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

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Edited by R. Michael Roberts, University of Missouri–Columbia, Columbia, MO, and approved January 13, 2016 (received for review October 15, 2015)

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.

mouse uterus | Notch1 | Pgr | methylation | infertility

Implantation is one of the most critical and highly regulated processes during mammalian reproduction. In reproductive aged women, there is only a 15% chance of pregnancy each cycle (1), and 75% of failed pregnancies are caused by implantation failure (2). The window of uterine receptivity is defined as the optimum time when the uterine endometrium is able to accept a blastocyst to implant. Uterine transition into the receptive phase requires priming with progesterone (P4) superimposed with estrogen [mainly 17β-estradiol (E2)], which has functions that are mediated primarily by nuclear receptors progesterone receptor (Pgr) and estrogen receptor isoform 1 (Esr1), respectively (3). Dysregulation of these two signaling pathways leads to defective uterine receptivity and failed implantation (4).

Notch signaling is a highly conserved pathway across species and present in most multicellular organisms. It plays vital roles in cellular survival, communication, and differentiation throughout development from embryonic to adult life (5). Canonical Notch signaling is initiated after the interaction of Notch transmembrane receptors (Notch1–Notch4) with cell-bound ligands (δ-like 1, 3, or 4 or Jagged 1 or 2), which leads to a cleavage cascade of Notch involving ADAM proteases and γ-secretase (6). Subsequently, the cleaved Notch intracellular domain (NICD) translocates to the nucleus, where it interacts with transcriptional repressor Recombination Signal Binding Protein Jκ (RBP-Jκ; also known as CBF-1) and converts it into a transcriptional activator of downstream target genes, such as hairy enhancer of split and hairy enhancer of split-related transcription factor families (7, 8). However, recent studies have revealed the existence of several other modes of Notch signaling generally referred to as noncanonical Notch signaling (6).

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 (N1ICD) 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.

Author contributions: R.-W.S., J.-W.J., L.M., and A.T.F. designed research; R.-W.S. and M.R.S. performed research; R.-W.S. and A.T.F. analyzed data; and R.-W.S. and A.T.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1520441113/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1520441113

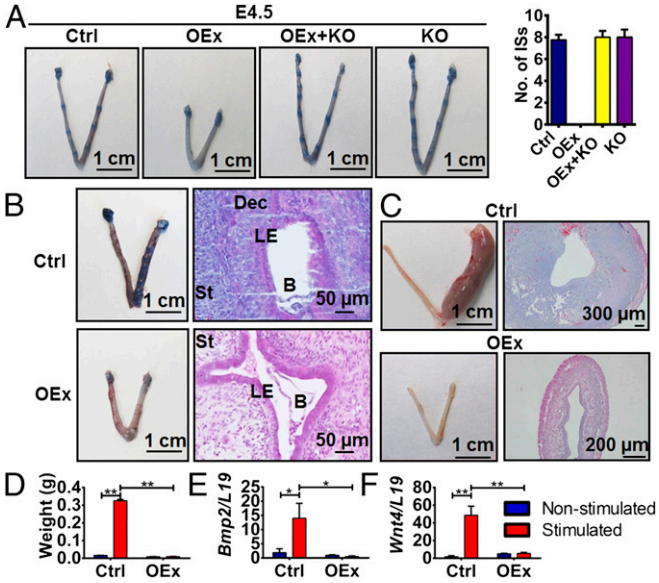

Fig. 1. Defective uterine receptivity in N1ICD OEx mice. (*A*) No implantation sites (ISs) are detected in N1ICD OEx mice at 3.5 dpc, whereas control, OEx + KO, and *Rbpj* KO mice have normal numbers of ISs. The number of ISs ($n = 3$ of each group) is shown in the histogram. (*B*) The right uterine 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 (*E*) *Bmp2* and (*F*) *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, decidua; 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 ($Pgr^{cre/+}Rosa26^{N1ICD/+}$) is shown in Fig. S1*A*. 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. 1*A*). 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. 1*B*, 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. 1*B*, OEx). Furthermore, OEx females showed no response to an artificial decidualization stimulus, whereas control mice exhibited a clear decidual response (Fig. 1*C*). Uterine weight and expression of decidualization 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. 2*A*). 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. 2*B*). 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. 2*C*). At 3.5 dpc, expressions of *Lif* mRNA (Fig. S2*D*) and Lif protein (Fig. 2*D*) in OEx mice were comparable with those of controls as measured by quantitative PCR (qPCR) and Western blot.

