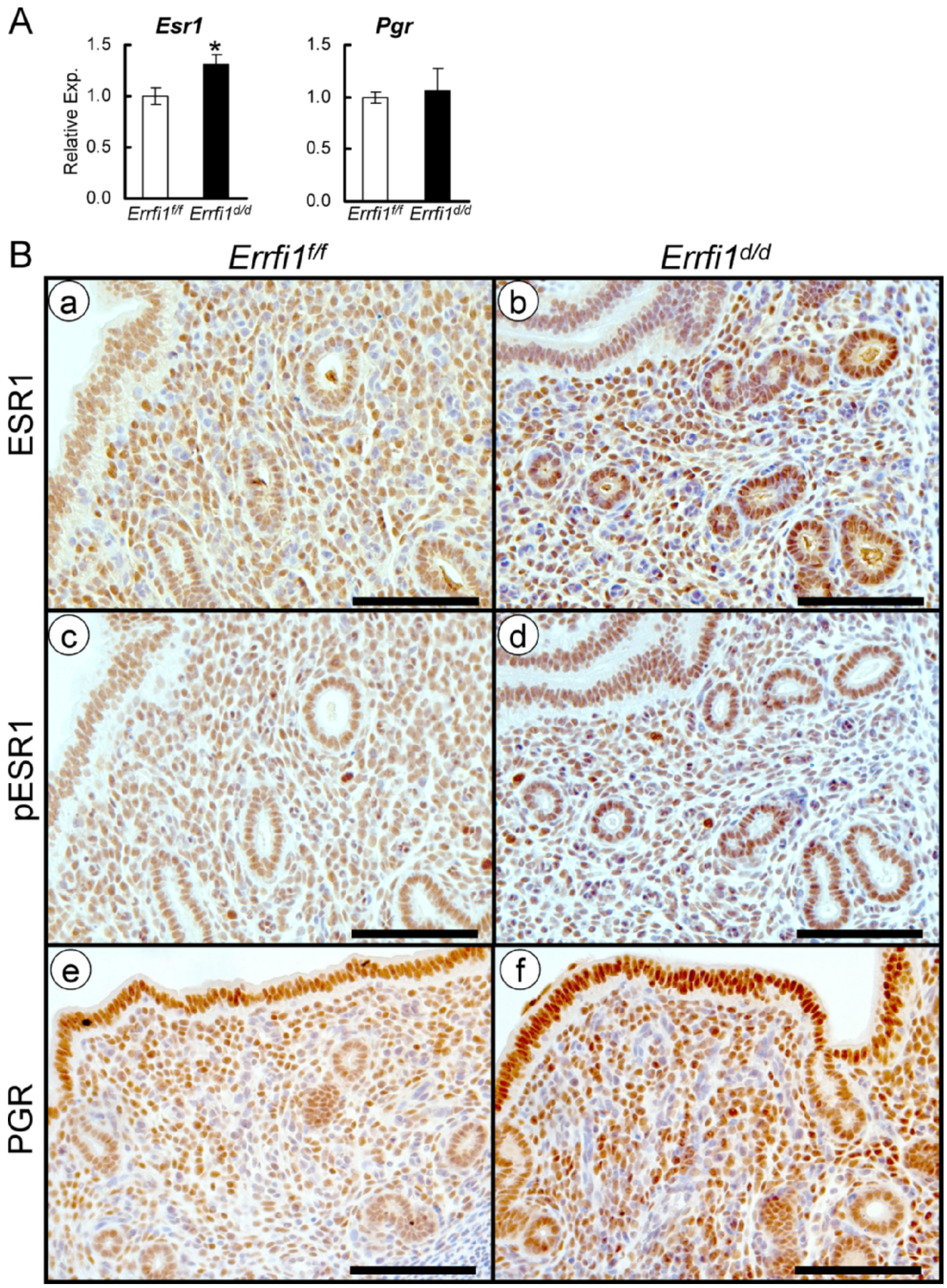

FIG. 5. An increase in E2 receptor activity in the uterine of *Errfi1*$^{d/d}$ mice. **A**) Real-time RT-PCR analysis of *Esr1* and *Pgr* was performed on uteri of *Errfi1*$^{f/f}$ and *Errfi1*$^{d/d}$ mice at 3.5 dpc. The results represent the mean ± SEM of six independent mouse sets. *$P < 0.05$. **B**) Immunohistochemical analysis of ESR1 (a and b), phospho-ESR1 (c and d), and PGR (e and f) in uteri of *Errfi1*$^{f/f}$ and *Errfi1*$^{d/d}$ mice at 3.5 dpc. Bar = 200 μm.

examined whether uterine *Esr1* and *Pgr* levels are altered in the *Errfi1*$^{d/d}$ mice. The expression of *Esr1* mRNA was significantly increased in the whole-uterine tissues of *Errfi1*$^{d/d}$ mice by real-time RT-PCR (Fig. 5A). Because the uterus consists of heterogeneous cell types that undergo dynamic changes to support embryo development and implantation, the spatial expression of ESR1 was examined by immunohistological staining using an ESR1-specific antibody. The results showed increased expression of ESR1 in the epithelial compartment of *Errfi1*$^{d/d}$ mice (Fig. 5B). To further determine whether these receptors were activated or not, we examined the Ser-122 phosphorylation status of ESR1 using antiphosphorylated ESR1 antibody and observed increased phosphorylation of ESR1 in the uterine epithelium of *Errfi1*$^{d/d}$ mice (Fig. 5B).

