Aberrant activation of canonical Notch1 signaling in the mouse uterus decreases progesterone receptor by hypermethylation and leads to infertility

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In mammalian reproduction, implantation is one of the most critical events. Failure of implantation and the subsequent decidualization contribute to more than 75% of pregnancy losses in women. Our laboratory has previously reported that inhibition of Notch signaling results in impaired decidualization in both women and a transgenic mouse model. In this study, we generated a Notch gain-of-function transgenic mouse by conditionally overexpressing the Notch1 intracellular domain (N1ICD) in the reproductive tract driven by a progesterone receptor (Pgr) -Cre. We show that the overexpression of N1ICD in the uterus results in complete infertility as a consequence of multiple developmental and physiological defects, including the absence of uterine glands and dysregulation of progesterone and estrogen signaling by a Recombination Signal Binding Protein Jκ-dependent signaling mechanism. We further show that the inhibition of progesterone signaling is caused by hypermethylation of its receptor Pgr by Notch1 overexpression through the transcription factor PU.1 and DNA methyltransferase 3b (Dnmt3b). We have generated a mouse model to study the consequence of increased Notch signaling in female reproduction and provide the first evidence, to our knowledge, that Notch signaling can regulate epigenetic modification of the Pgr.

Notch signaling is critical for maternal–fetal communication during implantation and placentation (9). Our laboratory has previously reported that both the conditional deletion of Notch1 in the mouse uterus and NOTCH1 silencing in Human Uterine Fibroblasts (HuFs) inhibit decidualization (10, 11). In the pathological condition of endometriosis, the decrease in NOTCH1 in eutopic endometrium results in impaired decidualization of endometrial stromal cells from patients with the disease (12). NOTCH2 has also been shown as a regulator of decidualization (13). Subsequently, expression levels of Notch1 are decreased in the mouse endometrial stroma and HuF cells on completion of transition to the decidual phenotype (10, 11). It is unclear whether down-regulation of Notch1 expression is required for completion of decidualization. To determine the mechanism(s) of Notch1-mediated effects in endometrial physiology, we generated a reproductive tract-specific, constitutively activated Notch1 mouse model, in which the intracellular domain of Notch1 (NICD) is overexpressed specifically in Pgr-positive cells within the reproductive tract.

In this study, we show that constitutively activated Notch signaling in the mouse uterus compromises uterine receptivity through multiple mechanisms, including the loss of uterine glands and the inhibition of P4 signaling. We further show that the suppression of P4 signaling is a result of the hypermethylation of its receptor Pgr. Our findings further indicate the importance of Notch signaling during early pregnancy.

Significance

These studies show a physiological role for Notch signaling in female reproduction. The fact that both loss and gain of function of Notch signaling result in the impairment of early pregnancy identifies Notch1 signaling as a critical regulator of endometrial function. We also provide the first evidence, to our knowledge, that Notch signaling can regulate methylation of exon 1 of the progesterone receptor (Pgr) gene through its target PU.1, which provides novel insight into the role of Notch in steroid hormone regulation. This mechanism also provides an opportunity for future studies in identifying the cause of progesterone resistance in gynecological pathologies in women, such as endometriosis and adenomyosis, in which the hypermethylation of Pgr has been reported.

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markers bone morphogenetic protein 2 (Bmp2) and wingless-type MMTV integration site family member 4 (Wnt4) in the stimulated horn of control mice were significantly increased compared with those of the nonstimulated horn, but no difference was evident between the stimulated and nonstimulated horns of OEx mice (Fig. 1 D–F). Collectively, N1ICD OEx mice are infertile because of defective uterine receptivity and impaired ability of uterine stromal cells to undergo decidualization.

N1ICD OEx Mice Fail to Develop Uterine Glands. The uterine size and weight of N1ICD OEx mice were significantly lower than those of control mice at 3.5 dpc (Fig. S2 A and B). Histological analysis revealed that the uteri of the OEx mice were completely devoid of uterine glands, whereas their control littermates had normal glandular structures (Fig. 2A). Forkhead box A2 (Foxa2), a marker of uterine glands (14, 15), was used to confirm the loss of glands in the OEx mice. The glandular epithelial (GE) cells of control mice expressed Foxa2 protein as expected, but surprisingly, luminal epithelial (LE) cells of OEx mice showed strong Foxa2 staining (Fig. 2B). Uterine gland secretion of leukemia inhibitory factor (Lif) is critical for implantation in mice (16, 17). The LE staining of Foxa2 in OEx mice suggested a glandular phenotype of LE cells, which we further confirmed by mRNA expression of Lif by in situ hybridization. Lif mRNA was observed in the luminal epithelium of OEx mice, whereas only GE cells expressed Lif mRNA in control mice (Fig. 2C). At 3.5 dpc, expressions of Lif mRNA (Fig. S2D) and Lif protein (Fig. 2D) in OEx mice were comparable with those of controls as measured by quantitative PCR (qPCR) and Western blot.

