

Uterine Epithelial Cell Estrogen Receptor Alpha-Dependent and -Independent Genomic Profiles That Underlie Estrogen Responses in Mice¹

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ABSTRACT

Estrogens exert their activity through estrogen receptor alpha (ERalpha) to stimulate hypertrophy and hyperplasia in the uterus. A uterine epithelial ERalpha conditional knockout mouse model (*Wnt7a*^{Cre+}; *Esr1*^{fl/fl} or cKO) demonstrated that ERalpha in the epithelial cells was dispensable for an initial uterine proliferative response to 17beta-estradiol (E₂) but required for subsequent uterine biological responses. This study aimed to characterize the differential gene expression patterns induced by E₂ in the presence or absence of epithelial ERalpha. RNA microarray analysis revealed that approximately 20% of the genes differentially expressed at 2 h were epithelial ERalpha independent, as they were preserved in the cKO uteri. This indicates that early uterine transcripts mediated by stromal ERalpha are sufficient to promote initial proliferative responses. However, more than 90% of the differentially expressed transcripts at 24 h were not regulated in the cKO, indicating that the majority of later transcriptional regulation required epithelial ERalpha, especially those involved in mitosis. This shows that loss of regulation of these later transcripts results in blunted subsequent uterine growth after 3 days of E₂ treatment. Additionally, progesterone's ability to inhibit E₂-induced epithelial cell proliferation was impaired, consistent with a uterine receptivity defect that contributes to cKO infertility. These transcriptional profiles correlate with our previously observed biological responses, in which the initial proliferative response is independent of epithelial ERalpha and thus dependent on stromal ERalpha, yet epithelial ERalpha is essential for subsequent tissue responsiveness.

epithelium, estradiol/estradiol receptor, proliferation, stroma, uterus

INTRODUCTION

Mammalian uterine tissues undergo tissue remodeling during ovarian cycles and pregnancy [1]. The ovarian steroid hormone estrogen acts through estrogen receptors (ERs) to stimulate cell proliferation, differentiation, and growth in both reproductive and nonreproductive tissues [2]. In rodents, ER alpha (ERα) is expressed primarily in female reproductive tissues, including theca cells in the ovary, oviduct, uterus, and vagina as well as the mammary gland, whereas ER beta (ERβ)

is expressed primarily in the granulosa cells in the ovary [3]. ERα is expressed in all uterine cell layers, including the epithelial cell layer and the mesenchymal (stromal and myometrial) cell layer [4].

In rodent uteri, 17β-estradiol (E₂) treatment rapidly increases nuclear occupancy and ERα recruitment to targeted gene promoter regions [5]. Subsequently, early-phase gene products involved in fluid uptake, hyperemia, and immune cell infiltrations are synthesized [6, 7]. Later-phase physiological responses include waves of DNA synthesis and mitosis, changes in epithelial cell morphology from single cuboidal into columnar secretory epithelial cells, and increased immune cell infiltration [8]. The subsequent increase in cell number (hyperplasia) and cell size (hypertrophy) leads to an increase in total uterine wet weight. It has been shown that E₂ stimulates cell proliferation strictly in the epithelial cells of ovariectomized adult mouse uteri [9]. We have previously shown that acute dosing of E₂ induces two distinct genomic profiles in the uterus, including early and late phases [10]. Female mice with a global deletion of ERα (ERαKO) lack these E₂-induced early and late genomic responses, whereas ERβKO mice show similar uterine responses to wild type (WT) [10]. Our previous findings indicate that ERα, not ERβ, regulates the genomic responses that are responsible for biological actions of E₂ in the rodent uterus.

Epithelial-mesenchymal interaction is known to be important not only for embryonic development but also for cellular growth and differentiation of adult mammary gland, uterus, and prostate tissues [11]. Several studies used a tissue recombination strategy to demonstrate that E₂ stimulated a rapid production of paracrine mitogenic signals, such as insulin-like growth factor 1 (*Igf1*) or other growth factors, through ERα in the stromal cells that subsequently induced uterine epithelial cell proliferation [12–15]. Recently, we generated a conditional deletion of epithelial ERα in the mouse uterus by crossing *Wnt7a*^{Cre+} mice with floxed *Esr1*^{fl/fl} mice (referred to as conditional knockout or cKO) to observe the physiological responsiveness in uterine tissue in vivo. Our findings demonstrated that the epithelial ERα is not necessary for the epithelial cell proliferative response of the uterus after 24 h of E₂ treatment [16], which is consistent with the findings from developmental tissue recombination studies [17] but also illustrates that the same developmental paracrine signaling mechanism is retained in adult tissue. Normal levels of CCAAT enhancer binding protein beta (*Cebpb*, a transcription factor important for uterine proliferation [18]) and *Igf1* are preserved in the absence of ERα in the epithelial cells [16]. However, lacking uterine epithelial ERα leads to an aberrant apoptotic event in epithelial cells after a prolonged (three consecutive days) E₂ treatment [16]. We found that baculoviral inhibitors of apoptosis repeat-containing 1 (*Birc1a*) is absent in cKO uteri after prolonged E₂ treatment, which may contribute

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to an increase in epithelial cell apoptosis. Additionally, female mice lacking epithelial ER α are infertile, in part due to an implantation defect [16]. These findings suggest that ER α , specifically in the uterine epithelial cells, is essential for complete uterine biological responses and establishment of successful pregnancy.

We hypothesized that loss of epithelial ER α in the uterine epithelial cells may not play a critical role in regulating expression of early uterine transcripts (2 h) that are responsible for initial E₂-mediated events (24 h) in the uterus, including mitogenesis, but may disrupt late-phase transcriptional profiles (24 h) that lead to aberrant subsequent uterine responses (72 h). To test the hypothesis, we used our epithelial ER α cKO mice and treated with vehicle or E₂ for 2 and 24 h to stimulate the early and late transcripts in the uteri. To understand the molecular mechanisms of downstream signaling of epithelial ER α during early and late time points, we analyzed our microarray data set from cKO and compared it with WT uterine samples using Ingenuity Pathway Analysis. Herein, we show that epithelial ER α was not involved in E₂-induced early transcription events that contribute to epithelial proliferation. However, more than 90% of gene products induced by E₂ at 24 h were missing in the cKO uteri, and these genes were found to be involved in cell cycle regulation, especially mitosis. Lacking these cell cycle-regulated genes may contribute to a blunted epithelial cell proliferation during the subsequent uterine growth observed in the cKO uteri.

MATERIALS AND METHODS

Animals and Experimental Procedures

A uterine epithelial cell-specific ER α deletion cKO mouse model was generated by crossing *Wnt7a*^{Cre+} mice with *Esr1*^{fl/fl} mice [16, 19]. Females with *Wnt7a*^{Cre+}, *Esr1*^{fl/fl}, and *Esr1*^{+/+} genotypes were considered as WT control littermates. Adult females (8–10 wk old) were ovariectomized and housed for 10 days to eliminate endogenous ovarian steroids prior to the study. Animals were randomly assigned into groups and treated with sesame oil (vehicle) or 0.25 μ g E₂ in 100 μ l sesame oil and euthanized for tissue collection at 2-, 24-, and 72-h time points as previously described [16]. For the 72-h time point, mice were injected with E₂ every 24 h for three consecutive days. In some experiments, the ovariectomized females were treated with a series of E₂ and progesterone (P₄) injection with a nidatory dose of E₂ prior to sacrifice (called “E+Pe”) to mimic the hormonal profile during the implantation window, as shown previously [20]. Briefly, animals were subcutaneously injected with E₂ (100 ng/mouse in 100 μ l sesame oil) for two consecutive days (D1 and D2). Mice were allowed to rest on D3–D5. On D6–D8, P₄ at a dose of 1 mg/mouse in 100 μ l sesame oil was injected for three consecutive days. At D9, a combination of P₄ (1 mg/mouse) together with E₂ (50 ng/mouse) was administered 18 h prior to tissue collection on D10. One uterine horn from each mouse in all treatment groups was snap frozen and stored at –80°C for RNA extraction. The contralateral uterine horn from the 24- and 72-h and E+Pe time points was also collected in 10% buffered formalin for hematoxylin and eosin (H&E) and immunohistochemical (IHC) analysis. Animals were handled according to National Institute of Environmental Health Sciences (NIEHS) Animal Care and Use Committee guidelines and in compliance with NIEHS-approved animal protocol.

