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Research Article

Notch effector recombination signal binding protein for immunoglobulin kappa J signaling is required for the initiation of endometrial stromal cell decidualization†

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Abstract

The Notch signaling pathway is required for reproductive success. This pathway activates its transcriptional effector, recombination signal binding protein for immunoglobulin kappa J (Rbpj), to induce transcription of its target genes. This signaling pathway is required for successful decidualization, implantation, and uterine repair following parturition. To identify the compartmental specific roles of the Notch signaling pathway in the establishment of pregnancy, we generated epithelial and decidual stromal cell specific knockouts of *Rbpj* utilizing *lactoferrin iCre* and *Prl8A2 iCre*, respectively. Both conditional knockout mouse models were fertile. The *Rbpj* epithelial knockout mice displayed 27% resorption sites at E15.5, but this did not significantly impact the number of live born pups compared with controls. In addition, the *Rbpj* epithelial knockout mice displayed increased estrogen signaling in their stromal compartment. Given that both mouse models exhibited fertility comparable to control animals, the epithelial and stromal specific nature of the iCre recombinases utilized, and previously published *Rbpj* total uterine knockout mouse models, we conclude that Notch effector Rbpj signaling is required at the initiation of pregnancy to support decidualization in stromal cells, but that Rbpj is not required in the epithelial compartment nor is it required for post-implantation pregnancy success.

Summary Sentence
Notch effector Rbpj signaling is required at the initiation of pregnancy to support decidualization in stromal cells, but Rbpj is not required in the epithelial compartment nor is it required for post-implantation pregnancy success.

Keywords: Notch signaling, implantation, decidualization, pregnancy

Introduction

The endometrium is essential for female reproductive function. This innermost layer of the uterus is comprised of several cell types, most notably the epithelial and stromal cells that facilitate implantation and support and maintain pregnancy. Specifically, the luminal epithelial cells at the surface of the endometrium coordinate embryo attachment and apposition, whereas the branching glandular epithelium secretes factors to support implantation. These populations of uterine epithelial cells crosstalk with the adjacent differentiating stromal cells, known as decidual cells, that also help facilitate implantation while also supporting endometrial remodeling [1, 2].

Endometrial stromal cell decidualization is critical for reproductive success in species that undergo interstitial implantation [3]. Stromal cell decidualization is the terminal differentiation process of stromal cells regulated by increased progesterone (P) and cyclic adenosine monophosphate (cAMP) signaling and low levels of estrogen (E) [4]. In humans, decidualization begins spontaneously in the latter half of the secretory phase of the menstrual cycle, whereas in mice decidualization is initiated in response to an implanting embryo [2, 3]. Studies surrounding implantation and decidualization in vivo in humans are limited due to obvious ethical concerns, whereas studies in the non-human primate and mouse have identified some of the critical molecular mechanisms responsible for successful implantation and decidualization such as those mediated by steroidal and embryonic signals [4]. Although the central mechanisms regulating decidualization have been identified, many pathways interact in this incredibly complex differentiation process [3]. Furthermore, the specific mechanisms by which these pathways affect the decidualization response have yet to be uncovered indicating a significant need to investigate the precise mechanisms at play in decidualization.

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The Notch signaling pathway is a ubiquitous juxtacrine signaling pathway that plays key roles in cell proliferation, cell fate, differentiation, and death [5, 6]. The Notch signaling pathway consists of four transmembrane Notch receptors and five transmembrane Delta-like and Jagged ligands [7]. Initial proteolysis of the Notch receptor is initiated by ligand binding leading to activation and release of the extracellular domain. Proteolytic cleavage of the transmembrane portion of the Notch receptor releases the Notch intracellular domain that translocates to the nucleus to interact with the Notch signaling pathway transcriptional effector, Rbpj, and its cofactors to induce transcription of target genes such as the *Hes* and *Hey* family of genes [5]. The Notch signaling pathway regulates processes that are critical in decidualization like differentiation, cell fate, and cell death indicating a potential role in the decidualization response. Indeed, the Notch signaling pathway plays significant roles in female reproduction including regulation of decidualization, implantation, and uterine repair [8–13].

