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Research Article

Notch1 is crucial for decidualization and maintaining the first pregnancy in the mouse†

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Abstract

The endometrium undergoes a pregnancy-delivery-repair cycle multiple times during the reproductive lifespan in females. Decidualization is one of the critical events for the success of this essential process. We have previously reported that Notch1 is essential for artificial decidualization in mice. However, in a natural pregnancy, the deletion of Notch1 (*Pgr*$^{Cre/+}$*Notch1*$^{f/f}$, or *Notch1*$^{d/d}$) only affects female fertility in the first 30 days of a 6-month fertility test, but not the later stages. In the present study, we undertook a closer evaluation at the first pregnancy of these mice to attempt to understand this puzzling phenomenon. We observed a large number of pregnancy losses in *Notch1*$^{d/d}$ mice in their first pregnancy, which led to the subfertility observed in the first 30 days of the fertility test. We then demonstrated that the initial pregnancy loss is a consequence of impaired decidualization. Furthermore, we identified a group of genes that contribute to Notch1 regulated decidualization in a natural pregnancy. Gene ontogeny analysis showed that these differentially expressed genes in the natural pregnancy are involved in cell–cell and cell–matrix interactions, different from genes that have been previously identified from the artificial decidualization model, which contribute to cell proliferation and apoptosis. In summary, we determined that Notch1 is essential for normal decidualization in the mouse uterus only in the first pregnancy but not in subsequent ones.

Summary sentence

Notch1 is essential for normal decidualization in the mouse uterus only in the first pregnancy but not in subsequent ones; the initial pregnancy loss is a consequence of impaired decidualization.

Key words: Notch1, decidualization, pregnancy loss, first pregnancy, extracellular matrix.

Introduction

The uterus undergoes the estrous or menstrual cycle and is under the control of ovarian steroid hormones [1]. During a short period termed the window of implantation, the endometrium becomes receptive, which permits the blastocysts to implant and further support its development [2, 3]. After delivery, the uterus is rapidly repaired and undergoes another estrus or pregnancy cycle [4, 5]. In most mammals, the uterus undergoes multiple pregnancy cycles during their reproductive lifespan.

The Notch pathway is highly conserved across many species [6]. In mammals, there are four Notch receptors (Notch1–Notch4), and five cell-bound ligands (Delta-like 1, 3, or 4 or Jagged 1 or 2). In the past decade, many studies have linked Notch signaling with female reproduction, which expands the role of this pathway in regulating physiological and pathological processes in reproduction [4, 7–11]. The lossing of *Notch1* results in impaired decidualization in both human and mouse artificial decidualization models [7, 8]. Similar to NOTCH1, NOTCH2 also plays a role in the decidualization process [12]. Furthermore, conditional deletion of Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (*Rbpj*) driven by progesterone receptor Cre ($Pgr^{Cre/+}Rbpj^{f/f}$, $Rbpj^{d/d}$) leads to decidualization impairment [4]. Eutopic endometrium from patients with endometriosis expresses lower levels of Notch1 compared with disease-free women and is associated with impaired decidualization [9]. Interestingly, besides the loss of function, Notch1 gain of function ($Pgr^{Cre/+}Rosa26^{N1ICD/+}$, $N1ICD^{OEx}$) also leads to decidualization failure in mouse [10]. In addition, hyperactivation of Notch signaling is observed in the ectopic endometrium from women with endometriosis [13]. This increase in Notch1 signaling in ectopic lesions is regulated by IL-6 [14].

In a previous study, we have reported that only 14.3% of *Pgr-Cre* driven *Notch1* conditional knockout ($Pgr^{Cre/+}Notch1^{f/f}$, $Notch1^{d/d}$) mice could give birth to a litter in the first 30 days of the fertility test, compared with 100% in $Notch^{f/f}$ mice [7]. However, in the subsequent stages of the 6-month trial, there is no significant difference in the number and size of litters produced by the two groups of mice. Considering a 19–20 day of the gestation period, we hypothesized that the $Notch1^{d/d}$ females that did not give birth in the first 30 days after being paired with fertile males were pregnant but miscarried later during the first pregnancy. In this study, we undertook a more detailed study of the $Notch1^{d/d}$ mice and found that a large percentage of pregnancy loss occurred between E11.5 and E15.5 of the first pregnancies, which contributes to the observation that only a few of $Notch1^{d/d}$ mice had pups in the first 30 days of a fertility test. Furthermore, we showed that the loss of first pregnancy was a consequence of impaired decidualization. Finally, we compared the transcriptome of the decidua on E7.5 from the first and the subsequent pregnancies and identified the *Notch1* regulated gene networks that are crucial for the decidualization.