Uterine Epithelial Cell Height Measurement and Immunohistochemical Analyses

Formalin-fixed uterine samples collected at 24- and 72-h time points were sectioned and stained with H&E using standard histological protocol. Epithelial cell heights were measured in Image-Pro Plus Software, version 4.1.0.0 for Windows (Media Cybernetics, Inc.). Epithelial cell heights were measured using a “length measurement tool” by drawing lines from the basement membrane to the tip of the epithelial cells. Averages of nine points per uterine section were calculated. Fold increase of uterine epithelial cell heights from each group was calculated relative to WT vehicle-treated group. Uterine sections were also stained with ER α , Ki-67, and phosphohistone H3 and PR antibodies for IHC analyses as previously described [16, 21]. The ER β antibody was purchased from Calbiochem (#PC168; EMD Millipore). The

staining was performed on the Discovery XT Automated System (Ventana Medical Systems) with the OmniMap DAB anti-Rabbit Detection Kit according to the manufacturer’s protocol (Ventana Medical Systems). The anti-ER β was diluted at 1:500 and incubated for 1 h. HAND2 antibody (SC-9409; Santa Cruz) was diluted at 1:50 and incubated with uterine sections at 4°C overnight. All sections were dehydrated and coverslipped.

Microarray Analysis

For microarray analysis, adult WT and cKO females (8–10 wk old) were ovariectomized and treated with vehicle or 0.25 μ g E₂. The uterine samples were collected at 2 and 24 h after E₂ administration and immediately frozen in liquid nitrogen. RNA was extracted using the RNeasy Mini Kit (Qiagen) and treated with DNaseI (Qiagen) according to the manufacturer’s protocol. Gene expression analysis was conducted using Agilent Whole Mouse Genome 4 \times 44 multiplex format oligo arrays (no. 014868; Agilent Technologies) as described previously [21]. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus [22] and are accessible through GEO Series accession number GSE23072 (WT samples) and GSE53812 (cKO samples). The gProcessedSignal data were obtained using Agilent Feature Extraction software (ver. 9.5), using the one-color defaults for all parameters. The Agilent Feature Extraction Software was used to perform error modeling, adjusting for additive and multiplicative noise. The resulting data were processed using Partek Genomics Suite (ver. 6.6Beta 6.11.1115). The data were log base 2-transformed and quantile normalized. ANOVA was used to detect differentially expressed genes between groups. Gene lists were generated using the following criteria: $P < 0.01$ and absolute value fold change ≥ 2 . Hierarchical clustering was generated using the Partek default clustering method (shift genes to mean of zero and scale to standard deviation of one).

Verification of Microarray Results by Real-Time RT-PCR

RNA was extracted from the whole uteri using Trizol Reagent (Invitrogen, Life Technology) according to the manufacturer’s protocol. Semiquantitative PCR and the analysis were carried out as previously described [16]. The expression values were calculated as fold change normalized to ribosomal protein L7 (*Rpl7*) expression, relative to WT vehicle. The primer sequences were designed using Primer Express (ver. 2.0; Applied Biosystems) and are listed in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org).

Image Analysis

Quantification of Ki-67 and phosphohistone H3 immunohistochemical analysis was determined using Definiens Tissue Studio version 3.6.1 (Definiens Inc.) and ImageJ version 1.47 (Wayne Rasband, National Institutes of Health), respectively. The amount of Ki67 signal in 1) uterine luminal epithelial cells and 2) uterine stromal cells (excluding the uterine glands) was determined by the algorithm in Definiens. This algorithm detected signals within designated regions of interest in the image and quantified the areas of stains within these regions of interest defined as marker areas. Using the accumulated data of immunohistochemical- and hematoxylin-stained areas from the regions of interest, the algorithm then calculated the percentage of marker present within the selected region(s) of interest. Phosphohistone H3-positive cells are not conducive to imaging by Definiens due to the punctate nature of positive nuclear regions. To quantify phosphohistone H3-positive cells, we used ImageJ software. Three images of positive cells from each sample were captured in the luminal and subepithelial stromal cell areas using Aperio ImageScope version 12.0.0.5039. The total number of nuclei within epithelial and stromal areas was counted and recorded as total cell count in each region using the Cell Counter Tool in ImageJ Plugins. Then the number of phosphohistone H3-positive cells was counted in the same region, and the percentage of phosphohistone H3-positive cells was calculated.

Statistical Analysis

Data were graphed and analyzed using GraphPad Prism version 6.0a for Mac OS X. All data are presented as mean \pm SEM and were evaluated for statistically significant differences ($P < 0.05$) using a two-way ANOVA with the Tukey post hoc test, unless otherwise indicated.

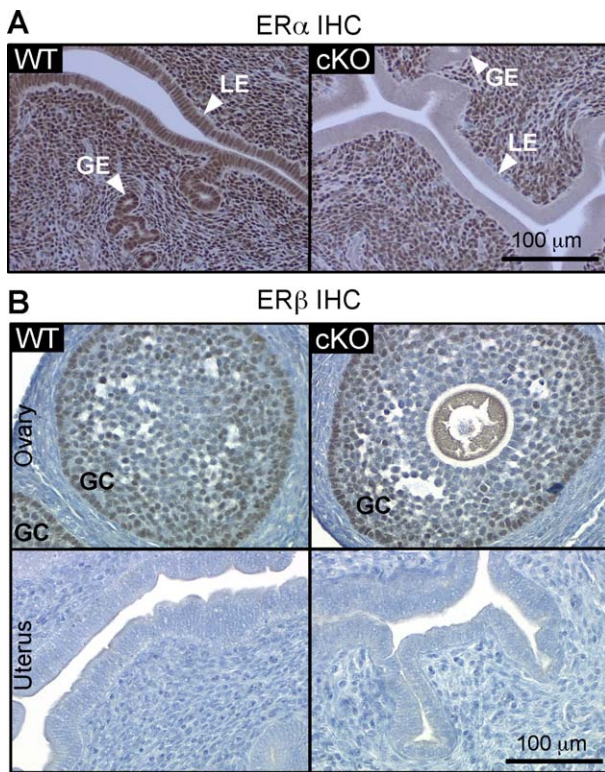


FIG. 1. Mice lacking ER α in uterine epithelial cells showed no alteration in uterine ER β expression. ER α (A) and ER β (B) immunohistochemistry (IHC) of WT control littermate (WT) and cKO animals. Representative images of uterine cross section (A) and ovarian and uterine cross section (B) are shown. Both WT and cKO ovarian sections, ER β protein was detected in the granulosa cells. GE, glandular epithelium; LE, luminal epithelium; GC, granulosa cells. Bar = 100 μ m.

