Juxtacrine Activity of Estrogen Receptor α in Uterine Stromal Cells is Necessary for Estrogen-Induced Epithelial Cell Proliferation

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Aberrant regulation of uterine cell growth can lead to endometrial cancer and infertility. To understand the molecular mechanisms of estrogen-induced uterine cell growth, we removed the estrogen receptor α (Esr1) from mouse uterine stromal cells, where the embryo is implanted during pregnancy. Without ESR1 in neighboring stroma cells, epithelial cells that line the inside of the uterus are unable to grow due to a lack of growth factors secreted from adjacent stromal cells. Moreover, loss of stromal ESR1 caused mice to deliver fewer pups due in part due to inability of some embryos to implant in the uterus, indicating that stromal ESR1 is crucial for uterine cell growth and pregnancy.

In female mammals, 17β-estradiol (E2), an endogenous estrogen, is primarily produced by the granulosa cells of the ovaries. E2 exerts its activity through estrogen receptors α and β (ESR1 and ESR2)1. Upon E2 binding, ESRs are dimerized, translocated from the cytoplasm into the nucleus, recruited onto targeted DNA sequences, where they initiate or repress transcription of E2-target genes in both non-reproductive and reproductive organs2. Female reproductive tissues including mammary glands, ovaries, oviducts, and the uterus, express both ESR1 and ESR2. In the uterus, ESR1 is the major subtype and is expressed in all cell layers: epithelia (monolayer of cells lining the uterine lumen), stroma (connective tissue in the endometrial lining between epithelia and myometrium), and myometrium (muscle cell layer).

Estrogens induce cell proliferation and growth in both reproductive and non-reproductive tissues (such as osteoblasts and hepatocytes). It has been shown that E2 selectively stimulates proliferation of uterine epithelial cells in adult ovarioctomized mice4–6. Tissue recombination studies using isolated epithelial and stromal cells from wild-type or Esr1−/− neonatal uterine tissues transplanted under the kidney capsule showed that ESR1 is not required in uterine epithelial cells for their proliferation. We have confirmed this observation using an adult epithelial cell specific knockout mouse model (Wnt7aCre/+; Esr1fl/fl)7, 8, with an intact (no tissue disruption and recombination) uterine tissue structure. The results from these two studies support a mechanism in which E2 treatment and activation of ESR1 in the stromal cells produces mitogenic factors, including insulin-like growth factor 1 (IGF1)9, 10, and transforming growth factor 11, which stimulate their subsequent signaling cascades in uterine epithelial cells, leading to cell proliferation. However, a functional requirement of stromal ESR1 in the normal uterine environment in vivo has not yet been explored.

In addition to cell proliferative events accompanying E2 treatment, we also evaluated the role of stromal ESR1 in female reproductive functions in this study. In normal mouse reproduction, the presence of a copulatory plug is observed the morning after mating is designated 0.5 days post coitus (dpc). At 0.5 dpc, the oocytes are fertilized by the sperm and during 3.0 dpc the embryos develop into morulas or blastocysts within the oviducts (known as Fallopian tubes in humans). In rodents, the blastocysts transit the oviduct to the uterus where they implant exclusively onto the anti-mesometrial pole of the uterine wall at approximately 4.0 dpc12. Embryo attachment requires secretion of leukemia inhibitory factor (LIF), an implantation facilitating cytokine, from uterine glands located in the anti-mesometrial pole of the uterus13. After embryo implantation, the uterine endometrium undergoes a

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decidual response (called decidualization), in which the stromal cells proliferate and differentiate into decidua. The decidual cells surrounding the embryos provide nutrients and support for the developing fetus before the placenta starts to fully function. The placenta forms on the mesometrial pole of the uterus, where the blood vessels are supplied via the uterine broad ligament. These implantation and decidualization processes are orchestrated by ovarian steroid hormones (E2 and progesterone; P4) through ESR1 and progesterone receptor (PGR).

We previously showed that female mice with a global deletion of ESR1 (Esr1−/−) are infertile, in part due to an implantation defect. Using a female reproductive tract epithelial cell ESR1 null mouse model (Wnt7aCre/−; Esr1−/−), our group and others have demonstrated that uterine epithelial ESR1 is crucial for embryo implantation, decidual response, and fertility. From these previous findings, we hypothesized that a lack of stromal ESR1 could lead to aberrant uterine cell proliferation, while not affecting embryo implantation or uterine decidual response. To test our hypothesis, we have generated a mouse model lacking stromal ESR1, specifically at the anti-mesometrial pole of the uterus. Here we report that stromal ESR1 is required for epithelial cell proliferation. Surprisingly, stromal ESR1 in the uterine anti-mesometrium is also crucial for optimal embryo implantation and artificially induced-decidualization.

Results

Stromal ESR1 underlying uterine epithelial cells is essential for E2-induced proliferative response. Amhr2Cre+/− mice were bred with Esr1f/+ to specifically delete ESR1 in uterine stromal cells. Deletion of Esr1 in the uterine tissues was confirmed using ESR1 immunohistochemical (IHC) analysis in 12-week-old mice. In the control uteri (Esr1f/+), ESR1 protein was detected throughout uterine cross-sections, including epithelial, stromal, and muscle cell layers (Fig. 1). In the Amhr2Cre+/−; Esr1f/+ uteri, ESR1 protein was ablated in the stromal cells of the uterine anti-mesometrial pole whereas the expression of ESR1 remained intact in the epithelial cell layer as well as in the stromal cells in the mesometrial pole (Fig. 1). We observed variable degrees of ESR1 deletion in individual Amhr2Cre+/−; Esr1f/+ mice. This is likely due to uneven expression of the Cre-recombinase amongst stromal cells in the Amhr2Cre+/− mouse line. Therefore, the extent of deletion of ESR1 in the circular smooth muscle cells varied between individual animals (Supplementary Fig. S1).