Notch signaling activation is required for successful implantation, decidualization, and uterine repair following parturition [14]. Our laboratory has extensively studied the specific roles of the Notch signaling pathway in female reproduction [8–12, 15]. Utilizing a *progesterone*-driven *Cre recombinase* mouse model, we conditionally deleted *Notch1, Rbpj* and overexpressed the *Notch1 intracellular domain* (*N1ICD*) all of which resulted in impaired decidualization [8, 10–12]. We first determined that Notch1 is critical for endometrial stromal cell decidualization utilizing a $Pgr^{cre/+}Notch1^{Fl/Fl}$ conditional deletion mouse model [8]. These mice exhibit an impaired decidualization response, decreased stromal cell proliferation, and increased decidual cell apoptosis indicating that Notch1 signaling is an important mediator of stromal cell proliferation and fate [8]. A $Pgr^{cre/+}Rbpj^{Fl/Fl}$ conditional deletion mouse model exhibits a decreased decidualization response compared with wild-type controls caused by decreased progesterone receptor expression and signaling and reduced glucose transporter, *Slc2a1*, expression that is important for stromal cell differentiation [10]. Importantly, these mice also exhibit compromised implantation orientation leading to embryonic death highlighting the importance of *Rbpj* in maternal–fetal communication at the initiation of pregnancy [13]. Additional studies on the importance of the Notch signaling pathway demonstrated that overexpression of *N1ICD* in the uterus resulted in a glandless phenotype and a completely impaired decidualization response [12]. Functional analyses revealed that overexpression of the *N1ICD* in conjunction with *Rbpj* knockout induces hypermethylation of the progesterone receptor through the PU.1-Dnmt3b complex compromising P4 signaling and enhancing E2 signaling [12]. Importantly, these data indicate that epithelial and stromal compartmentalization and cross-compartment Notch signaling and hormone signaling have profound effects on the decidualization response. Furthermore, these conditional deletion and overexpression mouse models provide significant evidence for the role of Notch1 signaling in endometrial stromal cell decidualization. To further elucidate these mechanisms, we induced decidualization artificially in vitro in human uterine fibroblasts [16] utilizing 17b-estradiol, medroxyprogesterone acetate, and dibutyryl cyclic AMP with *NOTCH1* knockdown by shRNA before and after the initiation of decidualization. *NOTCH1* inhibition prior to decidualization stimulus inhibits the decidualization response, whereas *NOTCH1* inhibition following the decidualization stimulus has no effect on the decidualization response [15]. These in vitro data combined with our extensive in vivo data suggest important roles for Notch signaling in both the epithelial and stromal compartments of the endometrium during early pregnancy events and specifically at the initiation of decidualization.

Given the importance of the Notch signaling pathway and its transcriptional effector, RBPJ in female reproductive function in both the epithelial and stromal compartments and its significant roles in decidualization, we sought to investigate the compartment specific roles of *Rbpj* in uterine epithelial cells and separately in decidual stromal cells utilizing transgenic mouse models. We generated an epithelial conditional knockout mouse model of *Rbpj* ($Ltf^{iCre/+}$ $Rbpj^{Fl/Fl}$, e-KO) and a decidual stromal conditional knockout mouse model of *Rbpj* ($Prl8A2^{iCre/+}$ $Rbpj^{Fl/Fl}$, ds-KO) to selectively inhibit all Notch signaling in each of these compartments (Figure 1). We hypothesized that given the severe phenotypes in the $Pgr^{cre/+}$ $Rbpj^{Fl/Fl}$ and $Pgr^{cre/+}$ $Rosa26^{N1ICD/+}$ mouse models, we would see infertility phenotypes resulting from decidualization failure or lack of uterine repair in the compartmental specific knockouts. Surprisingly, we observed a normal decidualization response and fertility in both models suggesting a role for Notch signaling in early decidualization, in line with our previous in vivo and in vitro data [8–10, 12, 15].