RESULTS

Blunted Late Proliferative-Phase Response to E₂ in cKO Uteri

To evaluate the function of ER α specifically in the epithelial cells during uterine proliferation, we generated mice lacking ER α in uterine epithelial cells using *Wnt7a*^{Cre+} mice crossed with *Esr1*^{fl/fl} mice as previously described [16]. IHC analysis of ER α demonstrated that ER α is expressed in all cell layers of WT uteri (Fig. 1A). Deletion of ER α was observed only in the luminal and glandular epithelial cells of cKO uteri (Fig. 1A) as previously reported [16]. Expression of ER α in the stromal cell layer was comparable between WT and cKO uteri (Fig. 1A). In the ovary, ER β protein in the granulosa cells is expressed comparably in WT and cKO (Fig. 1B). Expression of ER β was not detected in WT or cKO uterine samples (Fig. 1B), which suggests that loss of uterine epithelial ER α did not alter the expression level of ER β in the cKO uteri.

E₂ significantly increased the uterine epithelial cell height in both WT and cKO uteri after 24 and 72 h compared with the WT vehicle-treated group (Fig. 2, A and B). The increase of epithelial cell height was comparable between WT and cKO uteri 24 h after E₂ treatment. However, the fold increase in cKO uterine epithelial cell height 72 h after E₂ treatment was significantly lower than that of WT uteri (Fig. 2B).

Dampened E₂-Induced Uterine Transcripts in the Absence of Epithelial ER α

Our previous findings demonstrated that ER α -dependent genomic responses in the uterus mirrored the biphasic

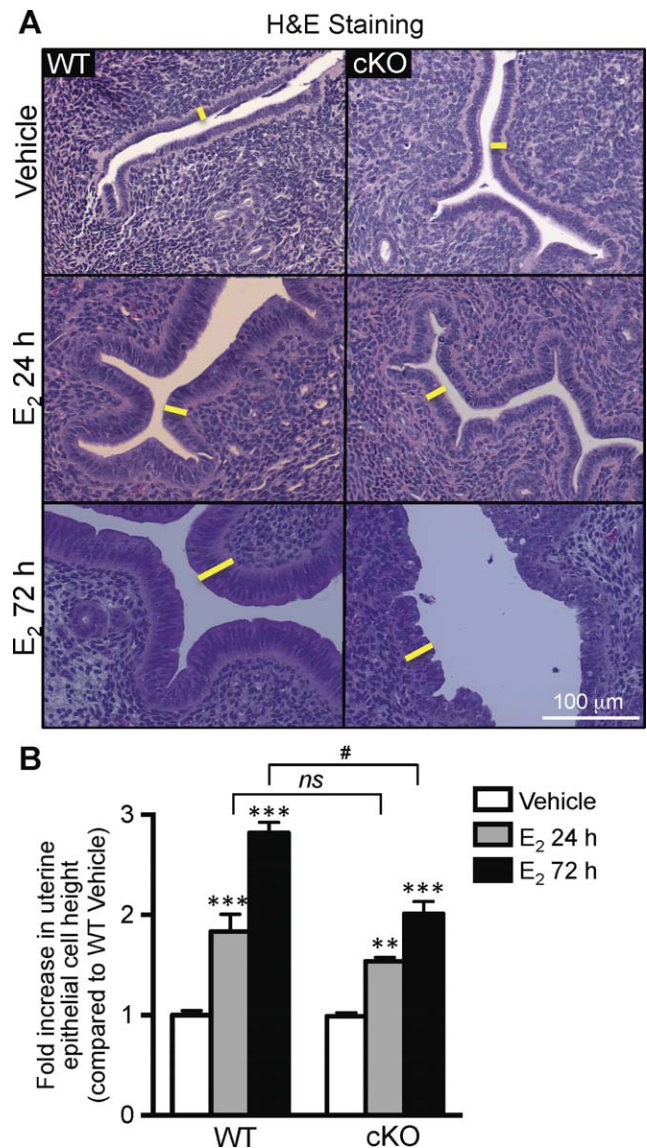


FIG. 2. Loss of uterine epithelial ER α led to a blunted late-phase uterine proliferative response after E₂ treatment. A) H&E staining of uterine sections from WT and cKO mice treated with vehicle or E₂ for 24 or 72 h. Adult females were ovariectomized and treated with E₂ (0.25 μ g/mouse) for 24 h or every day consecutively for 3 days (72 h). Uterine sections were stained with H&E, and epithelial cell heights were measured using ImagePro software as indicated in *Materials and Methods*. Yellow lines indicate examples of epithelial cell height measured. B) Uterine epithelial cell height after E₂ treatment of WT and cKO animals. Fold increase of uterine epithelial cell heights from each group was calculated relative to WT vehicle-treated group (arbitrary unit). Bar graphs represent mean \pm SEM, n = 4–7 mice/group. ** and ****P* < 0.01 and 0.001, respectively; significant difference when compared to vehicle-treated group within genotype. ns, no significant difference when compared between WT E₂ 24 h and cKO E₂ 24 h. #*P* < 0.0001, significant difference when compared between WT E₂ 72 h and cKO E₂ 72 h.

physiological response to E₂ treatment [10]. Because we observed comparable E₂-induced uterine responses between WT and cKO uteri at 24 h but a significantly lower response in cKO uteri at 72 h, we hypothesized that the early transcriptional profile (2 h) would not be altered in the absence of epithelial ER α , whereas the late transcripts (24 h) would be disrupted and lead to aberrant subsequent uterine responses in cKO uteri. To test our hypothesis, a microarray analysis was performed. We used uterine samples from WT and cKO treated