Fig. 1. Defective uterine receptivity in N1ICD OEx mice. (A) No implantation sites (ISs) are detected in N1ICD OEx mice at 3.5 dpc, whereas control, OEx + KO, and Aby KO mice have normal numbers of ISs. The number of ISs (n = 3 of each group) is shown in the histogram. (B) The right uterine horns, which received WT embryos, have ISs in control mice but not in OEx mice, whereas the left horns served as controls without embryo transfer. Histological staining shows an implanted blastocyst with decidualized stromal cells surrounding it in control mice and a free-floating blastocyst in uterine lumen without decidualization in OEx mice. (C) Artificial decidualization stimulus induces a decidual response in control mice vs. no decidualization response in OEx mice. Histological staining shows cross-sections of stimulated horns (right horns) in both two groups. Left horns are nonstimulated controls. (D) Weight of stimulated horns in control mice is >20 times higher than that of nonstimulated horns in control mice, but there is no difference between stimulated and nonstimulated horns of OEx mice (n = 4 of each group). Decidualization markers Bmp2 and Wnt4 are significantly increased in the stimulated horns of control mice, whereas no significant induction is evident in OEx mice (n = 4 of each group); B, blastocyst; Ctrl, control; dec, decidual; LE, luminal epithelium; St, stroma. *P < 0.05, **P < 0.01.

Results
N1ICD Overexpression Mice Are Completely Infertile Because of Impaired Uterine Receptivity. Generation of N1ICD overexpression (OEx) mice (Pgr<sup>+/-</sup>Rosa<sup>26</sup>MMTV<sup>Cre</sup>) is shown in Fig. S1A. After confirming the overexpression of N1ICD (Fig. S1 B–F), we tested the fertility of N1ICD OEx mice. For control females, both homozygous and heterozygous animals had normal litters (n = 5 and n = 3, respectively). However, both homozygous and heterozygous OEx female mice are completely infertile as determined with a 6-mo breeding test (n = 4 in both genotypes) (Table S1). Furthermore, N1ICD OEx mice displayed no visible implantation sites at 4.5 d postconception (dpc) in contrast to control mice, which displayed normal implantation (Fig. 1A). Successful implantation requires both a competent embryo and a receptive uterus (3). To bypass potential embryotrophic causes for infertility, we transferred blastocysts collected from WT donors into the uterine lumen of pseudopregnant OEx and control mice. Two days after transfer, the control recipients displayed evidence of implantation; blastocysts were attached to the uterine luminal epithelium, and the decidual reaction could be observed surrounding the implanted embryo (Fig. 1B, control). In contrast, no implantation sites were observed in the OEx recipients. Blastocysts remained free-floating in the uterine lumen, and the stromal cells showed no evidence of decidualization (Fig. 1B, OEx). Furthermore, OEx females showed no response to an artificial decidualization stimulus, whereas control mice exhibited a clear decidual response (Fig. 1C). Uterine weight and expression of decidualization

E2 and P4 Signaling Is Altered in N1ICD OEx Mice. Uterine receptivity in the mouse is mainly regulated by two ovarian steroid hormones, E2 and P4, which act by binding to their cognate receptors, Esr1 and the Pgr, respectively. The expression patterns of both receptors are critical for controlling receptivity in the mouse uterus. In OEx mice, expression of Pgr was markedly decreased in both epithelial and stromal cells compared with control mice, and expression of Esr1 was significantly increased in LE cells, especially its active phosphorylated form (p-Esr1) (Fig. 3 A and B) at 3.5 dpc. Pgr mRNA was significantly reduced in OEx mice, whereas Esr1 mRNA expression was not changed (Fig. 3 C and D). Immunostaining for antigen Ki67 (Ki67) also showed that glandular cell proliferation was significantly decreased in N1ICD OEx mice, whereas epithelial cell proliferation was significantly increased compared with control mice.