Materials and methods

Animal models

$Ltf^{iCre/+}$ [17], $Prl8A2^{iCre/+}$ [18], and $Rbpj^{Flox/Flox}$ [19] mice were housed and maintained in a designated animal care facility at Michigan State University or South China Agricultural University on a 12-hour light/dark cycle with free access to food and water. $Ltf^{iCre/+}$ mice were crossed with $Rbpj^{Flox/Flox}$ mice to produce uterine epithelial deletion of Rbpj in mature female mice (Figure 1A) [17]. $Prl8A2^{iCre/+}$ mice were crossed with $Rbpj^{Flox/Flox}$ mice to produce mice containing a deletion of *Rbpj* in decidualized stromal cells (Figure 1B) [20]. Females were placed with proven fertile males in the evening for timed mating experiments. Seminal plugs were checked each morning with day of plug designated as embryonic day (E) 0.5. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University and South China Agricultural University.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde, dehydrated in ethanol and xylene, and embedded in paraffin. Sections (6 μm) were deparaffinized and rehydrated in a graded alcohol series followed by antigen retrieval (Vector Laboratories, Burlingame, CA) and hydrogen peroxide treatment. Next, sections were blocked and incubated with antibodies against Rbpj, Esr1, p-Esr1, or Ki67 overnight at 4°C (see Supplementary Table 3). On the following day, sections were incubated with biotinylated secondary antibodies followed by incubation with horseradish peroxidase conjugated streptavidin. Immunoreactivity was detected using the DAB substrate kit (Vector Laboratories) and visualized as brown staining by light microscopy. Incubation with secondary antibody only served as a negative control. Alternatively, after dehydration, slides were stained with hematoxylin and