Using our previous mouse model in which Esr1 is selectively deleted in uterine epithelial cells, we reported that uterine epithelial ESR1 was not required for E2 to induce uterine epithelial cell proliferation. No E2 induced epithelial proliferation occurs in global Esr1-null uteri, demonstrating that uterine ESR1 is needed to mediate epithelial cell proliferation, thus we hypothesized that stromal ESR1 was required for paracrine regulation of epithelial cell proliferation. We collected the tissues 24 h after E2 treatment of ovariectomized 8–12-week-old females to observe E2-induced uterine wet weight increase, which reflects uterine growth (late response). In Esr1f/+ uteri, E2 significantly increased the uterine wet weight compared to the vehicle treated controls (Fig. 2A). However, there was no uterine weight increase in the E2 treated Amhr2Cre+/−; Esr1f/+ uteri compared to the vehicle control. Evaluation of cellular proliferative responses to E2 as reflected by expression of Ki67 proliferative marker, revealed few Ki67 positive cells in vehicle treated uteri of both genotypes. As expected, the luminal epithelial cells of E2 treated Esr1f/+ uteri were positive for Ki67 (Fig. 2B). However, the luminal epithelial cells exclusively in the mesometrial but not in the anti-mesometrial pole of the E2 treated Amhr2Cre+/−; Esr1f/+ uteri were positive for the Ki67 staining (Fig. 2B and higher magnification in Fig. 2C). A limited number of glandular epithelial cells were positive for Ki67 staining in both Esr1f/+ and Amhr2Cre+/−; Esr1f/+ uteri (Fig. 2B to C). Therefore, the Ki67-positive cells in the glandular epithelia were excluded from quantification of Ki67-positive cells. We found that the percentage of luminal epithelial cells that were Ki67-positive in the anti-mesometrial pole after 24 h E2 treatment was significantly less in Amhr2Cre+/−; Esr1f/+ than in Esr1f/+ uteri, whereas the percentage of epithelial cells that were proliferating in the mesometrial pole was similar in both Amhr2Cre+/−; Esr1f/+ and Esr1f/+ uteri (Fig. 2D). To determine whether the expression level of ESR1 in mesometrial and anti-mesometrial uterine stromal cells of Amhr2Cre+/−; Esr1f/+ corresponds with the E2-induced proliferative response of epithelial cells, we compared ESR1 and Ki67 in adjacent sections. We found that ESR1 was ablated in anti-mesometrial stromal cells next to non-proliferating epithelial cells in Amhr2Cre+/−; Esr1f/+ uteri (Fig. 2C and Supplementary Fig. S2). This finding suggests that loss of ESR1 in stromal cells prevents proliferation of immediately adjacent epithelial cells, indicating that signals emanating from stromal cells directly adjacent to responding epithelial cells transduce the required stimulus for proliferation. This stimulus is apparently unable to diffuse throughout the tissue, but rather works in a juxtacrine manner on neighboring epithelial cells.

Loss of stromal ESR1 leads to blunted E2-induced cell cycle-related transcripts and proteins. We previously reported that several genes associated with cell-cycle progression, including Igf1, CCAAT enhancer binding protein beta (Cebpβ), cyclin-dependent kinase inhibitor 1a (Cdkn1a), and mitotic arrest deficient 2-like protein 1 (Mad2l1), were E2 responsive genes and that their expression was epithelial ESR1-independent, therefore, potentially mediated by stromal ESR1. To confirm that the expression of these transcripts and encoded proteins were stromal ESR1-dependent, we euthanized ovariectomized 8–12-week-old animals and collected the uterine tissues 6 h after the injection of E2, Igf1, Mad2l1, and Cdkn1a were significantly increased by E2 treatment compared to vehicle treated uteri in both Esr1f/+ and Amhr2Cre+/−; Esr1f/+ groups (Fig. 3A). However, the E2 induction of these transcripts in Amhr2Cre+/−; Esr1f/+ uteri was blunted in comparison to the response of E2 treated Esr1f/+ uteri; the Igf1 was the most blunted (Fig. 3A). Mantena et al. have demonstrated that Cebpβ expression is rapidly induced in uterine stromal cells by E2, and contributes to uterine epithelial cell proliferation. Therefore, we reasoned that the deletion of stromal ESR1 would alter Cebp expression in the uterus. However, we found that E2 induced similar levels of Cebpβ transcript in Esr1f/+ and Amhr2Cre+/−; Esr1f/+ groups (Fig. 3A). To evaluate whether expression of the Cebpβ gene in the whole uterus masked any differences in induction in stromal cells, expression of CEBPB protein was examined in uterine sections using IHC analysis. After E2 treatment, CEBPB was highly expressed in both epithelial and stromal cells in both the mesometrium and anti-mesometrium of the uterus.
Esr1\(^{f/-}\) uteri (Fig. 3B). In Amhr2\(^{2cre/+}\); Esr1\(^{f/-}\) uteri, CEBPB was highly induced in the mesometrial area, whereas the expression was minimally detected in the anti-mesometrial area (Fig. 3B).

In addition to these E\(_2\)-responsive genes, other factors including kruppel like factor 4 (Klf4) and minichromosome maintenance complex components (Mcm2 and Mcm4) are also involved in E\(_2\)-induced uterine proliferation.\(^{24}\) We found that Klf4 was significantly induced by E\(_2\) treatment in both Esr1\(^{f/-}\) and Amhr2\(^{2cre/+}\); Esr1\(^{f/-}\) uteri (Fig. 3A). However, Mcm2 and Mcm4 transcripts tended to be induced by E\(_2\) but not at significant levels. As expected, Klf15 was not increased by E\(_2\) treatment as Klf15 expression was previously shown to be regulated by P4.\(^{24}\) These results suggest that uterine stromal ESR1 mediates the expression of some cell-cycle regulated genes and protein in response to E\(_2\) treatment.

We previously reported that deletion of ESR1 from epithelial cells had no effect on the expression of progesterone receptor (PGR), a hallmark E\(_2\)-induced protein in the uterus (after 24 h of treatment\(^{6}\)). We collected uterine tissues and evaluated the PGR protein levels using IHC analysis to determine how loss of anti-mesometrial stromal ESR1 affected uterine PGR expression. We found that E\(_2\) treatment compared to vehicle significantly increased PGR signal intensity in the cytoplasmic compartment in the mesometrial pole of both Esr1\(^{f/-}\) and Amhr2\(^{2cre/+}\); Esr1\(^{f/-}\) animals (Fig. 4A; yellow arrowheads and Fig. 4B). In Esr1\(^{f/-}\) uteri, E\(_2\) had a tendency to increase anti-mesometrial cytosolic PGR signal intensity (\(p = 0.2437\)). However, in the absence of anti-mesometrial stromal ESR1 in Amhr2\(^{2cre/+}\); Esr1\(^{f/-}\) animals, anti-mesometrial cytosolic PGR signal intensities...
in vehicle and E₂ treatment were similar (p = 0.5765). In addition, the proportion of PGR-positive stromal cells was significantly increased in E₂-treated Esr1⁻⁻ uteri in both mesometrial and anti-mesometrial poles (Fig. 4C). However, E₂ treatment only increased PGR-positive cells in the mesometrial stromal cells of Amhr2Cre/++;Esr1⁻⁻.