PGR in the stroma has been implicated as a critical modulator of *Esr1* activity in the epithelium [6, 7]. Because the activity of *Esr1* is enhanced in the *Errfi1*$^{d/d}$ mice, we wanted to determine if ablation of *Errfi1* altered the expression of stromal PGR. To address this possibility, we used quantitative PCR and PGR-specific immunostaining to assess the expression of PGR in the uterus of *Errfi1*$^{d/d}$ mice. The results show that the expression level of *Pgr* mRNA was not changed in the whole uterus and that the level of PGR protein was not reduced in any compartment of the *Errfi1*$^{d/d}$ mice (Fig. 5, A and B). These results suggest that *Errfi1* mediates epithelial E2 activity by regulating ESR1 levels and activation.

DISCUSSION

P4, acting through its cognate nuclear receptors, is critical for normal uterine functions associated with the establishment and maintenance of pregnancy [7, 49, 50]. Studies [6, 10] of the mouse model utilizing a null mutation of the *Pgr* gene (*Pgr*KO) demonstrated the essential roles that these receptors have in P4-mediated response. Thus, the identification of P4-regulated pathways in the uterus is crucial for understanding

the impairments that underlie the complex phenotype of the *Pgr*KO mice. In previous studies [20, 21], we have identified *Errfi1* as a *Pgr*-regulated gene. Herein, we show that the expression of *Errfi1* was increased from 0.5 dpc to 5.5 dpc, reaching statistical significance after 2.5 dpc (Fig. 1), which correlates with elevated P4 levels also seen on 2.5 dpc. Together, these results validate our previous findings that *Errfi1* is a *Pgr* target, as its expression correlates with both an increase in serum P4 levels and *Pgr* expression [20, 21]. We also showed that acute E2 treatment up-regulated *Errfi1* expression (Fig. 2). Notably, we observed synergistic induction of *Errfi1* mRNA expression upon chronic E2 and P4 treatment in the uterus. Recently, DNA microarray analysis has been used to compare the global expression pattern of endometrial genes in the eutopic endometrium of women with and without endometriosis [38]. Comparative gene expression analysis of P4-regulated genes in secretory-phase endometrium confirmed the observation of attenuated P4 response in endometriosis. This microarray analysis showed that *Errfi1* is induced in the secretory phase when P4 is high and that the expression of *Errfi1* is reduced in the endometrium of women with endometriosis [38]. These results suggest that *Errfi1* is also a P4-regulated gene expressed in the human endometrium and that its expression is altered in endometriosis.

Because the PR^{Cre} ($Pgr^{tm2(cre)Lyd}$) mouse shows Cre recombinase activity in the pituitary, ovary, uterus, and mammary gland, the cause of infertility in these mice may be due to a defect in any of these tissues [45]. However, $Errfi1^{d/d}$ mice did not show any alterations in ovarian morphology, ovulation, or fertilization and exhibited a normal estrous cycle and normal levels of serum P4 and E2 [20]. These results show that ovarian morphology, steroidogenesis, and function were unaffected in the $Errfi1^{d/d}$ females. These data suggest that the defects observed in the $Errfi1^{d/d}$ mice are inherent to the uterus. However, $Errfi1^{d/d}$ mice did not show any decidualization defect [20] but have an implantation defect. Implantation requires interactions between the embryo and the uterus. The PR^{Cre} ($Pgr^{tm2(cre)Lyd}$) mouse does not show Cre recombinase activity in the embryo. However, to exclude any possible role for the lack of *Errfi1* in the embryo, we transferred wild-type embryos to the uteri of $Errfi1^{f/f}$ and $Errfi1^{d/d}$ mice. The embryo-transferred $Errfi1^{d/d}$ mice also showed implantation defects compared with $Errfi1^{f/f}$ mice (data not shown). These data suggest that the implantation defect in the $Errfi1^{d/d}$ mice is due to *Errfi1* ablation in the uterus and not in the embryo.