Fig. 2. N1ICD OEx mice have no uterine glands. (A) Unlike control, OEx + KO, and KO mice, there are no glands in the uteri of N1ICD OEx mice at 3.5 dpc as detected by H&E staining. (B) The gland marker Foxa2 is expressed in the glandular epithelium of control, OEx + KO, and KO mice, whereas its expression is only present in the luminal epithelium of OEx mice. (C) Expression of Lif mRNA in GE cells of control mice and LE cells of N1ICD OEx mice at 3.5 dpc. (D) N1ICD OEx mice express comparable levels of Lif protein to control mice as measured by Western blot at 3.5 dpc. Ctrl, control. (Scale bar: 100 μm.)
at 3.5 dpc (Fig. 3 A and B). The expression of Pgr-regulated genes Indian hedgehog (Ihh) (18), amphiregulin (Areg) (19), homobox A10 (Hoxa10) (20), nuclear receptor subfamily 2, group F, member 2 (Nr2f2) (18), and heart and neural crest derivatives expressed transcript 2 (Hand2) (21) were significantly decreased, and E2 target genes mucin 3 (Muc1), Muc4, lactotransferrin (Ltf), and complement component 3 (C3) (21, 22) were significantly increased in OEx mice compared with control mice (Fig. 3 C and D). Protein levels of Muc1 and chicken ovalbumin upstream promoter transcriptional factors II (COUP-TFI) (encoded by the Nr2f2 gene) were detected by immunohistochemistry on 3.5 dpc. Muc1 was observed on the luminal surface of the uterus in OEX mice but not control mice, and COUP-TFI expression was lower in stromal cells of OEx mice (Fig. S3 A and B). The expression pattern of target genes in N1ICD OEx mice uteri indicated that P4 signaling was inhibited and that E2 signaling was enhanced, which correlates with the expressions of their respective receptors, suggesting that overexpression of N1ICD in the mouse uterus effects uterine receptivity.

However, decreased expression of Pgr is not the only factor that contributes to the inhibition of P4 signaling in N1ICD OEx mice. Serum P4 levels in OEx mice at 3.5 dpc were significantly lower than those of control mice, whereas E2 levels were not altered (Fig. S4A). These data show that both ligand and receptor for P4 signaling were altered in N1ICD OEx mice, although ovulation and the fertilization ability of oocytes are not different between the two groups of mice (Fig. S4B). Supplemental P4 injections from 2.5 to 3.5 dpc significantly up-regulated expression of P4 target genes compared with vehicle-treated OEx mice but were still significantly lower than those of vehicle-treated control mice (Fig. S4C). Supplemental P4 injections had no effect on E2 targets (Fig. S4D). Exogenous hormones (P4 and E2) were used to prime the uterus of ovarioectomized mice to mimic the receptivity of the uterus at 3.5 dpc, independent of the effects of ovarian factors (22). This treatment was capable of inducing a decidualization response after an artificial stimulation. All ovarian steroid hormone receptor target genes displayed the same expression pattern as those observed in the OEx and control pregnant mice at 3.5 dpc (Fig. S4 E and F), indicating that the disordered expression pattern of Pgr and Esr1 is sufficient for the dysregulation of their target genes and the failure of uterine receptivity in N1ICD OEx mice.

**PU.1 Mediates Hypermethylation of the Pgr Promoter in N1ICD OEx Mice.** To determine the mechanism by which Pgr is down-regulated in N1ICD OEx mice, transcription factor binding sites on the Pgr promoter were identified using MotifMap (motifmap.ics.uci.edu). Potential binding sites for the transcription factor PU.1 [encoded by the spleen focus forming virus (SFFV) proviral integration oncogene (Sfi1)] were identified on the promoter region of mouse Pgr (~660 bp 5′ of the transcription start site (TSS)] (Fig. 4A). PU.1 has been reported to be a direct target of Notch signaling (23). In our study, expression of PUI.1 was increased in OEx mice as well as Sfi1 mRNA (Fig. 4B and C and Fig. S3C). Previous studies suggested that PU.1 can hypermethylate its target genes by recruiting DNA methyltransferase 3b (DNMT3b) to the promoter region of target genes in the human during osteoclastogenesis (24). Therefore, we hypothesized that methylation of the Pgr promoter in N1ICD OEx mice could be altered by a PU.1/Dnmt3b-mediated pathway and further led to decreased Pgr expression.