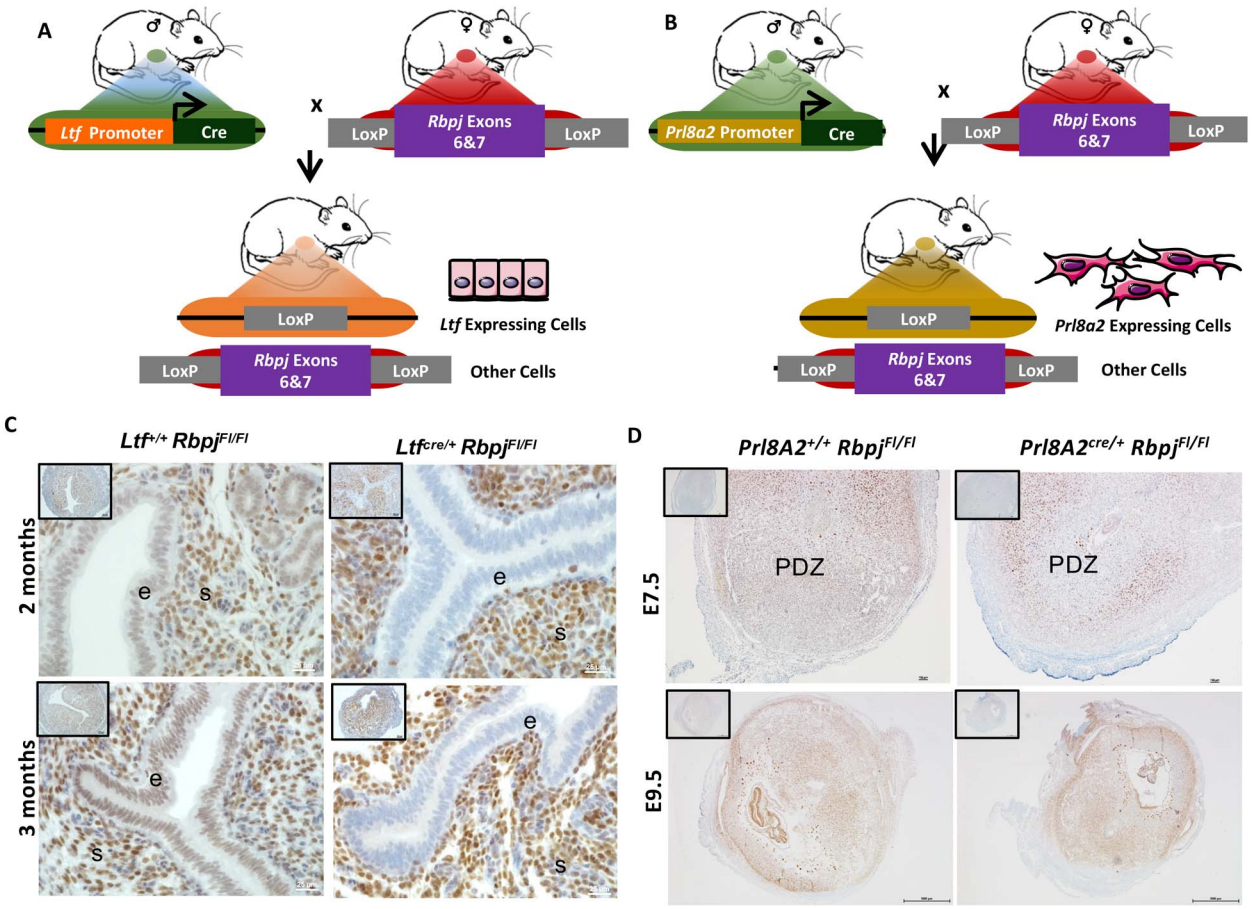

Figure 1. Mouse Models. (A) *Ltf cre* mice were crossed with *Rbpj Fl/Fl* mice to generate epithelial specific deletion of *Rbpj* in mature female uteri (e-KO). The DNA-binding domain coding region in exons 6 and 7 of *Rbpj* is excised with cre activation, resulting in an inactivated protein. (B) *Prl8A2 cre* mice were crossed with *Rbpj Fl/Fl* mice to generate decidual stromal cell deletion of *Rbpj* (ds-KO). (C) e-KO of Rbpj shown by IHC at 2 and 3 months of age in the luminal epithelium e: epitheliium, s: stroma. (D) ds-KO of *Rbpj* shown at E7.5 in the primary decidual zone (PDZ) and return of Rbpj expression at E9.5. Negative controls shown in upper corner.

eosin (H&E) followed by rehydration in a graded ethanol series then cover slipped and visualized by light microscopy. ImageJ image analysis software (NIH, v1.52s), was utilized to determine a digital HSCORE for staining intensity.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from frozen mouse tissue using TRIzol reagent (Invitrogen, Waltham, MA). About 1 μg of RNA was reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qPCR) was performed with SYBR Green PCR Master Mix (Applied Biosystems) using the ViiA7 qPCR system (Applied Biosystems). Primer sequences utilized are listed in Supplementary Table 4.

Statistical analysis

Data are expressed as mean ± SEM. Data were analyzed utilizing unpaired *t*-tests and two-way ANOVA followed by Sidak multiple comparison correction. Values were considered significant if $p < 0.05$. All statistical analyses were performed by GraphPad Prism (Graphpad Software, v9.0).

Results

Uterine epithelial and decidual stromal cell knockouts are fertile

We confirmed epithelial knockout of Rbpj by immunohistochemistry (IHC) in uterine cross-sections from sexually mature mice at 2 and 3 months of age (Figure 1C). No positive staining was observed in the luminal and glandular epithelium of the e-KO mice, whereas controls expressed positive Rbpj stain in both the luminal and glandular epithelium (Figure 1C). Knockout of Rbpj in stromal cells in the primary decidual zone at E7.5 in ds-KO mice was observed but we noted perfuse staining by E9.5 throughout the stromal compartment in the ds-KO and controls (Figure 1D). Rbpj e-KO mice exhibited Rbpj deletion after sexual maturity as expected, whereas the ds-KO mice showed transient deletion in decidual stromal cells post-implantation and a return to normal expression at the time of placentation, E9.5.

Next, we performed a 6-month breeding trial to assess fertility in these knockout models. Both the e-KO ($n = 9$) and ds-KO ($n = 5$) mice exhibited litter sizes consistent with controls during a 6-month breeding trial (Supplementary Tables 1 and 2). In addition, mixed-effects analysis showed that litter number has an effect on the number of pups per litter and genotype