Materials and methods

Animals and tissue preparation

All mice were maintained in a designated animal care facility at the Michigan State University and South China Agricultural University according to institutional guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committees of both Michigan State University and South China Agricultural University.

Notch1 uterine conditional knockout mice were generated as described previously [7]. Briefly, mice with floxed *Notch1* gene were crossed with Pgr-Cre mice to generate $Pgr^{cre/+}Notch1^{f/f}$ ($Notch1^{d/d}$) mice in which *Notch1* was deleted in the uterus. The $Pgr^{+/+}Notch1^{f/f}$ ($Notch1^{f/f}$) littermates served as controls. Females were paired with fertile males to induce pregnancy, and the morning of a vaginal plug was designated as E0.5. Pregnant females were sacrificed in a CO_2 chamber, and the uteri were collected on E7.5, E11.5, and E15.5, respectively. For subsequent pregnancies, females that had already produced three litters were paired with males, and uteri were collected on E7.5. Implantation sites (ISs) were weighed, flash-frozen, and stored at −80 °C for frozen sections and RNA isolation.

In situ hybridization

Total RNA was extracted by using TRZOL reagent (Invitrogen, CA, USA). Total RNA was then reverse transcribed, and the template of Prl3c1 probe was amplified with the primers 5′-GCCAC ACGATATGACCGGAA-3′ and 5′-TTTGCTCCCTCCAGAACGAC-3′. Digoxigenin-labeled antisense or sense cRNA probes were transcribed in vitro using an RNA labeling kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. In situ hybridization was performed as previously described [15]. Briefly, 10-μm frozen sections were fixed in a 4% paraformaldehyde solution for 1 h and permeabilized in 1% Triton X-100 for 20 min. The sections were then hybridized with an antisense probe (1:100) at 55 °C for 16 h in hybridization solution. Digoxigenin-labeled Prolactin family 3, subfamily c, member 1 (*Prl3c1*) sense probe was used as the negative control. Sections were then incubated in alkaline phosphatase conjugated anti-digoxigenin antibody at 4 °C (1:5000; Roche Applied Science, Penzberg, Germany) overnight, and the signal was visualized as a dark brown color after incubation with NBT-BCIP substrate solution (Ameresco, MA, USA). Levamisole (Sigma, MO, USA) was used to inhibit endogenous alkaline phosphatase activity.

High-through gene expression analysis

Both RNASeq and Microarray were used in this study to validate each other. For RNASeq, the RNA quality had an A260/A280 ratio ≥2.0 and RIN (RNA integrity number) value ≥7.0. The libraries were prepared using the TruSeq RNA sample preparation kit (Illumina, CA, USA) following the manufacturer's instructions. Furthermore, high-throughput sequencing was conducted on the Illumina HiSeq 2500 Rapid Run flow cell. The data were processed by Illumina Bclzfastq 1.8.4.

The Microarray was performed using GeneChip Mouse Gene 430.2.0 Arrays. Genes were considered as being differentially expressed with an adjusted *P*-value of <0.01 and an absolute fold change of ≥1.5. Data were analyzed with Ingenuity System Inc.

All the raw data of this study have been submitted to Gene Expression Omnibus (GSE153964).