*Pgr*KO mice [6] and conditional null mutant mice of *Ihh* [51] and *Nr2f2* (*COUP-TFII*) [52] are infertile. *Ihh* and *Nr2f2* are essential mediators of *Pgr* action in the uterus. Conditional *Ihh* and *Nr2f2* knockout mice are infertile because of implantation failure [51, 52]. The ablation of *Nr2f2* enhanced epithelial E2 activity, which impedes the maturation of receptive uterus [52]. Our finding that *Errfi1* suppresses *Esr1* action is intriguing. *Esr1* has been shown to regulate the expression of many glycoproteins during the periimplantation period [53, 54]. *Muc1* is an E2 target encoding an epithelial glycoprotein, and its expression is decreased at the time of implantation to facilitate epithelial remodeling [53, 55]. Persistent expression of *Muc1* during the periimplantation period prevents uterine receptivity and embryo attachment [55]. Real-time PCR showed high expression levels of *Muc1* in $Errfi1^{d/d}$ mice (Fig. 4A). In addition, immunohistochemistry detected high expression levels of MUC1 in the apical surface of mutant luminal epithelia (Fig. 4B). *Clca3* is a gene important for the overproduction of mucus protein [56] and a known E2 target gene in the uterine epithelium [57]. *Clca3* is also shown to be highly up-regulated in $Errfi1^{d/d}$ mice (Fig. 4). Using tissue recombination technique, Buchanan et al. [58] showed that epithelial *Ltf* expression is not only regulated by epithelial ESR1 but also regulated by stromal ESR1. *Ltf* was also elevated in the $Errfi1^{d/d}$ mice (Fig. 4A), indicating that E2 activity is up-regulated in the uterine luminal epithelium of $Errfi1^{d/d}$ mice. *Errfi1* was highly expressed in both stroma and epithelium. This raises the possibility that both stromal *Errfi1* and epithelial *Errfi1* are required to suppress epithelial *Esr1* function. Therefore, up-regulation of *Esr1* target genes suggests that *Errfi1* is essential for the *Pgr*-mediated suppression of *Esr1* activity in the epithelium, which is necessary for induction of the window of receptivity. These results suggest that abnormally increased E2 activity might be the underlying cause of the uterine receptivity defect displayed in $Errfi1^{d/d}$ mice.

Tissue recombinants composed of uterine tissues from *Pgr*KO and wild-type mice were also used to demonstrate an important role for stromal *Pgr* in the inhibition of uterine epithelial proliferation [59]. Decreased expression of stromal *Pgr* is observed in $Errfi1^{d/d}$ mice treated with E2 plus P4 for 3 days [20]. However, we observed no decrease in *Pgr* expression at 3.5 dpc in $Errfi1^{d/d}$ mice. The administration of E2 plus P4 treatment for 3 days mimics a later stage in pregnancy seen by increased stromal *Pgr*, whereas 3.5 dpc is during early pregnancy, which sees increased epithelial *Pgr* expression; therefore, the two conditions may not correlate. Although a detailed regulatory mechanism of this interdependence has yet to be defined, our results suggest a regulatory loop in which *Errfi1* acts as a mediator of *Pgr* function during periimplantation. *Errfi1* function then acts to repress E2 target genes, which may provide feedback and affect *Pgr* expression in the stroma.

We also showed that the expression of ESR1 and Ser-122 phosphorylated ESR1 is increased in the $Errfi1^{d/d}$ mice (Fig. 5). Enhanced expression of these molecules likely contributes to the observed increase in *Esr1* activity and the subsequent activation of the downstream *Esr1* targets. Increased phosphorylation of ESR1 appears proportional to the increase in expression levels seen in ESR1. The phosphorylation of ESR1 has been shown to couple with growth factor signaling and may be controlled through a paracrine mechanism and not likely through autophosphorylation; therefore, this finding supports the notion that the observed phenomena seen in the $Errfi1^{d/d}$ mice uterus are by dysregulation in epithelial-stromal communication. It is more likely that stromal *Errfi1* regulates *Pgr* to control an unknown paracrine signal, which acts through its epithelial receptor to suppress epithelial *Esr1* activity, as well as *Esr1* expression. Therefore, P4 suppression of epithelial E2 activity is initiated from the stromal compartment via *Errfi1* through a complex epithelial-stromal cross communication pathway. However, the compartment-specific functions of *Errfi1* and epithelial-stromal cross talk need to be addressed using tissue recombination techniques or compartment-specific ablation of *Errfi1* in the uterus.

In conclusion, *Errfi1* suppresses *Esr1* activity in the uterine epithelium, an event that is required for successful embryo implantation. Based on our previous observation that *Errfi1* is a mediator of the *Pgr*, these data suggest that *Errfi1* mediates P4′s suppression of E2 signaling during embryo implantation. Because increased E2 signaling and/or decreased P4 signaling is a hallmark of uterine diseases, including infertility, endometriosis, and endometrial cancer, understanding the mechanism by which *Errfi1* regulates the balance between these steroid hormone signaling pathways may provide valuable insight into these diseases.

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