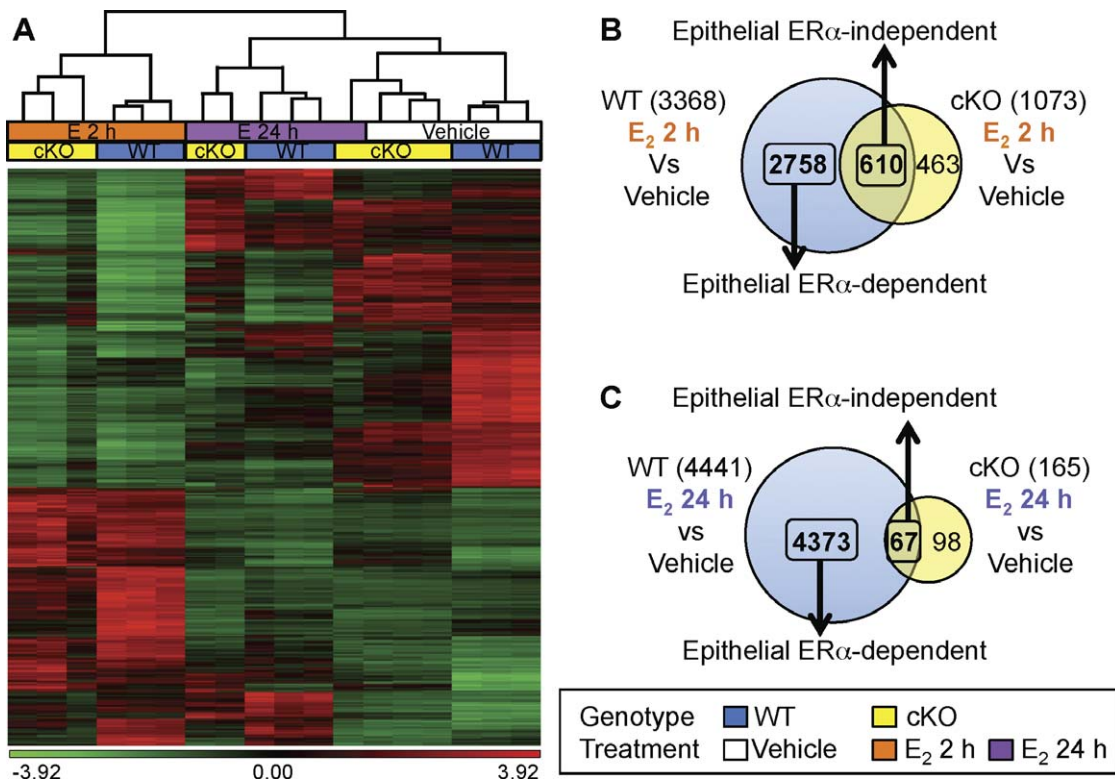


FIG. 3. Microarray analysis of uterine transcripts from WT versus cKO treated with vehicle, E₂ 2 h, or E₂ 24 h. **A**) Unsupervised hierarchical clustering of all replicates from all genotypes and treatment groups using Partek Genomic Suites with the following criteria: raw intensity of at least one sample of each probe ≥ 100 , gene is considered differentially expressed when $P < 0.01$, $|\text{fold change}| \geq 2$, $n = 3$ animals/group. Green or red represent normalized expression that is less or greater than the mean of all conditions, respectively. **B** and **C**) Venn diagram of transcripts differentially expressed relative to vehicle in WT and cKO at **(B)** 2 h and **(C)** 24 h after E₂ treatment using similar statistical criteria as **A**. Numbers in overlapping region are epithelial ER α -independent transcripts. Transcripts differentially expressed only in WT group are epithelial ER α -dependent transcripts.

with vehicle or E₂ for 2 h (early transcripts) or 24 h (late transcripts) to evaluate the functions of E₂-responsive transcripts that might underlie physiological end points at 24 and 72 h, respectively. Unsupervised hierarchical clustering demonstrated that the transcriptional profiles of WT and cKO were similar when treated with E₂ for 2 h, indicating similarities of transcripts regulated by E₂ regardless of the presence of epithelial ER α (Fig. 3A). Venn diagrams demonstrate that approximately only 20% of the transcripts regulated by E₂ at 2 h in WT were also E₂ regulated in cKO uteri (overlap of WT and cKO; Fig. 3B), indicating epithelial ER α -independency. More than 95% of the gene expression at 24 h was regulated by E₂ only in WT uteri, suggesting that the late phase was regulated primarily by epithelial ER α (Fig. 3C). These results indicate that uterine epithelial ER α plays crucial roles for E₂-induced gene transcription with varying degrees for the early (2 h) and late (24 h) responses, but the contribution of the epithelial cells is more pronounced at 24 h, which is consistent with morphological changes observed in the early- and late-phase responses, respectively.

Genes Involved in Proliferation of Epithelial Cells Expressed Independent of Epithelial ER α at 2 h

To characterize the molecular and cellular functions of ER α -independent genes in uteri 2 or 24 h after E₂ treatment, microarray data sets were analyzed using Ingenuity Pathway Analysis. Indeed, at 2 h, common E₂-regulated genes observed in both WT and cKO uteri were from signaling pathways for cellular growth and proliferation, gene expression, and cellular

development (Table 1). A list of genes involved in proliferation of epithelial cells is presented in Supplemental Table S2. Selected genes from the proliferation of epithelial cells category were validated using real-time PCR analysis. Mitotic arrest deficient 2-like protein 1 (*Mad2l1*), vascular endothelial growth factor A (*Vegfa*), inhibin beta B (*Inhbb*), progesterone receptor (*Pgr*), and FBJ murine osteosarcoma viral oncogene homolog (*Fos*) were significantly up-regulated, whereas patch 1 (*Ptch1*) was significantly down-regulated in WT and cKO uteri after 2 h of E₂ treatment (Fig. 4A). Microarray results also showed that cyclin-dependent kinase inhibitor 1a (*Cdkn1a*),

TABLE 1. Epithelial ER α -independent molecular and cellular functions that are commonly regulated in both WT and cKO uteri 2 or 24 h after E₂ treatment.

Categories	P-value	No. of molecules
At 2 h E ₂		
Cellular growth and proliferation	6.58E-11–3.71E-3	138
Gene expression	1.92E-10–3.97E-5	96
Cellular development	1.27E-9–3.73E-3	132
Cell death and survival	2.20E-8–3.53E-3	129
Molecular transport	6.42E-7–3.41E-3	71
At 24 h E ₂		
Cell death and survival	6.55E-8–9.55E-3	5
Cell cycle	1.11E-6–9.24E-3	5
Posttranslational modification	1.84E-5–8.04E-3	2
Cell morphology	6.89E-5–9.55E-3	6
Cellular function	6.89E-5–8.73E-3	4

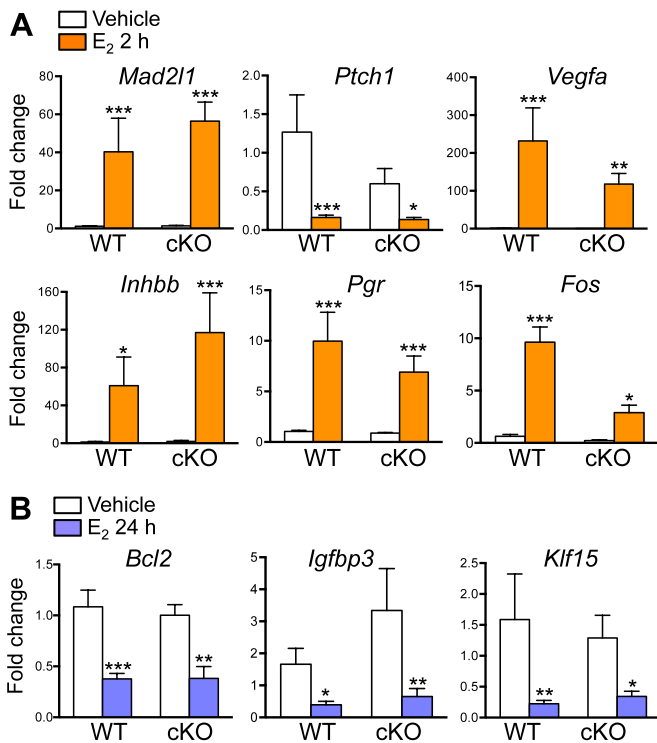


FIG. 4. Uterine epithelial ER α -independent transcripts 2 (A) and 24 (B) h after E₂ treatment. Validated gene expression using real-time PCR analysis of uterine samples from WT and cKO treated with E₂ and collected 2 (A) or 24 (B) h after the treatment, n = 4–7 animals/group. Bar graphs represent mean \pm SEM. *, **, and ***P < 0.05, 0.01, and 0.001, respectively; significant difference when compared to the vehicle-treated group within genotype.

Cebpb, and *Igf1* were increased in both WT and cKO uteri after 2 h of E₂ treatment (Supplemental Table S2), which is consistent with our previous findings [16]. This indicates that genes, most likely from the stroma, are involved in the proliferation of epithelial cells, remain E₂ responsive in cKO, and are sufficient to stimulate epithelial cell proliferation in the absence of epithelial ER α .