Gene ontology and pathway analysis

Gene ontology (GO) analysis was performed using the DAVID tool. GO terms with a gene count <10 were eliminated from further analysis. The significance cutoff for the false discovery rate (FDR) was set at 0.01. Spare GO terms were removed manually [16]. The wordcloud for significantly enriched GO and pathway terms was created by using the R package wordcloud. The STRING v10.0 database was used to construct the gene network. The minimum combined score of the hub gene network was set to 0.4 by default. The Cytoscape software was used to view and analyze the gene networks. The degree of distribution was calculated by the Cytoscape

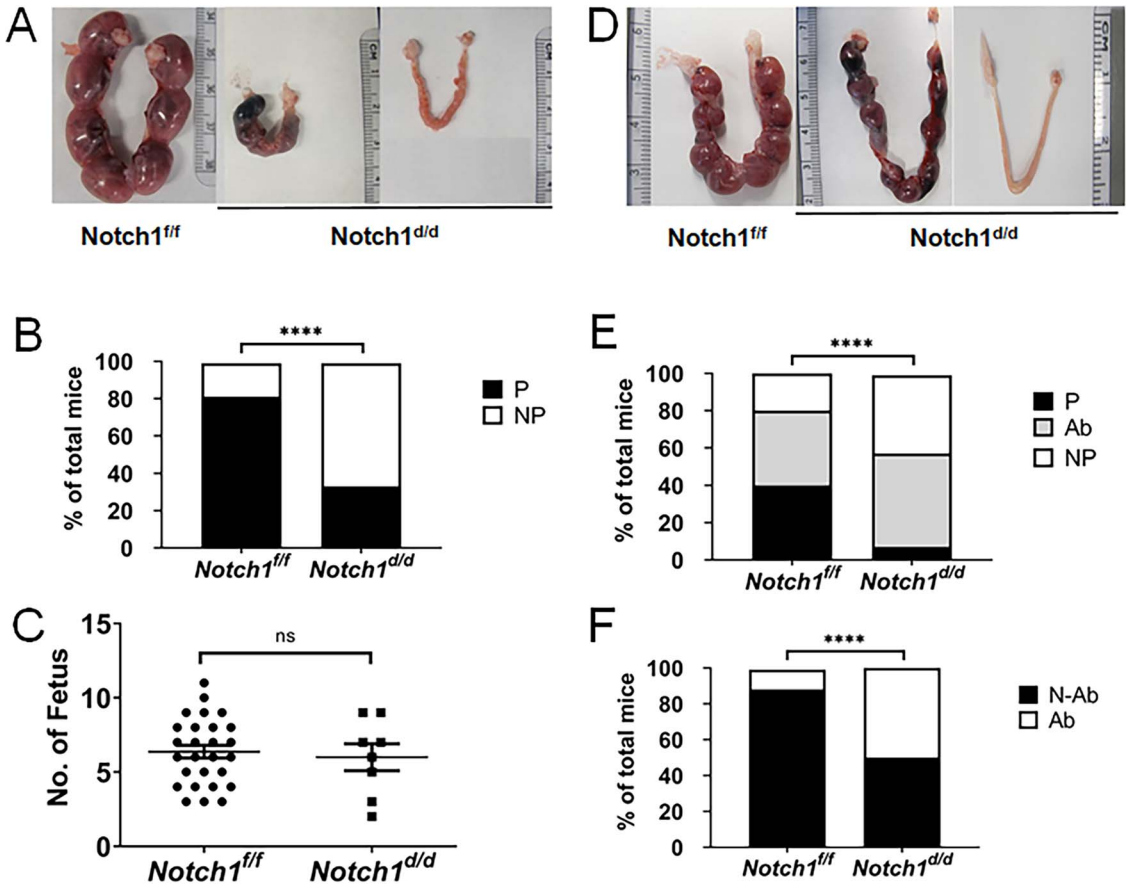

Figure 1. Number of pregnancy losses on E11.5 and 15.5 in *Notch1*$^{d/d}$ mice. (A) Representative uterine images of *Notch1*$^{f/f}$ and *Notch1*$^{d/d}$ mice on E15.5. (B) Percentage of mice with healthy pregnancies (P) or non-pregnant (NP) in *Notch1*$^{f/f}$ and *Notch1*$^{d/d}$ mice on E15.5. (C) The number of ISs in *Notch1*$^{f/f}$ and *Notch1*$^{d/d}$ mice on E15.5. (D) Representative pictures of uteri with absorbing implantation sits (middle) and non-pregnant uterus (right) in *Notch1*$^{d/d}$ group, and normal pregnant *Notch1*$^{f/f}$ mice on E11.5. (E) Percentage of mice with healthy pregnancy (P), with at least one absorbing IS (Ab), or non-pregnant (NP) in *Notch1*$^{f/f}$ and *Notch1*$^{d/d}$ mice on E11.5. (F) The number of ISs that are being absorbed (Ab) or not being absorbed (N-Ab) in *Notch1*$^{f/f}$ and *Notch1*$^{d/d}$ mice on E11.5. $^{****}P < 0.0001$; ns: no significance.