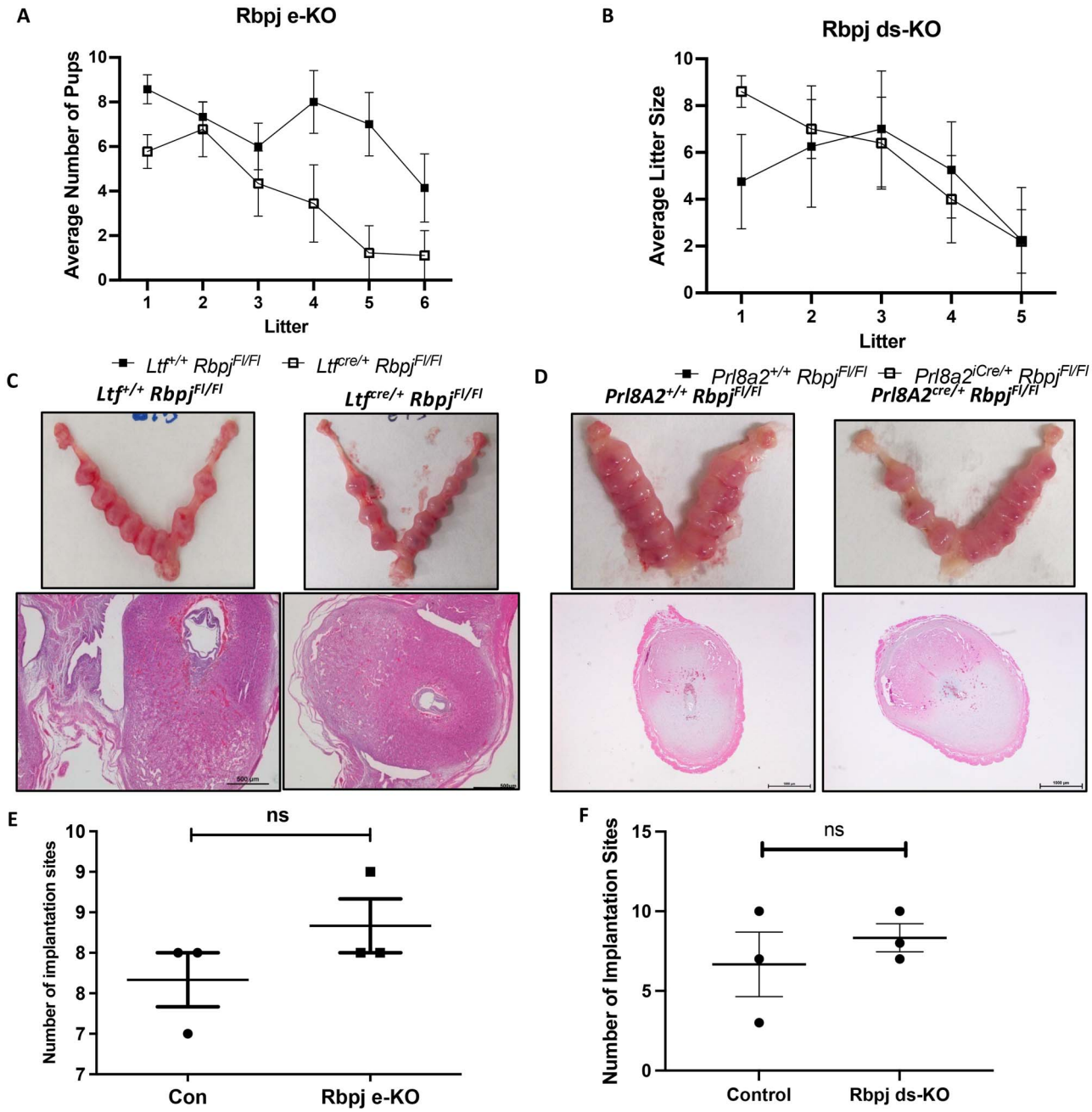

Figure 2. Rbpj epithelial and decidual stromal cell knockout mice are fertile. (A) e-KO of *Rbpj* results in fewer total pups compared with controls across a 6-month breeding trial but does not affect litter size. (B) ds-KO of *Rbpj* does not affect litter size compared with controls. Error bars represent standard error of the mean. (C) Normal implantation sites at E7.5 in control and e-KO mice shown by gross morphology and H&E staining. (D) Normal implantation sites at E7.5 in control and ds-KO mice shown by gross morphology and H&E staining. (E) e-KO mice implantation site quantity is not significantly different from controls at E7.5. (F) ds-KO mice do not have a difference in implantation site number compared with controls at E7.5.