At 24 h, there were fewer genes that were commonly regulated by E₂ in both WT and cKO uteri (Fig. 3C) compared to those at 2 h. Ingenuity Pathway Analysis demonstrated that limited numbers of overlapping E₂-regulated molecules were detected in both WT and cKO at 24 h (Table 1). Transcripts represented in these molecular and cellular functions were involved in cell death and survival, cell cycle, posttranslational modification, cell morphology, and cellular function (Table 1). Genes that were involved in cell death and survival network (Supplemental Table S3) were validated, including B-cell CLL/lymphoma 2 (*Bcl2*) and insulin-like growth factor binding protein 3 (*Igfbp3*), which were significantly down-regulated in both WT and cKO uteri (Fig. 4B). In addition, we also found that Krüppel-like factor 15 (*Klf15*), a progesterone (P₄)-regulated gene important for uterine epithelial cell proliferation [23], was also significantly suppressed in both WT and cKO uteri 24 h after E₂ treatment (Fig. 4B).

Lack of E₂ Regulation of Cell Cycle Genes After 24 h in the Absence of Epithelial ER α

Genes involved in cellular development, gene expression, cell morphology, cell signaling, and energy production were preferentially regulated in WT 2 h after E₂ treatment (Table 2).

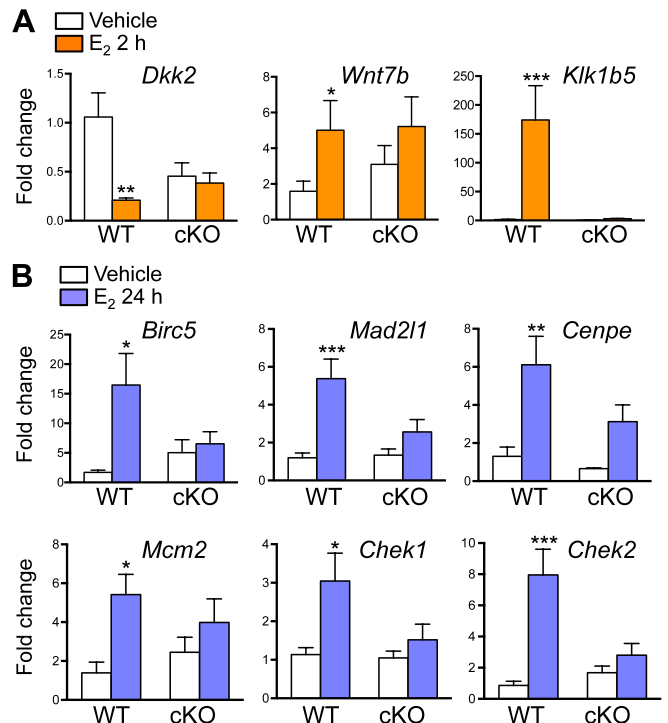


FIG. 5. Uterine epithelial ER α -dependent transcripts 2 (A) and 24 (B) h after E₂ treatment. Validated gene expression using real-time PCR analysis of uterine samples from WT and cKO treated with E₂ and collected 2 (A) or 24 (B) h after the treatment, n = 4–7 animals/group. Bar graphs represent mean \pm SEM. *, **, and ***P < 0.05, 0.01, and 0.001, respectively; significant difference when compared to the vehicle-treated group within genotype.

Interestingly, Wingless MMTV integration site (WNT)/ β -catenin signaling and regulation of the epithelial-mesenchymal transition pathway were categorized as epithelial ER α -dependent canonical pathways, as these genes were significantly regulated only in E₂-treated WT samples (Supplemental Table S4). Of note, there are some genes in cKO uteri that showed more than absolute twofold change; however, the P-values of those genes were greater than 0.01, which did not meet our statistical criteria. Real-time PCR analysis showed that dickkopf 2 homolog (*Dkk2*) was significantly down-regulated and that wingless-type MMTV integration site family, member 7B (*Wnt7b*), was up-regulated only in WT uteri at 2 h after E₂ treatment (Fig. 5A). Additionally, we found that kallikrein 1-related peptidase b5 (*Klk1b5*), an E₂-regulated gene [24, 25], was significantly up-regulated only in WT uteri. Expression levels of *Dkk2*, *Wnt7b*, and *Klk1b5* were unchanged in cKO uteri when treated with E₂ compared to vehicle control (Fig. 5A).

Genes that were not regulated after 24 h of E₂ treatment in the absence of epithelial ER α are listed by various molecular and cellular functions in Table 2. Mitosis was one of the top altered categories in the cellular cycle (Supplemental Table S5). We found that a number of genes in the mitosis category were unchanged in cKO after E₂ treatment, whereas all the genes in this category were up-regulated in WT uteri. Genes involved in mitosis include baculoviral IAP repeat containing 5 (*Birc5*), *Mad211*, centromere protein E (*Cenpe*), checkpoint kinase 1 (*Chek1*), and *Chek2* (Fig. 5B). In addition, we found that minichromosome maintenance deficient 2 mitotin (*Mcm2*), a molecule required for DNA synthesis [23], was up-regulated by E₂ treatment in WT but not in cKO uteri (Fig. 5B). Together, our findings suggest that a blunted uterine

TABLE 2. Molecular and cellular functions that are observed only in WT uteri 2 or 24 h after E₂ treatment.

Categories	P-value	No. of molecules
At 2 h E ₂		
Cellular development	1.24E-8–1.03E-2	166
Gene expression	7.48E-6–9.43E-3	163
Cell morphology	1.22E-5–1.03E-2	159
Cell signaling	1.75E-4–7.82E-3	81
Energy production	2.02E-4–6.73E-3	17
At 24 h E ₂		
Cellular cycle	1.01E-15–4.05E-2	207
Cellular assembly and organization	1.01E-15–3.68E-3	142
DNA replication, recombination, and repair	1.01E-15–3.57E-3	131
Lipid metabolism	2.33E-10–3.83E-3	160
Small molecule biochemistry	2.33E-10–3.83E-3	204

proliferative response occurs in the absence of epithelial ER α , which may be due to a lack E₂ regulation of genes selectively in epithelial cells required for mitosis.

Disrupted P₄ Inhibition of E₂-Induced Epithelial Cell Proliferation in cKO Uteri

Because a number of mitotic genes were unchanged in the cKO uteri 24 h after E₂ treatment, we reasoned that lack of

these gene products would lead to a proliferative defect in uterine stromal cells. It is known that the inhibition of epithelial cell growth and the induction of stromal cell proliferation are crucial for embryo implantation and decidual response. We previously determined that a lack of epithelial ER α causes female infertility partly due to defective implantation [16]. To mimic the hormonal profile during implantation, we administered E₂ followed by P₄ and then P₄ with a nidatory dose of E₂ 18 h prior to tissue collection (called “E+Pe”) to WT or cKO females. In the vehicle-treated group, both WT and cKO uteri showed minimal uterine epithelial proliferation due to a lack of hormones as reported previously [9, 26]. As expected, E+Pe significantly increased uterine weight in WT females (Fig. 6A). Evaluation of the presence of Ki-67 (proliferative marker) or phosphohistone H3 (mitotic marker) demonstrated that, in WT uteri, E+Pe treatment induced the proliferation of stromal cells, whereas basal proliferation in the epithelial cells remained unchanged (Fig. 6, B–E). In cKO uteri, treatment with E+Pe showed a dampened uterine weight increase when compared to the WT-treated group (Fig. 6A). Although there was a slight decrease of the proliferation index (Ki-67 and phosphohistone H3) in the stromal cells of cKO uteri compared with WT uteri (Fig. 6, B–E), there were no significant differences in the Ki-67 and phosphohistone H3-positive cells between E+Pe-treated WT and cKO stroma. However, the Ki-67- and phosphohistone H3-positive cells in the epithelia were significantly higher in cKO compared to WT uteri (Fig. 6, C and E). This suggests that lack of uterine epithelial ER α does not affect stromal cell