plugin Network Analyzer. The mean plus two standard deviations were chosen as the degree threshold value for hub genes.

Statistical analysis

Data are presented as mean±SEM unless stated otherwise. The unpaired student *t*-test was used to analyze the significance of data presented in Figures 1C and 2B and C. The Fisher exact test was used to analyze the significance of data presented in Figure 1B and F. The Chi-square was used to analyze the significance of data presented in Figure 1E. All statistical analyses were performed by GraphPad Prism 8.0 (GraphPad Software). $P < 0.05$ was considered to be significantly different.

Results

Notch1$^{d/d}$ mice lose their first pregnancy due to impaired decidualization

To test whether *Notch1*$^{d/d}$ mice lose their first pregnancy as hypothesized, we collected the uteri of both *Notch1*$^{f/f}$ and *Notch1*$^{d/d}$ mice on E15.5 after their first mating with fertile males. As expected, a significantly higher rate of pregnancy loss in *Notch1*$^{d/d}$ mice was observed compared with *Notch1*$^{f/f}$ control mice (Figure 1A). On E15.5, only 33.3% (8 out of 24) of *Notch1*$^{d/d}$ mice maintained a pregnancy compared with 81.8% (27 out of 33) in *Notch1*$^{f/f}$ mice (Figure 1B). However, for those mice which successfully maintained a pregnancy, the number of fetuses was comparable between *Notch1*$^{d/d}$ and *Notch1*$^{f/f}$ mice (Figure 1C). Next, we examined an earlier stage of pregnancy on E11.5. Similar to E15.5, up to 41.7% of *Notch1*$^{d/d}$ mice had no ISs, compared with only 20% in *Notch1*$^{f/f}$ group (Figure 1D and E). Moreover, in those *Notch1*$^{d/d}$ with ISs, a large number of absorbed ISs were observed (Figure 1D and E). Fifty percent of ISs were absorbed in *Notch1*$^{d/d}$ mice compared with only 11.6% in *Notch1*$^{f/f}$ group (Figure 1F). When we further analyzed each mouse individually, the absorption rate of ISs in each mouse ranged from 0 to 100% in *Notch1*$^{d/d}$ mice, with an average of 58.8%. Meanwhile, in *Notch1*$^{f/f}$ mice, this ranged from 0 to 42.9%, with an average of only 17.7%. These results suggested that some of the *Notch1*$^{d/d}$ mice were losing all the fetuses, which was consistent with the fact that a large percentage of *Notch1*$^{d/d}$ mice showed no pregnancies later than E15.5.

Decidualization is one of the essential events during early pregnancy. Decidulaization failure has been suggested as the reason for multiple disorders in the later stages of pregnancy, including placental impairment, pregnancy loss, and other pathological conditions such as preeclampsia [17–20]. Impairment of artificial decidualization in *Notch1*$^{d/d}$ mice [7] led us to test whether decidualization was affected in the *Notch1*$^{d/d}$ mouse during natural pregnancy.