Figure 2. Uterine response to E₂ treatment (24 h) in the absence of anti-mesometrial stromal ESR1. Adult (8–12-week-old) Esr1⁻⁻ and Amhr2Cre/++;Esr1⁻⁻ females were ovariectomized and treated with vehicle or E₂ for 24 h. (A) Uterine wet weight after 24 h of E₂ treatment. *p < 0.05; significant difference between vehicle and E₂ treated samples within genotype. N = 3 mice/genotype/treatment. (B) Uterine epithelial cell proliferation determined by Ki67 IHC staining in Esr1⁻⁻ and Amhr2Cre/++;Esr1⁻⁻ uteri. (C) Higher magnification of Amhr2Cre/++;Esr1⁻⁻ treated with E₂ for 24 h. Uterine sections were stained with Ki67 and ESR1 antibodies in adjacent sections. Note that epithelial cell proliferation, as indicated by the appearance of Ki67, is primarily observed in the M where ESR1 is expressed in the adjacent stromal cells. (D) Percentage of Ki67-positive cells of total luminal epithelial cells in M vs. AM regions. *p < 0.05; significant difference between vehicle and E₂ treated samples within genotype and region. #p < 0.05; significant difference between Esr1⁻⁻ and Amhr2Cre/++;Esr1⁻⁻ uteri after E₂ treatment in the AM region, unpaired t-test. N = 4–8 mice/genotype/treatment. All graphs represent mean ± SEM. M = Mesometrium, AM = Anti-mesometrium. Representative images shown.
Figure 3. Cell proliferation-related uterine transcripts and protein in ovariectomized 8–12-week-old Esr1\textsuperscript{f−} and Amhr2\textsuperscript{cre+}; Esr1\textsuperscript{f−} females treated with E\textsubscript{2} for 6 h. (A) Real-time PCR was performed and the relative expression values of Igf1, Mad2l1, Cdkn1a, Cebpb, Klf4, Mcm2, Mcm4, and Klf15 were normalized to Rpl7. *, ***p < 0.05, 0.001; significant difference between vehicle and E\textsubscript{2} treated samples within genotype. #p < 0.05; significant difference between E\textsubscript{2} treated samples between genotype; unpaired t-test. (B) CEBPB protein expression after 6 h of E\textsubscript{2} treatment in Esr1\textsuperscript{f−} and Amhr2\textsuperscript{cre+}; Esr1\textsuperscript{f−} uteri using IHC analysis. All graphs represent mean ± SEM. N = 3–5 mice/genotype/treatment. M = Mesometrium, AM = Anti-mesometrium. Representative images shown.
From these findings, we conclude that ESR1 must be present in all uterine stromal cells for \( E_2 \) to fully induce epithelial cell proliferation and properly regulate the pattern of PGR expression.

**Loss of stromal ESR1 in the uterus causes severe fertility defect.** To evaluate whether the anti-mesometrial stromal ESR1 is functionally required for female fertility, we determined the number of pups born to adult female mice during a 6-month period. The total number of pups delivered by Amhr2\(^{-/-}\)Esr1\(^{f/f}\) dams (0.7 ± 0.6 pups/dam) was significantly less than the number delivered by Esr1\(^{f/f}\) dams (38.3 ± 3.5 pups/dam) (Fig. 5A). Eight out of ten Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) dams evaluated did not deliver any pups. Because Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) was also expressed in the ovaries, we then investigated whether the subfertility phenotype in Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) females was due to impaired ovulation. Comparable numbers of oocytes were ovulated following gonadotropin stimulation of prepubertal Esr1\(^{f/f}\) and Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) females (Fig. 5B). Since ovulation occurs normally, and our recent findings indicate that blastocyst development is not affected by the deletion of stromal ESR1 in the oviducts, we reasoned that the subfertility phenotype in Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) females was due to impaired uterine function as a result of deletion of anti-mesometrial stromal ESR1. Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) adult females had significantly fewer implantation sites at 4.5 dpc, than Esr1\(^{f/f}\) females (Fig. 5C). However, Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) implantation sites exhibited no apparent morphological defects (Fig. 5D).

To determine the potential cause of impaired implantation observed at 4.5 dpc, we evaluated several key uterine receptivity genes expressed during early gestation (3.5 dpc), at which time nidatory levels of \( E_2 \) are secreted to prepare the uteri for embryo implantation. We found the hallmark implantation markers, Ltf and Indian hedgehog (Ihh), tended to be expressed at higher levels in Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) compared to Esr1\(^{f/f}\) (Fig. 5E). Surprisingly, significant elevation of \( E_2 \)-responsive genes such as munc1 (Muc1), lactotransferrin (Ltf), and chloride channel calcium-activated 3 (Clca3) was observed in Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) compared to Esr1\(^{f/f}\) uteri. These transcripts are normally suppressed during the 3.5 dpc pre-implantation period. Proper Wnt5a expression levels are crucial for embryo homing and optimal implantation. Here, we demonstrated that Wnt5a transcript was significantly elevated in Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) compared to Esr1\(^{f/f}\) uteri at 3.5 dpc. However, heparin-binding EGF-like growth factor (Hbegf, another implantation marker) was expressed at comparable levels in Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) and Esr1\(^{f/f}\) uteri. These findings indicate that stromal ESR1 in the anti-mesometrium is required for the suppression of some \( E_2 \)-regulated transcripts during implantation.