similarly has an effect on number of pups per litter, but did not show an interaction between litter number and genotype. Therefore, the e-KO mice had fewer pups in total indicating reduced fertile capacity compared with controls but no distinct difference in fertile capacity across time (Figure 2A). Indeed, the e-KO mice had significantly less litters per mouse compared with the controls, but the average litter size was not affected (Figure 2A and Supplementary Table 1). Unlike in the $Pgr^{cre/+}$ $Rbpj^{Fl/Fl}$ model in which Rbpj was conditionally deleted in both epithelial and stromal cells postnatally [11], the e-KO and ds-KO mice maintained litter sizes consistent with controls in subsequent litters (Figure 2A and B). Both the e-KO and ds-KO mice had normal implantation site morphology at E7.5 both grossly and in H&E-stained uterine cross-sections (Figure 2C and D). The number of implantation sites at E7.5 was also consistent with controls (Figure 2E and F). These data indicated that the ds-KO mice are fertile and do not display aberrations in early pregnancy, whereas the e-KO display reduced fertile capacity.

Epithelial Rbpj knockout mice exhibit increased E2 signaling and increased stromal proliferation

Esr1 expression in the e-KO mice at E3.5 was consistent with controls, but p-Esr1, indicative of active estrogen signaling,

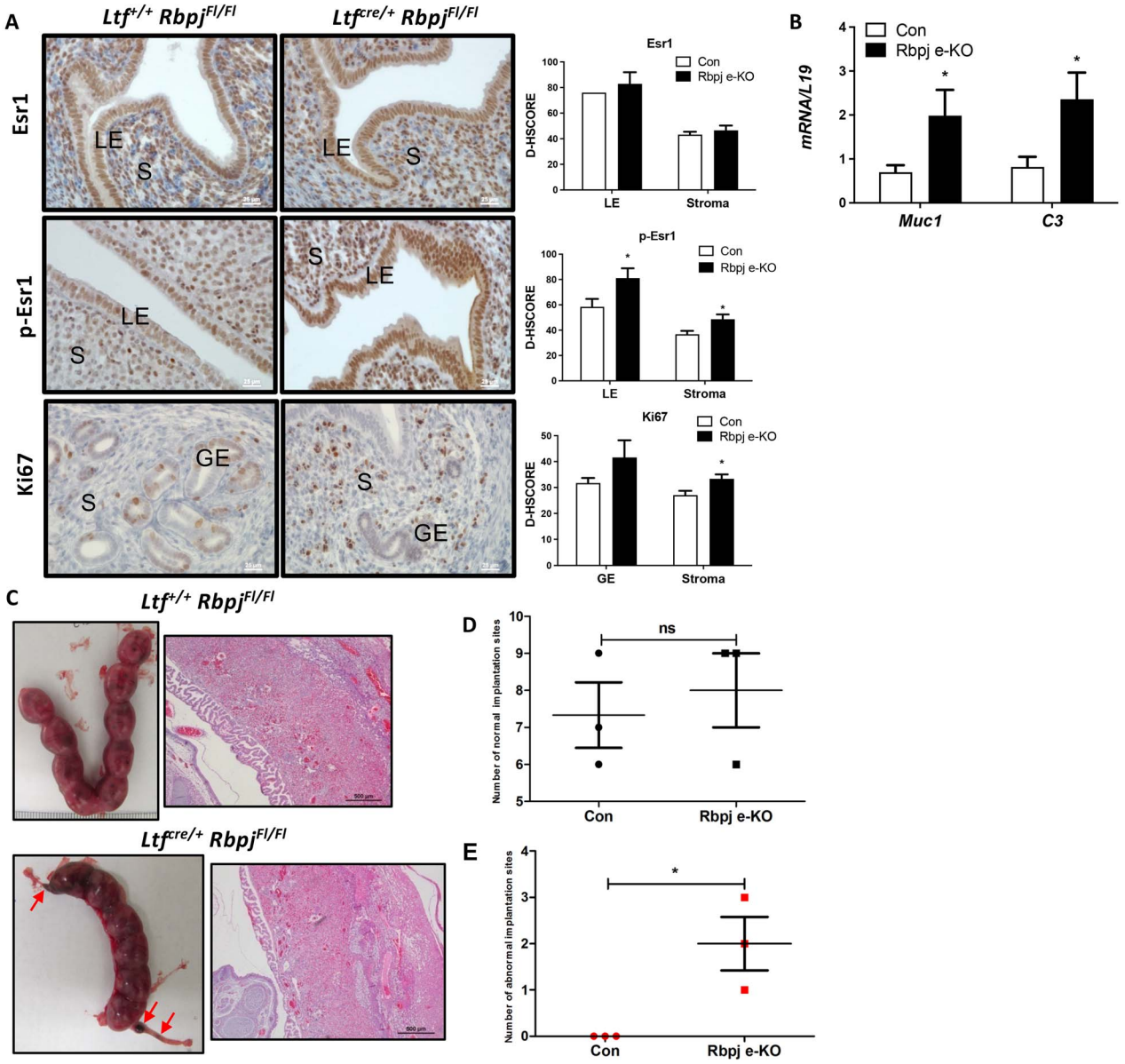

Figure 3. Epithelial Rbpj KO mice exhibit increased estrogen signaling at E3.5 and abnormal implantation sites at E15.5. (A) e-KO mouse uteri express increased p-Esr1 in the luminal epithelium (LE) and stroma (S) and increased Ki-67 in the stroma but not the glandular epithelium (GE) at E3.5. (B) Estrogen target genes *Muc1* and *C3* are increased in e-KO mice uteri at E3.5. (C) e-KO mice uteri (lower panel, red arrows) had resorption sites at E15.5 with abnormal histology (shown at right). (D) The total number of implantation sites in controls and e-KO mice was not different at E15.5 ($n = 3$). (E) e-KO mice had a significant number of abnormal implantation sites at E15.5 compared with controls ($n = 3$). e: epithelium s: stroma.