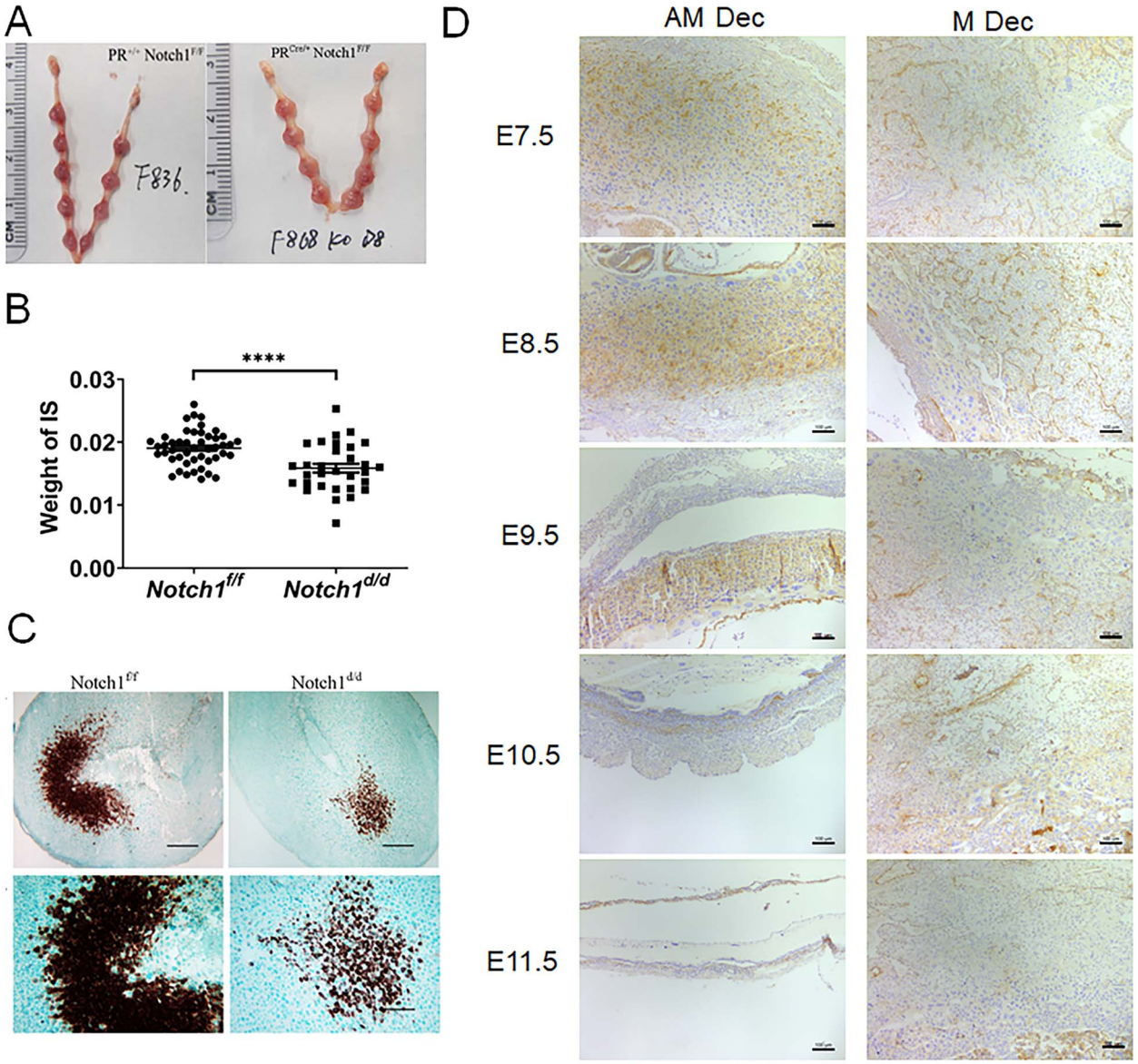

Figure 2. Impaired decidualization response in *Notch1*$^{d/d}$ mice. (A) Representative pictures of uteri from *Notch1*$^{f/f}$ and *Notch1*$^{d/d}$ mice on E7.5. (B) The number of ISs in *Notch1*$^{f/f}$ and *Notch1*$^{d/d}$ mice on E7.5, in which each spot represents one IS in each mouse. (C) The expression of *Prl3c1* mRNA in *Notch1*$^{f/f}$ and *Notch1*$^{d/d}$ mice on E7.5. $^{****}P < 0.0001$; $^{*}P < 0.05$; Bar = 400 μm (upper panel) or 200 μm (Lower panel).