**Stromal ESR1 ablation contributes to a lack of uterine stromal cell proliferation.** Successful embryo implantation requires the cessation of uterine epithelial cell proliferation and subsequent stromal cell proliferation. Because we observed impaired uterine receptivity, we investigated whether lacking ESR1 in the stromal cells contributed to defective uterine stromal cell proliferation or prevention of the cessation of the epithelial cell proliferation. To mimic the hormonal profile during embryo implantation, 8–12-week-old animals were ovarioctomized and treated with \( E + P_e \) (see Methods for detail). Mice lacking anti-mesometrial stromal ESR1 did not show a uterine weight increase after \( E + P_e \) treatment (Fig. 6A). Moreover, uterine stromal cell proliferation was blunted in the absence of stromal ESR1 in the anti-mesometrial region (Fig. 6B). However, the uterine epithelial cells ceased proliferation similarly in Esr1\(^{f/f}\) and Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) E+P treated uteri (Fig. 6B). In the

**Figure 4.** \( E_2 \)-induced progesterone receptor (PGR) expression in the uterus. Adult (8–12-week-old) female mice were ovarioctomized and treated with vehicle or \( E_2 \) for 24 h. (A) Top panel: Cross-sections of the whole uteri were stained with PGR antibodies. Bottom panels: PGR expression pattern in the mesometrial (M) vs. anti-mesometrial (AM) poles in Esr1\(^{f/f}\) and Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) uteri. Representative images shown. (B) Relative signal intensities of nuclear (Nuc) and cytosolic (Cyto) compartments in the uterine luminal epithelial cells of Esr1\(^{f/f}\) and Amhr2\(^{2+/+}\); Esr1\(^{f/f}\) uteri after vehicle and \( E_2 \) treatment for 24 h. (C) Percentage of PGR-positive cells of total stromal cells in M vs. AM regions. \(*p < 0.05\); significant difference between vehicle and \( E_2 \) treated samples within genotype and region. All graphs represent mean ± SEM. N = 3 mice/genotype/treatment.
absence of stromal ESR1, Lif expression was similarly induced in Esr1\(^{-/-}\) and Amhr2\(^{Cre+}\); Esr1\(^{-/-}\) E+Pe treated uteri (Fig. 6C). We then evaluated whether a lack of stromal cell proliferation was due to a loss of CEBPB expression. In the E+Poi control treatment group, there were fewer cells expressing CEBPB in the Amhr2\(^{Cre+}\); Esr1\(^{-/-}\) compared to Esr1\(^{-/-}\) uteri (Fig. 6D). However, upon E+Pe treatment, CEBPB was expressed similarly between Esr1\(^{-/-}\) and Amhr2\(^{Cre+}\); Esr1\(^{-/-}\) uteri (Fig. 6D). These results suggest that anti-mesometrial stromal ESR1 is required for the stromal cell proliferation and defective stromal cell proliferation is not due to a lack of CEBPB expression.

In addition, we evaluated 4.5 dpc implantation sites from 8–12-week-old mice to determine whether stromal ESR1 deletion in the anti-mesometrium altered CEBPB expression. At 4.5 dpc, CEBPB is expressed homogeneously in the uterine epithelial and stromal cells surrounding the implantation sites (Fig. 6E), regardless of the expression of Esr1 in the stroma. Expression of ESR1 was not detected in the primary decidual zone in either Esr1\(^{-/-}\) or Amhr2\(^{Cre+}\); Esr1\(^{-/-}\) uteri. These results indicate that CEBPB is expressed independently from ESR1 expression in the implantation sites and that the implantation defect observed in the Amhr2\(^{Cre+}\); Esr1\(^{-/-}\) females is not due to a lack of CEBPB.

Ablation of anti-mesometrial stromal ESR1 caused impaired uterine decidual response after artificial stimulation. Pawar et al. have shown that a lack of epithelial ESR1 impairs decidualization\(^1\). To
clarify whether anti-mesometrial stromal ESR1 was also required for uterine decidualization, we evaluated the decidual response using a well-established method of injection of inert oil into the uterine lumen to artificially stimulate decidualization\(^\text{17}\). In 8–12-week-old Esr1\(^{−/−}\) animals, artificial stimulation resulted in decidualization of 6 out of 10 uteri, whereas 2 out of 9 Amhr2\(^{2cre}\); Esr1\(^{−/−}\) females responded (Fig. 7A). The uterine weight increase resulting from decidual stimulation was significantly greater in Esr1\(^{−/−}\) than in Amhr2\(^{2cre}\); Esr1\(^{−/−}\) –uteri (Fig. 7B). Decidual markers and cell-cycle regulated genes, including bone morphogenenic protein 2 (Bmp2), prolactin family 8, subfamily a, member 2 (Prl8a2), cyclin B1 (Cdc2a), and cyclin dependent kinase A1 (Ccda2a), were significantly induced in stimulated horns compared to un-stimulated horns in Esr1\(^{−/−}\) –uteri (Fig. 7C). The expression of these genes tended to be increased in responding horns of Amhr2\(^{2cre}\); Esr1\(^{−/−}\) animals, however, statistical analysis could not be performed as only 2 of 9 Amhr2\(^{2cre}\); Esr1\(^{−/−}\) animals responded to the artificial stimulation. As we observed an impaired decidual response in Amhr2\(^{2cre}\); Esr1\(^{−/−}\) animals, we next evaluated whether stromal cell proliferation was affected by a lack of stromal ESR1 by using an EdU incorporation assay. EdU was mainly detected in the mesometrial pole of Esr1\(^{−/−}\) –uteri, whereas a smaller area of EdU incorporation was seen in the anti-mesometrial pole of the decidualized Amhr2\(^{2cre}\); Esr1\(^{−/−}\) uterus. (Fig. 7D). However, EdU incorporation was not or was minimally detected in stimulated non-responding Amhr2\(^{2cre}\); Esr1\(^{−/−}\) –uteri (Supplementary Fig. S3).

Because the uterus responds differently to artificial stimulation and embryo-initiated decidualization\(^\text{16}\), to determine whether stromal ESR1 is required for natural decidualization, we assessed the decidual response 5.5 and 7.5 dpc after natural mating of Esr1\(^{−/−}\) and Amhr2\(^{2cre}\); Esr1\(^{−/−}\) adult females. We found that PGR was expressed similarly in Amhr2\(^{2cre}\); Esr1\(^{−/−}\) and Esr1\(^{−/−}\) –uteri regardless of ESR1 expression status in the anti-mesometrium (Fig. 8A). Moreover, proliferation of the decidual cells in the anti-mesometrium was comparable between Esr1\(^{−/−}\) and Amhr2\(^{2cre}\); Esr1\(^{−/−}\) –uteri at 5.5 and 7.5 dpc using Ki67 IHC analysis (Fig. 8B). There was no significant difference in expression of decidual gene markers, including epidermal growth factor receptor (Egfr), FK506 binding protein 5 (Fkbp5), prostaglandin-endoperoxide synthase 2 (Ptgs2), wingless-type MMTV integration site family, member 4 (Wnt4), Bmp2, and Prl8a2 between Esr1\(^{−/−}\) and Amhr2\(^{2cre}\); Esr1\(^{−/−}\) –uteri at 5.5 dpc (Fig. 8C). These findings suggest that ESR1 expression in anti-mesometrial stromal cells is required for normal decidualization in response to artificial stimulation, but that decidualization can occur after implantation.