To confirm our hypothesis, we first showed an interaction of PU.1 and Dnmt3b proteins in our mice by immunoprecipitation. PU.1 and Dnmt3b interacted with each other in uterine tissues of N1ICD OEx mice, and this interaction was much stronger than that observed in control mice (Fig. 4D). Using ChIP, binding of both PU.1 and Dnmt3b on predicted DNA regions on the Pgr promoter was verified. Binding efficiency of both PU.1 and Dnmt3b was significantly higher in OEx mouse than in control mouse (Fig. 4E, −755 to −644). Next, we identified the presence of cytose–phosphate–guanine (CpG) islands by using the UCSC Genome Browser (https://genome.ucsc.edu/). Surprisingly, the CpG islands appear in exon 1 of the Pgr gene instead of the promoter (Fig. S5). The relative positions of the PU.1 binding site, Pgr TSS, and CpG islands are diagrammatically illustrated in Fig. 4F. The methylation of CpG islands on exon 1 of Pgr gene was identified by bisulfite sequencing. Our data showed that Pgr is hypermethylated in the uterus of N1ICD OEx mice compared with control mice at 3.5 dpc (Fig. 4G).
However, the hypermethylated CpG islands were localized at ~1,300 bp downstream from the PU.1/Dnmt3b binding site (Fig. 4F). To further investigate if there are more PU.1/Dnmt3b binding sites close to the hypermethylated CpG islands, additional ChIP primers were designed to scan PU.1 and Dnmt3b binding along the region in proximity to the predicted binding site and the CpG islands (Fig. 4E and F). Interestingly, the presence of another PU.1/Dnmt3b binding site in the region of the CpG islands was detected, supporting our hypothesis that the hypermethylation of Pgr exon 1 is associated with the DNA binding of PU.1/Dnmt3b complexes (Fig. 4E, 649–735).

### Ablation of RBP-Jκ in N1ICD OEx Mice Rescues Infertility

To determine whether the overexpression of the N1ICD-induced inhibitory phenotype was mediated solely by RBP-Jκ-dependent signaling mechanisms, we performed the N1ICD OEx experiments in the absence of RBP-Jκ [Pgrαcre/Rosa26N1ICD+/Rbpj−/−] (OEx + KO; Fig. S2C). The implantation failure observed in the N1ICD OEx mice was rescued in the absence of RBP-Jκ. The number of implantation sites at 4.5 dpc in OEx + KO mice was comparable with that in control mice and markedly higher than that in OEx mice with intact RBP-Jκ (Fig. 4F). Smaller uterine size, as seen in N1ICD OEx mice, was also rescued in OEx + KO mice (Fig. S2A and B). Glandular development was detected in uteri of OEx + KO mice with Foxa2 staining evident (Fig. 2A and B). Expression patterns of Pgr and Esr1 showed no difference in OEx + KO mice compared with control mice at 3.5 dpc, both of which were significantly different from the OEx mice (Fig. 3A and B). Expression levels of all target genes of P4 and E2 signaling were also rescued when RBP-Jκ was deleted (Fig. 3C and D and Fig. S3A). The altered proliferation pattern of OEx mice was also rescued by the deletion of RBP-Jκ (Fig. 3A and B). Most importantly, the expression of Sfpi1 (PU.1) also returned to the basal level when Rbpj was deleted in N1ICD OEx mice (Fig. 4C). These data in the OEx + KO mice collectively show that the phenotype associated with N1ICD overexpression occurs in an RBP-Jκ-dependent manner.