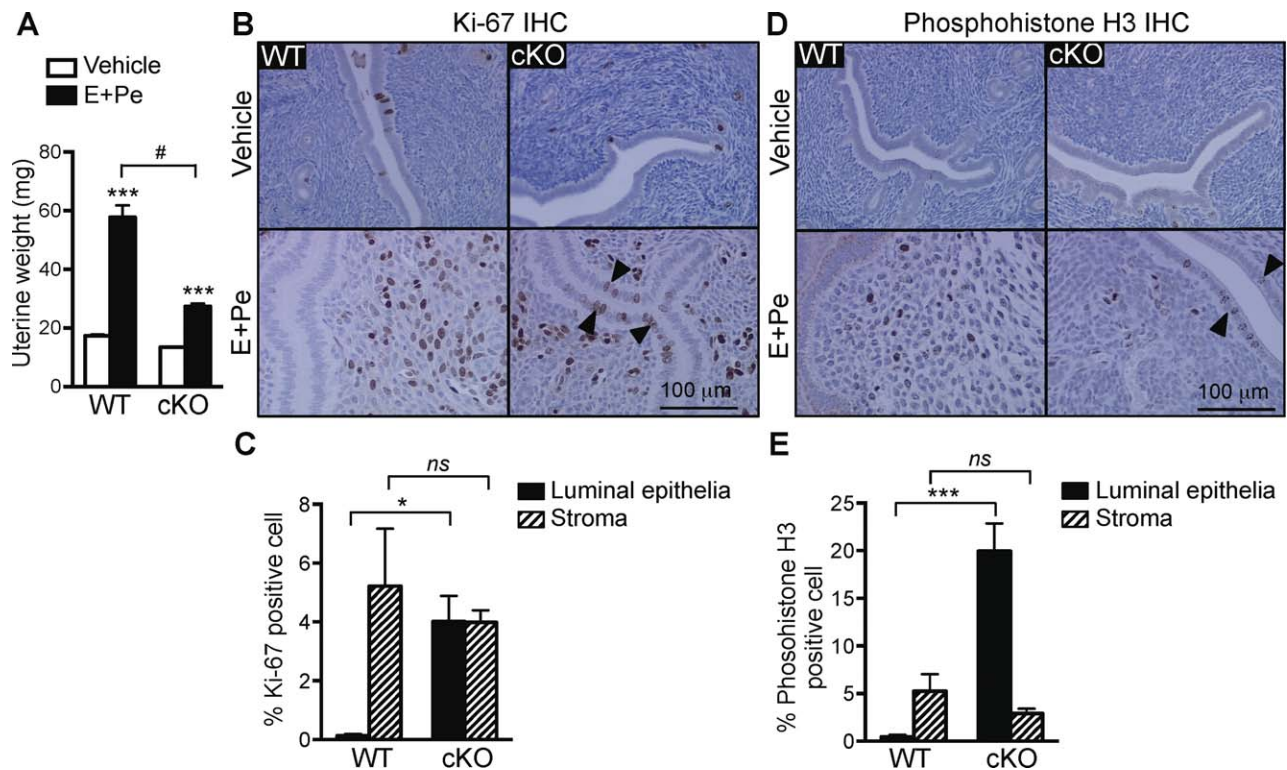


FIG. 6. Epithelial ER α mediates P₄ inhibition of E₂-induced epithelial cell proliferation. **A**) Uterine weight increased in WT and cKO ovariectomized animals after a series of E₂ and P₄ with nidatory E₂ treatments (called “E+Pe”), as described in *Materials and Methods*, compared to vehicle control, n = 5–8 animals/group. Bar graphs represent mean \pm SEM. ****P* < 0.001; significant difference when compared to vehicle control within genotype. #*P* < 0.0001, significant difference when compared between WT E+Pe and cKO E+Pe. **B–D**) Immunohistochemical analyses and quantification of Ki-67 (**B** and **C**) and phosphohistone H3 (**D** and **E**) in WT and cKO uterine cross sections. Representative images are shown (n = 5). Bar = 100 μ m. **C** and **E**) Cells with positive staining of Ki-67 and phosphohistone H3 in the E+Pe-treated groups were counted (detail described in *Materials and Methods*) and presented as percentage of total cell count (luminal epithelial vs. stroma). Ki-67-positive cells were quantified from the whole uterine cross section. Phosphohistone H3-positive cells were quantified from the luminal and subepithelial stromal cells area. * and ****P* < 0.05 and 0.001, significant difference between designated groups. ns, no significant difference.

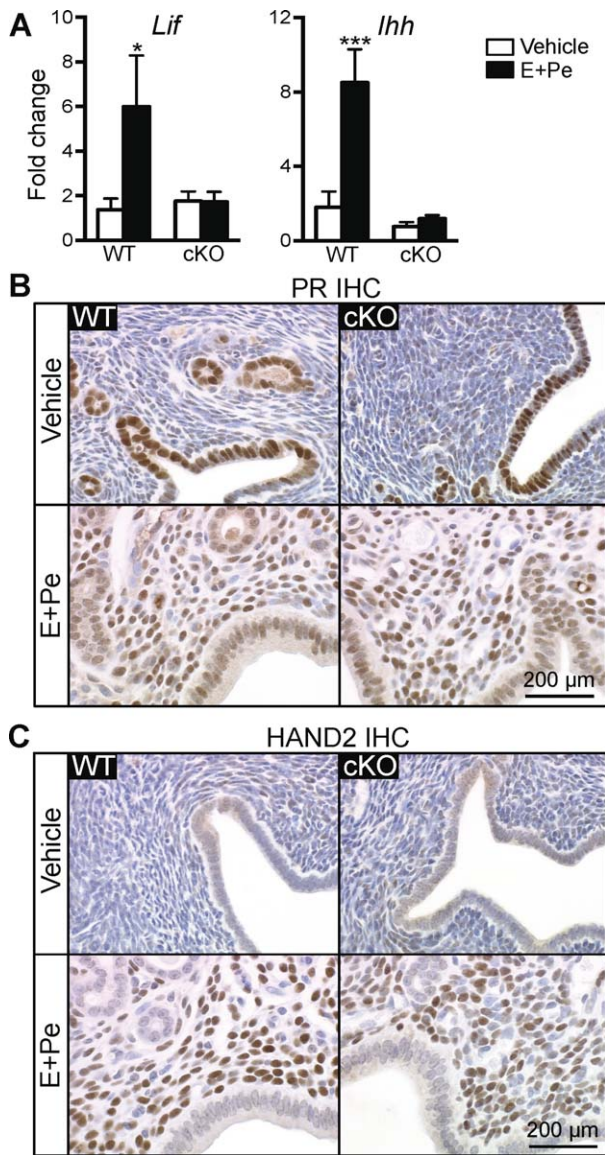


FIG. 7. Epithelial ER α mediates A) *Lif* and *Ihh* induction in WT and cKO ovariectomized animals after E+Pe treatments. Bar graphs represent mean \pm SEM, $n = 5-8$ animals/group. * and *** $P < 0.05$ and 0.001 , respectively; significant difference when compared to vehicle control within genotype. Loss of epithelial ER α does not alter expression pattern of PR (B) and HAND2 (C) when treated with E+Pe. Representative images are shown ($n = 5$).

mitosis and proliferation but leads to an inability to appropriately arrest epithelial cell proliferation. Additionally, leukemia inhibitory factor (*Lif*) [27] and Indian hedgehog (*Ihh*) [28, 29], both required for uterine receptivity and induced in WT uteri, were not induced in the cKO (Fig. 7A). In the vehicle-treated WT and cKO uteri, PR is expressed exclusively in the luminal and glandular epithelial cells (Fig. 7B). Treatment with E+Pe induced the stromal PR and decreased epithelial PR expression in both WT and cKO uteri when compared to vehicle treatment (Fig. 7B). Moreover, expression of HAND2, a PR-regulated transcription factor expressed in uterine stromal cells during implantation [30], was comparably expressed in cKO and WT uteri (Fig. 7C). This suggests that the expression of PR and its downstream effector (HAND2) in the stromal cells were not disrupted by a lack of epithelial ER α . In summary, loss of epithelial ER α disrupted progesterone's

ability to inhibit E₂-induced epithelial cell proliferation but did not affect uterine stromal cell proliferation.