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 stromal cell proliferation was significantly decreased in N1ICD OEx mice, whereas epithelial cell proliferation was significantly increased compared with control mice

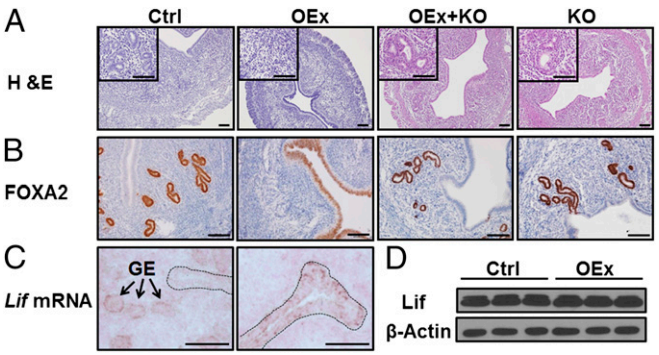

Fig. 2. N1ICD OEx mice have no uterine glands. (*A*) Unlike control, OEx + KO, and KO mice, there are no glands in the uteri of N1ICD OEx mice at 3.5 dpc as detected by H&E staining. (*B*) The gland marker Foxa2 is expressed in the glandular epithelium of control, OEx + KO, and KO mice, whereas its expression 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 expressions 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 1 (*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-TFII) (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-TFII 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 correlate with the expressions of their respective receptors, suggesting that overexpression of N1ICD in the mouse uterus effects uterine receptivity.