### Discussion

Implantation is one of the most critical events in the establishment of pregnancy in rodents and primates. Successful implantation requires a competent blastocyst and a receptive uterus during a specific window of time during the cycle to initiate the bilateral communication and establish a successful pregnancy (3). Here, we show that uterine receptivity has been compromised by aberrant activation of Notch signaling in the mouse uterus. The uteri of OEx mice neither respond to artificial decidualization nor accept transferred WT embryos for implantation. The absence of uterine glands contributes significantly to the defective uterine receptivity. Brief exposure of female pups to P4 during neonatal days 2–10 results in the failure to develop uterine glands and further leads to infertility (25–27). Loss of certain genes, such as Foxa2, Cnnb1 (β-catenin), Wnt4, Wnt5a, Wnt7a, and Left1, also results in reduction or absence of uterine glands (reviewed in ref. 28). In this study, N1ICD OEx mice also failed to develop their uterine glands, similar to the studies described above. However, there is a marked difference between our OEx mice and data reported in previous studies: the LE cells of OEx mice exhibit a glandular phenotype, including the expression of GE markers Foxa2 and Lif. Uterine gland secretion of Lif is essential for blastocyst implantation in mouse (16, 17). Lif is also the main mediator of uterine receptivity failure in the absence of uterine glands: an intraluminal injection of Lif can partially rescue the lack of a decidual response in the Foxa2 null mouse uterus (14). Our N1ICD OEx mice completely failed to decidualize, although they produce a comparable amount of Lif to that of control mice. Therefore, we deduced that the lack of uterine glands is not the only component that contributes to uterine receptivity in OEx mice.

P4 signaling is a highly regulated cellular pathway that plays a critical role in the initiation and maintenance of pregnancy. Without functional P4 signaling, as seen in Pgr KO mice, pregnancy is unable to occur because of implantation and decidualization failure (29, 30). In our study, the expression of Pgr is significantly decreased in N1ICD OEx mice compared with control mice, which correlates with the dramatic down-regulation of P4 target genes (Ihh, Nr2f2, Areg, Hoxa10, and Hand2) in both epithelial and stromal cells. These data suggest that P4 signaling is significantly reduced when Notch signaling is aberrantly activated. During and after embryo implantation, the surrounding uterine stromal cells undergo decidualization, during which stromal cells proliferate and differentiate into decidual cells to promote the growth of the embryo (3). Stromal proliferation is significantly decreased in OEx mice as indicated by Ki67 staining, suggesting an impaired potential of these cells to decidualize. Furthermore, the inhibitory P4 signaling in N1ICD OEx mice is because of not only the decrease in Pgr but also, the lower serum P4 levels, which suggest impaired ovarian P4 synthesis. However, supplemental P4 can only rescue the expression of P4 target genes by 10–30% compared with the expression levels found in control mice. Dysregulated E2 signaling and E2-induced LE proliferation associated with infertility can result from a lack of P4 receptor and target gene expression, including Ihh, Nr2f2, and Hand2 (21, 29–32). In our study, decreased P4 signaling is associated with abnormally up-regulated E2 signaling in uterine epithelial cells of OEx mice as evidenced by increased expression of Esr1 and its phosphorylated form p-Esr1; up-regulation of target genes Muc1, Muc4, Lif, and C3; and the increased proliferation of epithelial cells, which is driven by E2.
signaling. These data suggest that the dysregulation of epithelial E2 signaling in OEx mice is, at least partially, caused by suppression of P4 signaling. However, loss of inhibition by decreased P4 signaling is not the only factor responsible for increased E2 signaling and its induced proliferation, because induction of target genes in OEx mice is much higher (~10 times) than induction of these genes in Ihh, Nv2f2, and Hand2 null mice (21, 31, 32). In ESR1-positive breast cancer cells, N1ICD stimulates ESR1-dependent transcription, even in the absence of E2, by recruiting p300 and IKKα to ESR1 binding sites on chromatin (33). This effect requires RBP-Jκ, because canonical Notch1 transcriptional complexes form in proximity to ESR1 binding sites (33). The possibility that overactivated Notch1 signaling may interact with E2 signaling directly in endometrial epithelial cells through a similar mechanism will be the focus of future studies.