was increased in both the luminal epithelium and stroma (Figure 3A). In addition, Esr1 targets *Muc1* and *C3* were increased in the e-KO mice (Figure 3B). Finally, we observed increased Ki67 expression in the stroma of e-KO mice indicative of increased stromal proliferation (Figure 3A). These results suggest that a lack of *Rbpj* expression encourages activation of E signaling and increased stromal proliferation.

Limited fetal resorption sites, between one and three ($n = 3$), were present in the e-KO mice at E15.5; however, this did not compromise the average number of pups per litter (Figure 3C lower panel). There were no differences in the quantity of normal implantation sites between the e-KO and control mice despite the presence of resorption sites (Figure 3D). The number of abnormal implantation sites in the e-KO mice was significant compared with controls with at least one resorption site per e-KO mouse ($n = 3$, Figure 3E). These results suggest that Rbpj e-KO induces fetal resorption but does not compromise the fecundity of these mice.

Discussion

Endometrial stromal cell decidualization is an early critical reproductive event that involves the coordinated transformation of endometrial stromal cells into decidual cells [3]. This process is regulated by P and cAMP signaling, but many pathways integrate to coordinate the success of decidualization. Previously, the Notch signaling pathway was implicated in this process and considered significant for the decidualization response [8–10, 12, 15]. This study shows that neither epithelial nor decidual stromal deletion of *Rbpj* has detrimental

effects on the decidualization process, nor on reproductive success. First, this suggests that the Notch signaling pathway has compartment specific roles in the uterus. Since epithelial deletion leads to increased resorption sites, this shows that Rbpj signaling may play a specific role in implantation and post-implantation development although the sample size in this group was small indicating a potential limitation. Indeed, Rbpj expression is required for appropriate embryo orientation during implantation [13]. Furthermore, deletion of Rbpj in stromal cells that have already decidualized has no effect on fertility which solidifies the importance of Notch-dependent Rbpj signaling during the initiation of decidualization but not decidualization maintenance.