**Loss of stromal ESR1 in uterine anti-mesometrium leads to an increased resorption of embryos post-decidualization.** In the absence of stromal ESR1 in the anti-mesometrium, there was a 50% increase in resorption sites compared to Esr1\(^{−/−}\) controls (Fig. 9A). To determine the cause of embryo resorption during this post-decidualization period of pregnancy, we assessed the expression of angiogenic genes in 10.5 dpc implantation sites. Several studies show that uterine decidual response is marked by an increase in uterine vasculature that subsequently provides nutrients to developing embryos\(^\text{44}\). Decidual vascularization is mainly

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**Figure 6.** Proliferation of the uterine stromal cells after a series of E\(_2\) and P\(_4\) treatments (E + Pe) to mimic the hormonal profile during implantation and at 4.5 dpc. (A) Uterine wet weights of Esr1\(^{−/−}\) and Amhr2\(^{2cre}\); Esr1\(^{−/−}\) –8–12-week-old females mice treated with E + Poil or E + Pe. \(p < 0.05\); significant difference between E + Pe treated Esr1\(^{−/−}\) vs. Amhr2\(^{2cre}\); Esr1\(^{−/−}\) females. (B) EdU incorporation assay of Esr1\(^{−/−}\) and Amhr2\(^{2cre}\); Esr1\(^{−/−}\) –female mice that were treated with E + Poil or E + Pe. Cells with green signal represent EdU positive (DNA synthesis) cells. Cells with blue signal represent the nuclei stained with Hoechst. (C) Real-time PCR analysis of Lif. Values were normalized to Rpl7. **\(p < 0.01\); significant difference between E + Poil and E + Pe treated samples within genotype. All graphs represent mean ± SEM. (D) CEBPB IHC staining of Esr1\(^{−/−}\) and Amhr2\(^{2cre}\); Esr1\(^{−/−}\) –uterine cross sections at 4.5 dpc using IHC analysis. Representative images shown. N = 3–4 mice/genotype/treatment. M = Mesometrium, AM = Anti-mesometrium. (E) Expression of CEBPB and ESR1 proteins of implantation sites from Esr1\(^{−/−}\) and Amhr2\(^{2cre}\); Esr1\(^{−/−}\) –uteri at 5.5 and 7.5 dpc using Ki67 IHC analysis (Fig. 8B). There was no significant differ -
regulated by vascular endothelial growth factors (VEGFs) and angiopoietins. The hallmark features of decidual vascularization consist of angiogenic genes, including Vegfa, Vegfb, Vegfc, FMS-like tyrosine kinase 1 (Flt1), also known as Vegf receptor 1 or Vegfr1), kinase insert domain protein receptor (Kdr, also known as Vegfr2), Flt4 (or Vegfr3), angiopoietin 2 (Angpt2), adrenomedullin (Adm), anti-angiogenic factors such as thrombospondin (Thbs1), and gap junction protein alpha 1 (Gja1, also known as connexin 43). At 10.5 dpc, the implantation sites of Amhr2Cre+;Esr1f− mice showed significantly higher levels of Vegfb, Kdr, and Thbs1 compared to those of Eomes−/− females, while other angiogenic genes (Vegfa, Vegfc, Flt1, Flt4, Angpt2, Adm and Gja1) were expressed at similar levels (Fig. 9B). This finding indicates that there is slight difference in the expression of angiogenic and anti-angiogenic markers in the absence of anti-mesometrial stromal ERα, which may perturb optimal angiogenesis, leading to a failure of embryo development, and increased embryo resorption.

**Discussion**

We report here that the expression of ERα in uterine stromal cells is necessary for E2-induced epithelial cell proliferation. Initially, based on the previous evidence that epithelial ERα regulated implantation and decidualization, we hypothesized that stromal ERα was not necessary for embryo implantation and decidualization. However, the results presented in this study demonstrate that loss of stromal ERα caused over-expression of E2-regulated genes that are normally suppressed during early pregnancy to provide a receptive uterine environment, leading to decreased embryo implantation. Stromal ERα also appeared to be required for uterine decidual response to artificial stimulation. Additionally, we observed aberrant expression of some angiogenic factors at 10.5 dpc, which could lead to disrupted angiogenesis in the absence of stromal ERα in the anti-mesometrium.
Together, our findings indicate that uterine epithelial cell proliferation is modulated by stromal ESR1 and that E₂ orchestrates its function through both epithelial and stromal ESR1 in order to provide the optimal uterine environment for embryo implantation and uterine decidualization.
Consistent with previous findings, the Cre activity of Amhr2\textsuperscript{Cre/+} animals was only active in the anti-mesometrial pole of the uterus\textsuperscript{8-20}, therefore, in Amhr2\textsuperscript{Cre/+}; Esr1\textsuperscript{−/−} animals, the deletion of stromal ESR1 was observed only in the anti-mesometrium leaving the expression of stromal ESR1 in the mesometrium intact. Such a model system has exceptional specificity, since in the same animal only a portion of the tissue is affected by Cre expression, while other portions essentially function as an internal control. Note that we observed a higher level of ESR1 expression in anti-mesometrial epithelial cells than in mesometrial epithelial cells, however, only in some animals (3/8 Amhr2\textsuperscript{Cre/+}; Esr1\textsuperscript{−/−} animals evaluated, Supplementary Fig. S1). This phenomenon might occur because luminal epithelial cells that developed in a region lacking stromal ESR1 were developmentally altered and had characteristics of glandular epithelial cells, which normally expressed more ESR1 than epithelial cells.