DISCUSSION

Our findings, together with our previous studies, demonstrate that stromal and not epithelial ER α is crucial for regulating gene expression in the acute or early phase of estrogenic responses in the uterus that lead to initial epithelial cell proliferation. Early transcriptional responses (2 h), which underlying initial biological response at 24 h, are preserved in the absence of epithelial ER α (Fig. 8A). However, the deletion of epithelial ER α disrupts the expression of E₂-regulated late transcripts involved in cell cycle progression and thereby impairs subsequent epithelial cell growth and proliferation. These findings indicate that late transcriptional responses (24 h), underlying subsequent or continuous growth (72 h), and depend on epithelial ER α (Fig. 8B). During the receptivity window, uterine epithelial cells ceases proliferation in order for the embryo to attach, while stromal cells undergo proliferation supporting embryo implantation. Our model demonstrated that the epithelial ER α is indispensable for P₄ inhibition of epithelial cell proliferation (Fig. 8C). We also showed that epithelial ER α is crucial for implantation markers expressed in the epithelial cells (*Lif* and *Ihh*). However, stromal PR and HAND2 expression were induced in the absence of epithelial ER α , indicating that epithelial ER α does not account for expression of PR and its downstream signaling molecule (HAND2) in the stromal cells. This model suggests that epithelial ER α also regulates other molecule(s) responsible for inhibition of epithelial proliferation regardless of HAND2 expression.

Epithelial-stromal (or epithelial-mesenchymal) interactions are crucial for tissue development, growth, and differentiation [11]. In mouse uterine tissues, genetic fate mapping has shown that progenitor stromal cells provide the replacement of epithelial cells during tissue remodeling after parturition [19]. In the oviduct, tissue recombination studies demonstrate that the origin of stromal cells underlying epithelium determines the epithelial cell development, characteristics, and ultimately the epithelial cell functions [31]. These studies indicate that stromal cells in the mesenchyme origin determine cell fate and functionality and stimulate the proliferative phase of the epithelial cell responses in female reproductive tracts. In our model, we analyzed the transcriptional profiles in whole uterine tissue in response to E₂ in the absence of epithelial ER α . The findings from this study indicate that early-phase E₂-induced transcriptional changes in the uteri are independent of epithelial ER α . We demonstrated that gene expression induced by E₂ was not due to compensation via ER β increased expression in the uterus due to the loss of ER α but more likely through the remaining stromal ER α . To date, ours is the first finding demonstrating in vivo that signals regulated by ER α in the epithelial cells were indispensable for optimal uterine transcript expression and physiological functions induced by E₂. Moreover, we also showed that epithelial ER α is required for P₄-mediated inhibition of epithelial cell proliferative responses, a process crucial for embryo implantation.

In the initial phase of E₂-induced uterine responses, we observed a comparable increase of epithelial cell height in this study and DNA synthesis and cell proliferation in our previous findings [16] in both WT and cKO animals 24 h after E₂ treatment. Herein, we explored in more detail the uterine transcripts during the early phase (2 h) and found that approximately 20% of differentially regulated transcripts were shared between WT and cKO uteri (ER α -independent

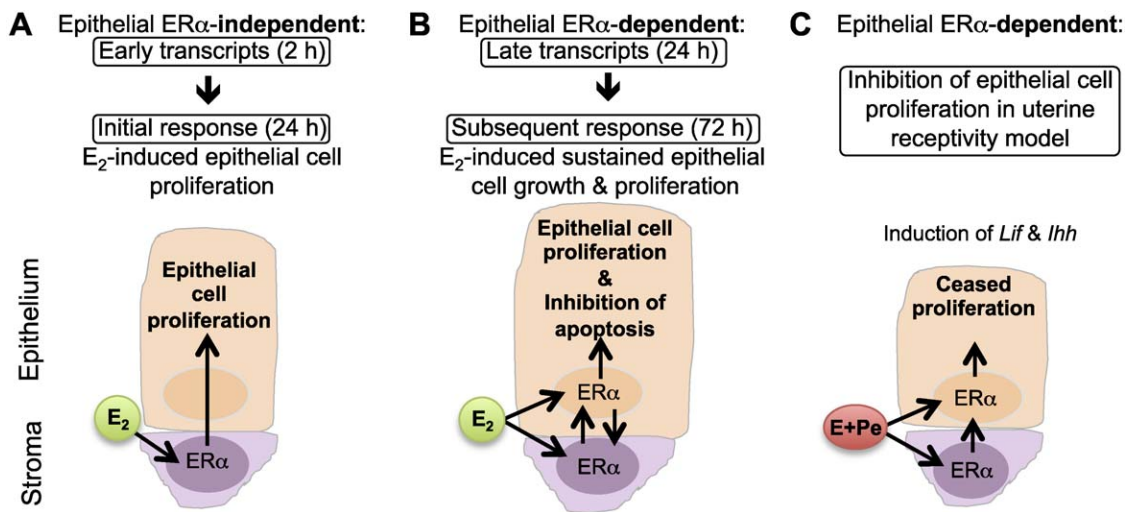


FIG. 8. Working model illustrating epithelial ER α function during uterine proliferation in response to E₂. **A**) Epithelial ER α -independent, early transcriptional phase (2 h) of E₂-induced initial epithelial cell proliferation (24 h). E₂ activates stromal ER α to produce and secrete mitogenic signals (such as growth factors and potentially other molecules shown in Supplemental Table S1) and stimulate uterine epithelial cell proliferation. This initial proliferative event is not regulated by epithelial ER α . **B**) Epithelial ER α -dependent, late transcriptional phase (24 h) of E₂-induced subsequent epithelial cell growth and proliferation (72 h). Both epithelial and stromal ER α are pivotal for a maximal uterine biological response regulated by E₂. At this stage, epithelial ER α may induce transcripts needed to generate autocrine signals or provide positive feedback to the stromal cells to induce gene products involved in mitosis and cell cycle progression as well as inhibition of apoptosis that led to an ultimate physiological end point induced by E₂. **C**) Inhibition of epithelial cell proliferation and induction of implantation markers during the uterine receptivity window require the expression of epithelial ER α . Stromal cell proliferation during this window appears to be independent from epithelial ER α expression.

transcripts). Molecules involved in proliferation of epithelial cells and transcription of RNA, such as *Mad211*, *Vegfa*, *Inhbb*, *Pgr*, and *Fos*, as well as other transcripts shown in Supplemental Table S2, were regulated similarly in WT and cKO uteri. Additionally, our previous findings showed that IGF-1 treatment stimulates uterine epithelial proliferation in the cKO as similar to WT uteri [16]. Basic transcription element binding protein 1 (*Bteb1*, also called *Klf9*), a growth factor-regulated gene [32], was expressed comparably in cKO and WT uteri that were treated with IGF1 (our unpublished data), indicating epithelial ER α -independent activation of growth factor-mediated signals. Together, these findings suggest that epithelial ER α is not responsible for E₂-induced early gene response (2 h) and that signals produced from genomic action of E₂ in cKO uteri at 2 h is dependent on stromal ER α and sufficient to induce epithelial cell proliferation.