To our knowledge, regulation of DNA methylation by Notch signaling has not been reported. Spi1 or transcription factor PU.1, a direct target of Notch1 (23, 34), has been reported as a DNA methylation mediator through interaction with DNA methyltransferase Dnmt3b (24). For the first time, to our knowledge, we show that overactivation of Notch signaling can induce hypermethylation of exon 1 of the Pgr gene and further lead to the inhibition of P4 signaling. This process is associated with increased binding of PU.1 as well as its interacting protein Dnmt3b, which directly mediates DNA methylation. According to a previous study, the PU.1/Dnmt3b complex can methylate DNA regions within 500 bp of their binding site (24). In the case of our uterine-specific N1ICD OEx mouse, the verified PU.1 binding motif predicted by MotifMap exists about 1,300 bp away from the hypermethylated CpG islands, which is farther than previously reported. However, the presence of a second binding site for the PU.1/Dnmt3b complex in the DNA region of CpG islands showed the correlation between PU.1/Dnmt3b binding and CpG island hypermethylation in N1ICD OEx mice. Furthermore, the binding of Dnmt3b to the CpG island region (649–735) is much stronger than its binding to the PU.1 binding motif on the Pgr promoter (755 to –644). Similarly, the binding of PU.1 to its binding motif (755 to –644) is significantly higher than that of the CpG island region (649–735). These data suggest that Dnmt3b directly binds to the CpG island region (649–735) and binds to the PU.1 binding motif (755 to –644) indirectly through its interaction with PU.1. However, the PU.1 binding that we observed from the –755 to –644 region is likely different from interactions with the CpG islands binding site occurs indirectly through interaction with Dnmt3b. Therefore, we created a spatial model based on our findings (Fig. 5B). First, PU.1 binds to the Pgr promoter and enriches Dnmt3b through their direct interaction. Second, the enriched Dnmt3b binds to the CpG island 1.3 kb away from the promoter through a looped DNA structure and hypermethylates this region.

Notch signaling occurs through canonical or noncanonical pathways. In canonical Notch signaling, the NICD translocates to the nucleus and binds directly to RBP-Jκ, converting it from a transcriptional suppressor to an activator and inducing the transcription of downstream target genes (7). In contrast, noncanonical Notch signaling does not require activation of RBP-Jκ (6). In this study, the fact that the deletion of Rbpj completely rescues the phenotypes of N1ICD OEx mice indicates that the abnormalities that we observe in N1ICD mice are responsible for the phenotype of N1ICD OEx mice.

In summary, we have investigated the effects of constitutively activated Notch signaling in female reproduction. We found that increased Notch signaling in the mouse reproductive tract leads to infertility because of the failure of multiple reproductive processes, including dysregulation of P4 and E2 signaling and absence of uterine glands, all of which combined contribute to decidualization failure in the N1ICD OEx mice. In pathological conditions, such as endometriotic ectopic lesions (36) and adenomyosis (37), our finding that Notch signaling hypermethylates the Pgr gene provides a novel direction for understanding gynecological pathologies, which could lead to therapeutic avenues for these diseases.
Dnmt3b and lower levels of P4 synthesized by ovaries. However, the defect of P4 signaling is not the sole cause for the implantation and decidualization failure. Other physiological defects, such as the hyperactivation of E2 signaling in part caused by the decrease in Pgr and the absence of uterine glands in N1ICD OEx mice, could also contribute to implantation and decidualization failure. The mechanisms associated with this process are summarized in Fig. 5. The mechanisms by which N1ICD OEx causes glandular development failure and hyperactivation of E2 signaling will be investigated in future studies.

Materials and Methods

All antibodies and primers used in this study are listed in Tables S2 and S3, respectively. More descriptions of methods are in SI Materials and Methods.

Animals. PgrCremtm mice (41), Rosα2CreERT2 mice (The Jackson Laboratory), and RbpαR26tm mice (42) were maintained in the designated animal care facility according to the Michigan State University institutional guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University.

Human Tissue Collection. Placental tissues were obtained with informed consent using a protocol approved by the Institutional Review Board at Michigan State University and Spectrum Health System.

Embyro Transfer. All embryos used were collected from WT C57BL/6 females. Pseudopregnant recipients were induced by mating with vasectomized males. Some blastocysts were transferred into the uterine lumen of one horn in the afternoon at 2.5 dpc, and implantation status was detected at 4.5 dpc by tail vein injection of Chicago Sky Blue (Sigma-Aldrich).

Artificial Decidualization Model. As described in ref. 10, mice were ovariec- tomized and treated with E2 and P4 (Sigma-Aldrich). A scratch stimulus was then performed on the uterine luminal epithelium of the mesometrium side of one uterine horn to induce decidualization. The unscathed horn served as a hormonal control. Animals were killed 5 d after the scratch, and both horns were collected for analysis.

Statistical Analysis. Data are expressed as means ± SEMs. Data were ana- lyzed using the Student’s t test and one-way ANOVA followed by Tukey’s posthoc multiple-range test. Values were considered significant if P was <0.05. All statistical analyses were performed by GraphPad Prism 5.0 (GraphPad Software).

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