Along with several groups, our laboratory, has demonstrated that E\textsubscript{2} mediates its proliferative effect through stromal ESR1 activity via paracrine activity by inducing secretion of growth factors, such as Igf1, and cell-cycle related proteins, including Mad2l1, Cdkn1a, and Cebpb\textsuperscript{−/−},\textsuperscript{11, 22, 23, 37, 38}. These findings are consistent with our previous report that the ablation of epithelial ESR1 does not affect the expression level of these growth factors and cell cycle regulated genes\textsuperscript{9}. However, in our stromal ESR1 deletion model, E\textsubscript{2}-induced Igf1, Mad2l1, Cdkn1a, and Cebpb were attenuated, but not absent. This discrepancy likely reflects selective deletion of anti-mesometrial stromal ESR1 and retention of mesometrial ESR1 to mediate the observed responses.

Here, we observed prominent epithelial cell proliferation in the mesometrium after E\textsubscript{2} treatment, whereas the proliferation was blunted in the anti-mesometrium. These findings illustrate the local requirement of stromal ESR1 activity for epithelial proliferation, indicating a juxtacrine mechanism in which the stromal factors have a localized action and primarily affect neighboring epithelial cells. This unique responsive pattern in the Amhr2\textsuperscript{Cre/+}; Esr1\textsuperscript{−/−} uteri was with Kid7, CEBPB, and PGR protein expression after E\textsubscript{2} treatment. By selectively deleting ESR1 in the anti-mesometrium, we have created a unique tool, in which both positive and negative controls are present in the same tissue, to test the effect of stromal ESR1 in E\textsubscript{2}-regulated cell proliferation.

Our studies provide compelling data regarding the role of ESR1 during normal uterine proliferation. Such findings have the potential to advance understanding of abnormalities of the endometrium, such as endometriosis and endometrial cancer. It is well-established that endometrial cancer type I is estrogen-dependent, ESR1-positive, and is the most common form of endometrial cancer (>80% of the endometrial cancer cases). Moreover, IGF1 has been shown to be the major driver of endometrial hyperplasia progression and endometrial cancer formation in women\textsuperscript{39}. Additionally, Ghazal \textit{et al}. has recently demonstrated that estrogen increases IGF1R expression and subsequently induces stromal cell proliferation in endometrial tissues from women with endometriosis\textsuperscript{40}. Our studies showed that local production of IGF1 was regulated by the stromal cells underlying luminal epithelial cells via the stimulation of estrogen signaling through stromal ESR1. Together, these findings advance general understanding regarding the roles of estrogen during normal endometrial growth. Furthermore, our studies indicate the potential source and location of growth factor production that could be targeted by therapeutic agents against endometrial growth abnormalities such as endometriosis, endometrial hyperplasia, and cancer.

Ablation of anti-mesometrial stromal ESR1 led to a decreased fecundity, without affecting the number of ovulated oocytes and blastocysts in the uterus\textsuperscript{32}. Pawar \textit{et al}. and our laboratory have demonstrated that epithelial ESR1 is necessary for embryo implantation\textsuperscript{18-17}. Implantation of embryos normally occurs exclusively in the anti-mesometrial pole of the uterus\textsuperscript{15}. We found that ablation of anti-mesometrial stromal ESR1 affected uterine receptivity, partly due to increased expression of E\textsubscript{2}-regulated genes, such as Muc1, Lif, and Clic3. This finding suggests that the stromal ESR1 is involved in regulating the gene expression in epithelial cells. Lacking stromal ESR1 in the anti-mesometrium decreases the number of implantation sites at 4.5 dpc by 50%. Cessation of uterine epithelial cell proliferation and increase stromal cell proliferation are also crucial steps for normal uterine receptivity\textsuperscript{32}. Using a hormonal profile mimicking implantation (E\textsubscript{2}+P\textsubscript{4}), we found that lacking ESR1 in the anti-mesometrial stromal cells caused diminished stromal cell proliferation. Together, these findings suggest that Amhr2\textsuperscript{Cre/+}; Esr1\textsuperscript{−/−} females are less receptive to embryo attachment/implantation due to a lack of stromal cell proliferation in the specific area of the uterus linked to the loss of anti-mesometrial stromal ESR1.

LIF, a key mediator of uterine receptivity, is expressed in glandular epithelial cells and regulated by epithelial ESR1\textsuperscript{8, 13, 17}. Loss of glandular LIF expression impairs receptivity\textsuperscript{33}. As expected, Lif transcript was comparable in both Esr1\textsuperscript{−/−} and Amhr2\textsuperscript{Cre/+}; Esr1\textsuperscript{−/−} uteri in the E\textsubscript{2}+P\textsubscript{4} model, but tended to be increased in the uterus at 3.5 dpc. This result suggests that ablation of stromal ESR1 does not significantly affect the production of Lif in the glandular epithelial cells. This comparable production of Lif in Amhr2\textsuperscript{Cre/+}; Esr1\textsuperscript{−/−} and Esr1\textsuperscript{−/−} uteri may facilitate the initiation of embryo attachment/implantation in the absence of proper stromal cell proliferation. However, uterine receptivity is not determined by the proliferation status of epithelial and stromal cells or the production of Lif alone, other factors such as HBEFG and WNT5A are also crucial implantation signals\textsuperscript{36, 31}. We found that Wnt5a transcript was significantly increased in Amhr2\textsuperscript{Cre/+}; Esr1\textsuperscript{−/−} uteri, which could potentially disrupt embryo homing and subsequently lead to implantation failure.

Mantena \textit{et al}. have demonstrated that both E\textsubscript{2} and P\textsubscript{4} treatment regulate uterine CEBPB expression\textsuperscript{34}. In their report, E\textsubscript{2} rapidly increased uterine CEBPB expression in ovariectomized mice within 1 h of treatment whereas P\textsubscript{4} induced expression after 24 h. However, the expression of CEBPB during decidualization on day 6 of pregnancy is solely regulated by PGR, as CEBPB protein is attenuated by a PGR antagonist (RU486). We also found rapid induction of CEBPB by E\textsubscript{2}, which is required for epithelial cell proliferation, but only in the mesometrial region, indicating that stromal ESR1 mediated the induction. However, CEBPB expression in the uterine anti-mesometrium as a result of E\textsubscript{2}+P\textsubscript{4} treatment or decidual response was independent of stromal cell ESR1 expression.