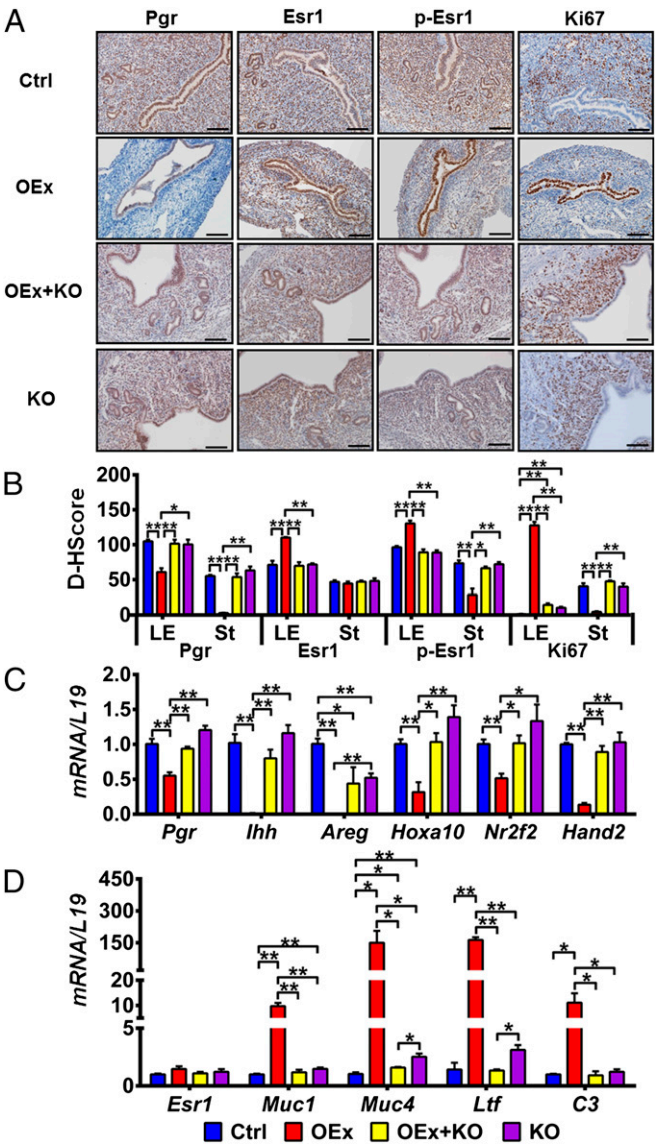

Fig. 3. Dysregulation of P4 and E2 signaling and proliferation pattern in N1ICD OEx mice at 3.5 dpc. (*A*) Immunohistochemistry shows that the expression patterns of Pgr, Esr1, p-Esr1, and Ki67 (proliferation marker) are altered in N1ICD OEx mice compare with control, OEx + KO, and KO mice. Quantitative expression levels of these proteins are shown in *B*. (*C*) mRNA levels of *Pgr* together with P4 target genes *Ihh*, *Areg*, *Hoxa10*, *Nr2f2*, and *Hand2* are significantly decreased in OEx mice compared with control mice but rescued, at least partially, in OEx + KO mice. Expression levels in KO mice are similar to those of OEx + KO mice. (*D*) Quantitative PCR expression of *Esr1* has no significant difference among the four different genotypes of mice, but E2 targets *Muc1*, *Muc4*, *Ltf*, and *C3* are dramatically up-regulated in OEx mice compared with control mice. The up-regulation of E2 targets is abolished in OEx + KO mice (n = 3–4 in each group). Ctrl, control; D-HScore, digital HScore; St, stroma. $^{*}P < 0.05$; $^{**}P < 0.01$. (Scale bar: 100 μm.)

Fig. S4*A*). 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. S4*B*). 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. S4*C*). Supplemental P4 injections had no effect on E2 targets (Fig. S4*D*). Exogenous hormones (P4 and E2) were used to prime the uterus of ovariectomized 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 patterns after this exogenous hormone treatment 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.

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 (

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 (*Sfpi1*)] were identified on the promoter region of mouse *Pgr* ~660 bp 5′ of the transcription start site (TSS) (Fig. 4*A*). PU.1 has been reported to be a direct target of Notch signaling (23). In our study, expression of PU.1 was increased in OEx mice as well as *Sfpi1* mRNA (Fig. 4 *B* and *C* and Fig. S3*C*). 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 leads 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. 4*D*). 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 mice than in control mice (Fig. 4*E*, −755 to −644). Next, we identified the presence of cytosine–phosphate–guanine (CpG) islands by using the UCSC (University of California Santa Cruz) 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. 4*F*. 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. 4*G*).