The decidualization process is a terminal differentiation of stromal cells that involves a coordinated change in morphology and function to prepare for and to support pregnancy. The Notch signaling pathway is known to regulate proliferation and indeed proliferation of endometrial stromal cells [7]. We have previously shown that Notch1 is required for the initiation of decidualization in vitro to induce proliferation of endometrial stromal cells and must be downregulated for differentiation to occur or cells undergo apoptosis [8, 15]. Utilizing in vitro decidualization studies, we determined that the NOTCH1 receptor is cleaved and therefore active until Day 6, but is inactive after that (unpublished). The temporality of Notch1 expression in vivo was unidentified until now. Our previous studies indicate that active Notch signaling through Rbpj is most important during the initiation of decidualization. The recent publication of the *Prl8a2 iCre* model showed that this Cre is most active at gestation day (GD) 7.5 [18]. Dickson et al. utilize *Prl8a2*$^{iCre/+}$ *Rosa26*$^{mTmG/+}$ *and Prl8a2*$^{iCre/+}$ *Sun1*$^{LsL/+}$ reporter models to show the progression of *Prl8a2 iCre* activation at GD 5.5, 7.5, and 9.5 and in artificially stimulated decidouma at Day 5 [18]. These data show strong fluorescent reporter activity at the antimesometrial pole at GD 7.5 and 9.5 and in decidouma and minimal fluorescent activity at GD 5.5 [18]. The activity of Prl8a2 iCre highlighted in Dickson et al. is reflected in our data which showed that *Prl8a2 iCre* was most efficient at deleting Rbpj in the primary decidualization zone at E7.5 but did not show any differences immediately post-implantation at E5.5 (data not shown) indicating that only cells that are already decidualized express Prl8a2. These results support the idea that once decidualization has already begun and the initial stromal cell proliferation events have occurred, inhibiting Notch signaling by deleting Rbpj expression has no effect. This confirms that Notch signaling is not important in the differentiation process of decidualization, but in the early proliferative initiation events.

Notch signaling has previously been shown to interact with steroid hormone receptor signaling in many contexts [14]. e-KO of Rbpj resulted in increased p-Esr1 expression and increased esr1 target gene expression resulting in increased proliferation, particularly in the stromal compartment. These results mimic those seen in the N1ICD overexpression model suggesting that regulated activation of the Notch signaling pathway is important for estrogen signaling in the uterus [12]. Indeed, total uterine deletion of Rbpj also resulted in significant decreases in *Pgr* and target gene expression during artificial decidualization indicating that Rbpj expression is important for P responsiveness in the uterine environment [10]. Our studies show that canonical Notch signaling through Rbpj leads to overactivation of estrogen signaling and inhibition of progesterone signaling in a coordinated manner between the epithelium and stroma [12]. This e-KO further supports these findings given that epithelial deletion of Rbpj increases p-Esr1 expression and proliferation in the stromal compartment. Other studies have also shown the importance of epithelial-stromal crosstalk in hormone signaling during epithelial proliferation, implantation, and decidualization (summarized in Marquardt et al. [21]). This further supports our previous findings that the Notch signaling pathway indirectly coordinates hormone signaling in the uterine environment through epithelial-stromal crosstalk.

In summary, we have shown that epithelial deletion of Rbpj does not compromise fertility, suggesting that Notch signaling in the epithelium is not required for the establishment of pregnancy but is required for pregnancy maintenance given the increased resorption sites noted in these mice. Complementary to this, deletion of Rbpj after decidualization has already begun has no effect on pregnancy success confirming that canonical Notch signaling activation in the stroma is required at the initiation of decidualization but is not required to maintain the decidualization response. Future studies will investigate the precise role of Notch dependent and independent Rbpj signaling in stromal cells prior to and at the beginning of the establishment of pregnancy and the compartmental specific roles of this pathway throughout pregnancy.

Author contributions

A.F., G.M., and Y.S. contrived the study design. G.M. and Y.S. performed the experiments. T.K., R.S., and J.J. contributed to murine care and experimental approach. A.G. and T.S. generated the *Prl8a2 iCre* genetically engineered mouse model. G.M. wrote the manuscript. All authors reviewed and edited the manuscript.

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Supplementary material

Supplementary material is available at *BIOLRE* online.

Conflict of interest

The authors declare no conflicts of interest.

Data availability

The data supporting this article are included in the manuscript and in its supplementary material.

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