Previous findings using the original Esr1\textsuperscript{−/−} mouse line\textsuperscript{45} showed that global deletion of ESR1 did not affect uterine decidual responses in an artificial decidualization model\textsuperscript{42, 43}. However, our recent unpublished data using the Ex3\textsubscript{α}ERKO mouse line\textsuperscript{44} indicates that global loss of Esr1 prevents decidualization. Additionally,
recent findings suggest that ESR1 in uterine epithelial cells is in fact modulating the decidualization process\(^1\). Protein expression analysis of ESR1 in mouse uteri during pregnancy clearly showed that ESR1 is not expressed in the primary decidual zone\(^4\), which suggests that stromal ESR1 in the uterine anti-mesometrium is not required for the decidual response. However, after artificial stimulation, uteri with stromal ESR1 deletion in the anti-mesometrium showed impaired decidual responses as measured by uterine weight increase and cell proliferation. These results confirm recent findings showing ESR1 is required for normal decidualization of cultured human stromal cells\(^4\).

From our findings, we surmise that the regulation of uterine epithelial cell proliferation in response to E\(_2\) mediated by ESR1 is through a local cell-cell communication between the stromal cells and adjacent epithelial cells. In addition, this communication is crucial for normal embryo attachment/implantation and decidual response to artificial stimulation.

**Methods**

**Animals and experimental procedures.** We generated a mouse model with stromal cell selective deletion of ESR1 (encoded by the *Esr1* gene) using *Amhr2\(^{2\text{Cretg/}+}\) mice\(^9\), 46 bred with our *Esr1\(^{1\text{f/f}}\)* animals\(^2\). Female *Esr1\(^{1\text{f/f}}\)* mice were considered control animals for experiments. *Amhr2\(^{2\text{Cretg/}+}\) mice exhibit higher expression levels of Cre activity in anti-mesometrial uterine stromal cells than in mesometrial uterine stromal cells\(^18\)-\(^20\).

Adult females (8–12-week-old) were ovariectomized and housed for 14 days to eliminate the endogenous circulating ovarian steroid hormones. Animals were randomly grouped and subcutaneously injected with vehicle control (sterile normal saline) or 17\%-estradiol (E\(_2\), Steraloids, Newport, RI) at a dose of 0.25 \(\mu\)g/mouse in saline. To evaluate transcript and protein expression initially regulated by E\(_2\) (early responses), we euthanized the mice 6 h after the injection of E\(_2\) and collected the uterine tissues. To determine the E\(_2\)-induced protein expression and uterine wet weight increase reflecting uterine growth (late responses), we collected the tissues 24 h after E\(_2\) treatment. In some experiments, animals were injected with E\(_2\) and P\(_4\) (Sigma) called “E+P” to mimic the hormonal profile during implantation as previously described\(^4\). The control group of this experiment was injected with the series of hormones similar to the E+P treatment group except the last ntidatory dose of E\(_2\) was replaced with sesame oil, called “E+Oil”. The animals were injected intraperitoneally with 100 \(\mu\)L of 5-ethyl-2-deoxyuridine (EdU, Invitrogen, Carlsbad, CA) at a dose of 2 mg/mL in phosphate-buffered saline (PBS) 2 h prior to sacrifice. At the time of collection, uteri were weighed, and one uterine horn was snap frozen and stored at \(-80^\circ\)C for RNA extraction. The contralateral horn was collected in 10% buffered formalin solution for histological 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. All methods were performed in accordance with the relevant guidelines and regulations.

**Artificial decidualization.** Adult (8–12-week-old) female mice were ovariectomized (\(n = 9–10\) animals/genotype). Two weeks after ovariectomy, the mice were treated with E\(_2\) (100 ng/mouse) subcutaneously for 3 consecutive days (Day 1 or D1) to D3. On D6-D11, the mice were treated daily with P\(_4\) (1 mg/mouse) together with E\(_2\) 6 h after the injection of E\(_2\), and collected the uterine tissues. To determine the E\(_2\)-induced protein expression and uterine wet weight increase reflecting uterine growth (late responses), we collected the tissues 24 h after E\(_2\) treatment. In some experiments, animals were injected with E\(_2\) and P\(_4\) (Sigma) called “E+P” to mimic the hormonal profile during implantation as previously described\(^4\). The control group of this experiment was injected with the series of hormones similar to the E+P treatment group except the last ntidatory dose of E\(_2\) was replaced with sesame oil, called “E+Oil”. The animals were injected intraperitoneally with 100 \(\mu\)L of 5-ethyl-2-deoxyuridine (EdU, Invitrogen, Carlsbad, CA) at a dose of 2 mg/mL in phosphate-buffered saline (PBS) 2 h prior to sacrifice. At the time of collection, uteri were weighed, and one uterine horn was snap frozen and stored at \(-80^\circ\)C for RNA extraction. The contralateral horn was collected in 10% buffered formalin solution for histological 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. All methods were performed in accordance with the relevant guidelines and regulations.

**Fertility study and collection of implantation sites.** To evaluate the ovulatory response, pubertal (3–5-week-old) *Esr1\(^{1\text{f/f}}\) and *Amhr2\(^{2\text{Cretg/+}}\); *Esr1\(^{1\text{f/f}}\)* females were injected with 5 U of pregnant mare's serum gonadotropin (PMSG, EMD Millipore, Billerica, MA) in sterile normal saline. Human chorionic gonadotropin (hCG, EMD Millipore) was injected 48h after PMSG injection. At 18h post hCG injection, the ovulated oocytes were collected from the oviduct, the number of oocytes were counted and recorded. In the fertility study, adult (8-week-old) *Esr1\(^{1\text{f/f}}\) and *Amhr2\(^{2\text{Cretg/+}}\); *Esr1\(^{1\text{f/f}}\)* females were mated with a male proven breeder (C57BL/6J, Jackson Laboratory) continuously for 6 months. Numbers of pups per litter per dam over the 6-month period were recorded.