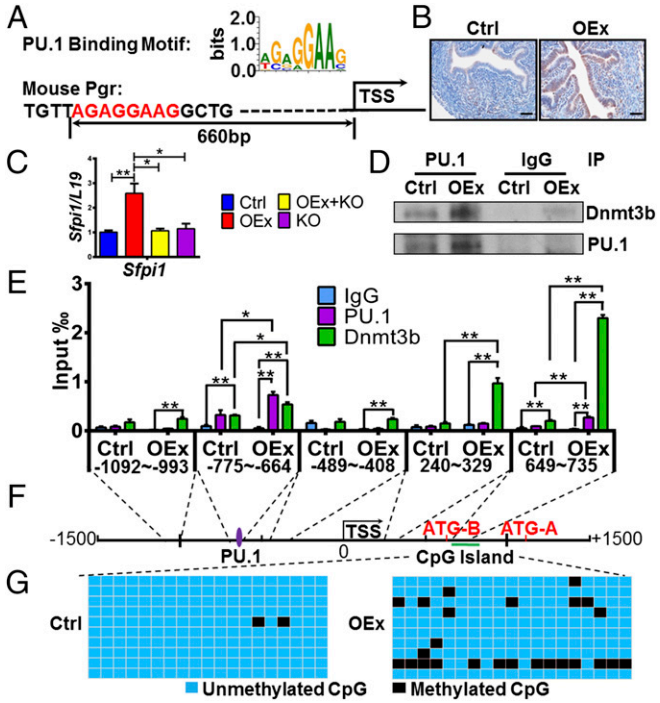

Fig. 4. DNA hypermethylation of the *Pgr* gene mediated by PU.1/Dnmt3b. (*A*) Predicted PU.1 binding site on the *Pgr* promoter. PU.1 is up-regulated in N1ICD OEx mice by (*B*) immunohistochemistry and (*C*) quantitative PCR; quantitative expression levels of PU.1 (immunohistochemistry) are shown in Fig. S3*C*. (*D*) The PU.1 and Dnmt3b interaction is stronger in OEx mice than that in control mice ($n = 3$). (*E*) Binding of PU.1 and Dnmt3b protein on the *Pgr* promoter was detected by ChIP ($n = 3$). Binding efficiency of both PU.1 and Dnmt3b is significantly higher in OEx mice than control mice at positions of the predicted PU.1 motif (–755 to –644) and the CpG island (649–735). (*F*) Relative positions of the PU.1 binding site, the *Pgr* transcription start site, and the CpG island. (*G*) The number of methylated CpGs (black cells) is much higher in OEx mice than in control mice. Blue cells are unmethylated CpGs. All data are collected at 3.5 dpc. Ctrl, control; IP, immunoprecipitation. $*P < 0.05$; $**P < 0.01$. (Scale bar: 100 μm.)

However, the hypermethylated CpG islands were localized at ∼1,300 bp downstream from the PU.1/Dnmt3b binding site (Fig. 4*F*). 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. 4 *E* 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. 4*E*, 649–735).

Ablation of RBP-Jκ in N1ICD OEx Mice Rescues Infertility. To determine whether the overexpression of the N1ICD-induced infertile phenotype was mediated solely by RBP-Jκ–dependent signaling mechanisms, we performed the N1ICD OEx experiments in the absence of RBP-Jκ [$Pgr^{Cre/+}Rosa26^{N1ICD/+}Rbpj^{flox/flox}$ (OEx + KO; Fig. S2*C*)]. The implantation failure observed in the N1ICD OEx 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. 1*A*). Smaller uterine size, as seen in N1ICD OEx mice, was also rescued in OEx + KO mice (Fig. S2 *A* and *B*). Glandular development was detected in uteri of OEx + KO mice with Foxa2 staining evident (Fig. 2 *A* 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. 3 *A* and *B*). Expression levels of all target genes of P4 and E2 signaling were also rescued when RBP-Jκ was deleted (Fig. 3 *C* and *D* and Fig. S3*A*). The altered proliferation pattern of OEx mice was also rescued by the deletion of RBP-Jκ (Fig. 3 *A* 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. 4*C*). 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*, *Ctnnb1* (β-catenin), *Wnt4*, *Wnt5a*, *Wnt7a*, and *Lef1*, 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 intrauterine 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*, *Ltf*, 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*, *Nr2f2*, 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. *Sfpi1* 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 direct binding, whereas the CpG islands binding site occurs indirectly through interaction with Dnmt3b. Therefore, we created a spatial model based on our findings (Fig. 5*B*). 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 when N1ICD is overexpressed are occurring through canonical pathway signaling. Deletion of *Rbpj* would result in derepression of genes normally repressed by RBP-Jκ in the absence of NICD. Our data indicate that genes actively transactivated by N1ICD through RBP-Jκ are responsible for the phenotype of N1ICD OEx mice. Recently, an N1ICD/RBP-Jκ cobinding site was identified ~2.6 kb upstream from the *Sfpi1* gene (supplemental data in ref. 35), suggesting that Notch signaling directly regulates PU.1 expression in an RBP-Jκ–dependent manner, which supported by our data that PU.1-mediated *Pgr* suppression is rescued by deletion of *Rbpj*.