WNT signaling regulates left-right patterning and cell development and differentiation through cell-cell communication [33]. In mouse uteri, E₂ increases expression of *Wnt4*, *Wnt5a*, and *Fzd2* (a WNT molecule receptor) but decreases secreted frizzled-related protein-2 (SFRP-2; negative regulator of WNT signaling) expression [34]. Overexpression of SFRP-2 inhibited E₂-induced uterine proliferation [34], indicating that the activation of the WNT/ β -catenin canonical pathway is involved in E₂-induced uterine epithelial cell proliferation. However, Hou et al. [34] showed global deletion of ER α did not affect E₂-regulated expression of WNT signaling molecules in the uteri. In contrast, we found that WNT/ β -catenin signaling was one of the top canonical pathways regulated in an epithelial ER α -dependent manner. We have validated that E₂-mediated regulation of *Dkk2* and *Wnt7b* (signaling molecules in WNT/ β -catenin pathway) was lost in the absence of epithelial ER α . However, lacking E₂ regulation of these molecules in WNT/ β -catenin pathway in cKO uteri did not alter the initial (24 h) proliferative response. Recently, a study using uterine stromal cell-specific knockout of β -catenin demonstrated that loss of stromal β -catenin does not affect

E₂-induced mitosis in the uterine epithelial cells [35]. Together with our results, this suggests that epithelial ER α regulates expression of WNT/ β -catenin signaling molecules, but these molecules were not the only E₂-induced signals needed for epithelial cell proliferation. It is possible that the ER α -independent regulation of uterine WNT/ β -catenin molecules observed in the Hou study was due to the residual splice variant of ER α in the original ER α KO model [36, 37].

Ingenuity pathway analyses of our microarray data sets demonstrated that the top cellular and molecular function at 24 h regulated by epithelial ER α was cell cycle progression. A total of 207 molecules in the cell cycle category that were differentially expressed in the WT were not significantly regulated in cKO uteri. Other E₂-regulated cell cycle mediators during mitosis that were selectively regulated in WT uteri were cyclin, cell division cycle, centromere protein, E2F transcription factor, and kinesin (*Kif*) family members. Previous studies in our laboratory demonstrated that expression of *Mad211*, a component of mitotic spindle assembly checkpoint, peaks 2 h after E₂ treatment and declines but remains elevated 24 h after E₂ treatment [10]. In the present study, we found that *Mad211* was induced in both WT and cKO uteri 2 h after E₂ treatment. However, loss of epithelial ER α led to a return of *Mad211* to basal level 24 h after E₂ treatment. It is possible that epithelial ER α may provide a positive feedback to stromal cells to generate a maximal paracrine mitogenic signal to stimulate epithelial cell proliferation (Fig. 8B). Alternatively, the *Mad211* pattern may also suggest that the initial rise in *Mad211* is due to stromal ER α and that epithelial ER α is required for the subsequent late-phase expression involved in the proliferative response. Overall, these results suggest that proper communication between epithelial and stromal cells is required to properly maintain the optimal proliferative response in the uterus.

We observed unique transcripts expressed only in cKO uteri, which may reflect differences resulting from tissue development in the presence or absence of epithelial ER α .

However, we did not observe any overt morphological defect in cKO uteri. In addition, we also reported that a lack of epithelial ER α cause an aberrant epithelial cell apoptosis subsequent to proliferation in part due to a lack of *Birc1a* expression [16]. In this study, we also showed that inhibitor of apoptosis, *Birc5*, was not significantly up-regulated in cKO uteri after 24 h of E₂ treatment (Supplemental Table S5). However, ingenuity pathway analysis did not indicate a significant change in the apoptotic signaling pathway in cKO uteri at either 2 or 24 h of E₂ treatment compared to WT uteri. It is possible that E₂-regulated transcripts involved in apoptosis may be significantly altered later than the 24-h E₂ treatment in cKO uteri or the changes in apoptosis gene(s) occurred only in the epithelial cells and were diluted by the whole uteri extract, which obscured the difference. Future analyses using laser capture microdissection to distinguish the alteration in expression profile exclusively in epithelial versus stromal cells in the absence of epithelial ER α would allow detection of such subtle differences.

Our previous findings showed that there are no implantation sites in female mice lacking epithelial ER α and that these females are sterile [16]. To understand the role of epithelial ER α and identify signaling molecules downstream of epithelial ER α during implantation, we administered ovarian steroids (E+Pe) to mimic the hormonal profile during the uterine receptive window. In the ovariectomized vehicle-treated mice, the labeling index is extremely low or at a minimally detectable level of cell activity in the absence of ovarian hormones [9, 26]. We have observed the similarly low Ki-67 and phosphohistone H3-positive staining in both WT and cKO uteri. In this experiment of P₄ inhibition of epithelial cell proliferation, we compared the uterine proliferative responses in epithelial versus stromal cells only in the animals that received the E+Pe treatment in the presence or absence of epithelial ER α . In the absence of uterine epithelial ER α , we observed an incomplete inhibition of epithelial cell proliferation compared to WT uteri. This finding indicates that the epithelial ER α is required for P₄ inhibition of E₂-induced proliferation. Due to the limitation of the software we used and the enrichment of the areas we analyzed as described in *Materials and Methods*, the direct comparison between the numbers of Ki-67 and phosphohistone H3-positive cells in epithelial versus stromal cells cannot be made from our analyses. The inhibitory effect on epithelial cells is needed to provide a receptive uterine environment for embryo attachment. Studies using uterine epithelial cell specific knockout of PR demonstrated aberrant P₄ inhibition of E₂-induced epithelial cell proliferation [38]. However, we have shown here that deletion of epithelial ER α has no effect on mRNA level or protein expression of PR [16]. Therefore, a lack of inhibitory effect on epithelial cell proliferation was not due to loss of PR expression.

We found that *Lif*, an E₂-regulated secretory molecule in uterine glandular epithelial cells [27, 39], was not induced during the uterine receptive window in the absence of epithelial ER α . In addition, global deletion of *Ihh*, a P₄-regulated uterine gene, caused an implantation defect and a lack of stromal cell proliferation during the preimplantation period [28]. However, recent studies from our laboratory and others reported that E₂ also induces *Ihh* expression in mouse uteri [29, 40]; however, the induction was minimal when compared to the response induced by P₄. We found that the uterine *Ihh* transcript was not increased in the cKO mice compared to a strong induction in the WT uteri. We also found that expression of *Ptch1*, the *Ihh* receptor, was comparable between WT and cKO uteri. This may be due to the fact that expression of *Ptch1* is restricted in

the uterine stromal cells [28] and would thus be regulated independently from epithelial ER α . Together, these findings suggest that E₂ induces the luminal and glandular epithelial ER α regulation of *Lif* and *Ihh* during implantation. Lack of *Lif* and *Ihh* alters the uterine receptivity and decreases uterine proliferation during implantation in the cKO uteri. This study provides a more complete understanding of an ER α cell-specific functionality and gene-selective expression regarding the interaction of epithelium and stroma in eliciting uterine E₂ responses.

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