To collect the uteri at different stages of pregnancy as well as implantation sites, adult (8–12-week-old) *Esr1\(^{1\text{f/f}}\) and *Amhr2\(^{2\text{Cretg/+}}\); *Esr1\(^{1\text{f/f}}\)* females were mated with the stud male (B6/D2F1/J, Jackson Laboratory) over night. The next morning, the observed presence of a copulatory plug was designated as 0.5 dpc. At 3.5, 4.5, 5.5, 7.5 and 10.5 dpc, uteri were collected from both *Esr1\(^{1\text{f/f}}\) and *Amhr2\(^{2\text{Cretg/+}}\); *Esr1\(^{1\text{f/f}}\)* mice. To visualize implantation sites at 4.5 dpc, embryos were injected into the blasocoel with 0.5 \(\mu\)L of blue dye (Sigma) and injected into the tail vein as previously described\(^4\). The visible blue bands indicate the implantation sites. Some of the implantation sites were collected for RNA extraction, the rest of the implantation sites were collected in formalin for histological analysis. At 5.5, 7.5 and 10.5 dpc, the implantation sites are visible without the blue dye injection. The sites were collected for RNA and histological analysis.

**Real-time RT-PCR analysis.** After collection, RNA was extracted from uteri (or implantation sites) using TriZol reagent (Invitrogen) according to manufacturer’s protocol. Genomic DNA contamination was eliminated by incubating the RNA samples with DNase (Invitrogen). Two \(\mu\)g of RNA was used as a template for cDNA synthesis using SuperScript II (Invitrogen). Real-time PCR and analysis was performed as described previously\(^8\). Expression values were normalized to ribosomal protein L7 (Rpl7) and calculated as fold change over vehicle control (or over E+Poil) of the *Esr1\(^{1\text{f/f}}\)* group. The primer sequences of Rpl7, Lif (Leukemia inhibitory protein),
Ihh (indian hedgehog), Igf1 (insulin-like growth factor-1), Mad21l1 (mitotic arrest deficient-like 1), Cdkn1a
(Cyclin-Dependent Kinase Inhibitor 1 A), Cebp (CCAAT Enhancer Binding Protein Beta), Klf4 and Klf15
(Kruppel like factors), Mcm2 (minichromosome maintenance complex component 2), and Ltf (lactotransferrin)
were reported previously. The primer sequences for Adm (adrenomedullin), Antpt2 (angipoiotin 2), Bmp2
(bone morphogenetic protein 2), Ccnb1 (cyclin B1), Cdc2a (cyclin dependent kinase A1), Clca3 (chloride channel
calcium-activated 3), Egfr (epidermal growth factor receptor), Fkbp5 (FK506 binding protein 5), FMS-like tyrosine
kinase 1 (Flt1 also known as vascular endothelial growth factor receptor 1 or Vegfr1), Flt4 (or Vegfr3), Gja
(gap junction protein alpha), Hbegf (heparin-binding epidermal growth factor), Kdr (kinase insert domain pro-
tein receptor also known as Vegfr2), Muc1 (mucin 1), Prl8a2 (prolactin family 8, subfamily a, member 2), Pigs2
(prostaglandin-endoperoxide synthase 2), Plasminogen activator inhibitor 1 (Plasminogen activator inhibitor 1),
Vegfa (vascular endothelial growth factor), Vegfb, and Vegfc.

Immunohistochemical (IHC) analysis. Formalin-fixed tissues were cross-sectioned (5 microns). The tissues
were stained with mouse primary antibodies against ESR1 (ImmunoTech, Beckman Coulter, Pasadena, CA,
#1545), PGR (ImmunoTech #1546), and Ki67 (BD Pharmingen, BD Biosciences, San Jose, CA, #550609) as indicated
previously. The stromal cells with brown staining (PGR-positive) and blue staining (hematoxylin or PGR-negative)
were counted with mouse primary antibodies against ESR1 (ImmunoTech, Beckman Coulter, Pasadena, CA,
#1545), PGR (ImmunoTech #1546), and Ki67 (BD Pharmingen, BD Biosciences, San Jose, CA, #550609) as indicated
previously. The images were taken using bright field microscopy (DMi8, Leica Microsystems, Buffalo Grove, IL) with 10x
and 100x objective lenses using Leica Application Suite (Leica Microsystems). Ki67- (10x) and PGR- (100x) positive
images were quantified using Image software with Cell Counter Plugins. The Ki67-positive epithelial cells were counted
and calculated as a percentage of total epithelial cells. The glandular epithelial cells were excluded as the majority of
the glands are embedded within the stroma of the anti-mesometrial pole. As a result, the proliferation rate of glandular epithelial cells
may vary based on the degree of ESR1 deletion in each animal. The total epithelial cell count ranged from 45–324 cells
per microscopic field from a total of 3–8 animals per genotype. To eliminate bias from selecting the compartment
doing or not expressing ESR1, we instead calculated the Ki67-positive luminal epithelial cells of the entire
mesometrial pole (top 50% of the uterus) and anti-mesometrial pole (bottom 50% of the uterus), regardless of
ESR1 expression status (dotted lines indicated in Supplementary Fig. S2A).

The stromal cells with brown staining (PGR-positive) and blue staining (hematoxylin or PGR-negative)
were counted per microscopic field by two different individuals. The number of brown and blue cells were summed
as total stromal cells per microscopic field. The PGR-positive cells were calculated as a percentage of the total
stromal cells. The total cells counted ranged from 131–285 cells per microscopic field from a total of 3 animals/
genotype/treatment.

Measurement of relative signal intensity of PGR in nuclear and cytosolic compartments. All
images were taken with a 100x objective lens with similar and software settings. To quantify the light intensities
of the nuclear and cytoplasmic compartment, the RGB images were converted into grayscale 8-bit images using Image
software and then were inverted. The darkest areas were converted to light white areas, and vice versa
(color values from black to white = 0–255). The value of grayscale value was measured in the nuclear or cytoplasmic
compartments of the epithelial cells using the wand tool to select an area of 1 × 1 pixel for each compartment
within the microscopic field. The relative intensities were calculated as a percentage relative to 255-white signal
(255 white signal = 100% relative intensity). The total cells counted ranged from 13–67 cells per image from a
total of 3 animals/genotype/treatment.
Statistical analysis. All graphs represent mean ± SEM. Statistical analysis was performed using GraphPad Prism version 6.0 h for Mac OS X (GraphPad Software, Inc., La Jolla, CA). Statistical significance is considered when p < 0.05 using two-way ANOVA with Tukey post hoc test, unless otherwise indicated.

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Author Contributions

W.W., S.C.H., and K.S.K. wrote the manuscript, W.W., S.L.L., and L.J.D. collected the data and prepared the figures. K.C.D. and S.R.S. performed quantitation analysis for data in Figures 2 and 4. All authors reviewed the manuscript.

Additional Information

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