Our study shows the first genetic evidence, to our knowledge, that overactivation of canonical Notch signaling leads to hypermethylation,

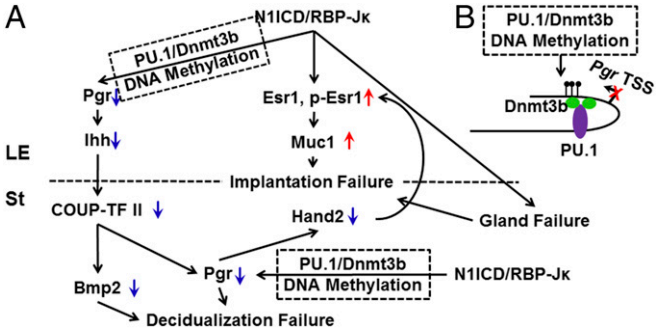

Fig. 5. Working model. (*A*) Overexpression of N1ICD, working through RBP-Jκ, inhibits P4 signaling and overactivates E2 signaling. Dysregulation of P4 and E2 signaling contributes to implantation and decidualization failure and further leads to defective uterine receptivity as a consequence of the altered expression of their target genes. Overactivation of canonical Notch signaling decreases Pgr through hypermethylation of exon 1 by the PU.1/Dnmt3b complex. (*B*) The PU.1/Dnmt3b spatial working model. PU.1 first binds on the promoter of *Pgr* and then recruits Dnmt3b through their direct interaction. Dnmt3b binds to the CpG island from 1.3 kb away from the PU.1 binding site through a looped DNA structure and hypermethylates this region.

suggesting that Notch signaling plays a role in regulating an epigenetic modification of gene expression. In addition, hypermethylation of *Pgr* has been reported to contribute to decreased expression of *Pgr* 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.

In previous studies, we reported that inhibition or deletion of Notch signaling results in impaired decidualization in both women and a transgenic mouse model because of failure of cell survival before differentiation (10, 11). In this study, we showed that constitutively active Notch1 signaling also impairs decidualization in mouse uterus as well as HuFs (Fig. S6). These studies suggest that Notch signaling plays two distinct roles during decidualization. Silencing of NOTCH1 during the initiation of decidualization inhibits stromal cell differentiation, indicating that activation of Notch1 signaling is required only at the initiation of the decidualization process, which is also associated with the induction of FOXO1 (12). As decidualization progresses, Notch1 is down-regulated (10, 11), and this down-regulation is necessary to permit differentiation into the decidual phenotype, which was shown in a previous study (11) and is also shown in this study. Decidualization is dependent on cAMP stimulation, sustained PKA activity, and cAMP response element-binding protein (CREB) activation (38, 39), and N1ICD sequesters nuclear CREB and inhibits cAMP/PKA-mediated signaling (40). When N1ICD is overexpressed, cAMP/PKA-mediated signaling is inhibited. This inhibition also prevents the ability of the stromal cells to differentiate and further illustrates the importance of Notch1 down-regulation in the physiological context during decidualization. Furthermore, in addition to preventing stromal cell differentiation and decidualization, N1ICD overexpression contributes to epithelial defects, such as the dysregulation of both P4 and E2 signaling and absence of uterine glands, all of which combined contribute to decidualization failure in the 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 through their nuclear receptors in a canonical RBP-Jκ–dependent manner. We also showed that the inhibition of P4 signaling is a consequence of both hypermethylation of *Pgr* by N1ICD complexing with PU.1 and

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. $Pgr^{Cre/+}$ mice (41), $Rosa26^{N1ICD/N1ICD}$ mice (The Jackson Laboratory), and $Rbpj^{flox/flox}$ 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.

Embryo Transfer. All embryos used were collected from WT C57BL/6 females. Pseudopregnant recipients were induced by mating with vasectomized males. Seven 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 ovariectomized and treated with E2 and P4 (Sigma-Aldrich). A scratch stimulus was then performed on the uterine luminal epithelium of the antimesometrium side of one uterine horn to induce decidualization. The unscratched 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 analyzed 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).

ACKNOWLEDGMENTS. The authors thank Dr. John Lydon (Baylor College of Medicine) and Dr. Francesco DeMayo (National Institute of Environmental Health Sciences) for providing the $Pgr^{Cre/+}$ mice and Dr. Nadia Carlesso (Indiana University) for providing the $Rbpj^{flox/flox}$ mice. The authors also thank Ms. Samantha Bond for excellent technical assistance and Mr. Mark Olson for his help in manuscript preparation. This study was funded by NIH Grants F30 HD082951 (to M.R.S.) and R01 HD042280 (to A.T.F.).

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