The results showed that the weight of the ISs was significantly lower in *Notch1*$^{d/d}$ mice compared with the *Notch1*$^{f/f}$ mice on E7.5 (Figure 2A and B). Histological evidence showed a remarkable delay of embryonic development and lower expression of the decidualization marker *Prl3c1* as detected by in situ hybridization (Figure 2C). All the results above suggested that in *Notch1*$^{d/d}$ mouse, impaired decidualization during the early stages of pregnancy led to fetal absorption and pregnancy loss in the middle and late stages of the first pregnancy and further contributed to the lower birth rate of *Notch1*$^{d/d}$ mice in the first 30 days of the fertility test.

Transcriptome analysis of *Notch1*$^{d/d}$ and *Notch1*$^{f/f}$ mice on E7.5 of the first and subsequent pregnancies

To better understand the mechanisms by which Notch1 affects decidualization on E7.5, ISs on E7.5 of the first pregnancy and subsequent pregnancies, which represent the fourth or fifth pregnancy in this study, were collected from both *Notch1*$^{d/d}$ and *Notch1*$^{f/f}$ mice. After removing the myometrium and embryonic tissue, the pure decidual tissues were used for RNA-sequencing. Comparisons of gene expression profiles were analyzed, and the data are shown in Figure 3A. In Comparison 1, we compared differentially expressed genes between *Notch1*$^{d/d}$ and *Notch1*$^{f/f}$ mice in their first pregnancy, and there were 165 up-regulated genes and 280 down-regulated genes. In Comparison 2, in the subsequent pregnancy, there were 83 genes up-regulated, and 122 genes down-regulated in *Notch1*$^{d/d}$ mice compared with control mice. However, when we mapped these two comparisons together, there were only 112 overlapping genes, suggesting a distinction in the gene regulation network by Notch1 between the first and subsequent pregnancies (Figure 3B). Furthermore, we analyzed comparisons 3 and 4, which reflect differential gene expression between the first and subsequent pregnancies in *Notch1*$^{d/d}$ and *Notch1*$^{f/f}$ mice, respectively. Surprisingly, only

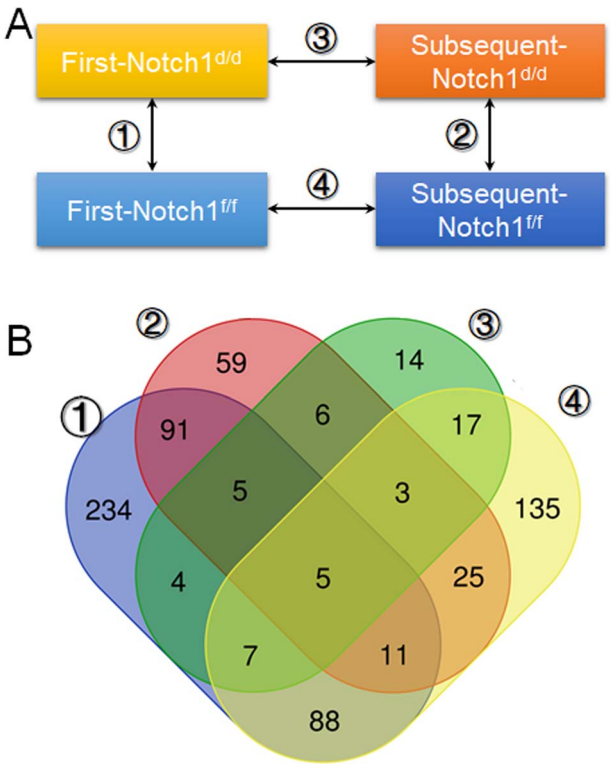

Figure 3. Determination of Notch1-regulated genes during decidualization on E7.5.(A) The four physiologically relevant comparisons used to identify genes regulated by *Notch1* deletion and decidualization: comparison 1, $Notch1^{d/d}$ vs. $Notch1^{f/f}$ in first pregnancy; comparison 2, $Notch1^{d/d}$ vs. $Notch1^{f/f}$ in subsequent pregnancies; comparison 3, first pregnancy vs. subsequent pregnancy in $Notch1^{d/d}$ mice; comparison 4, first pregnancy vs. subsequent pregnancy in $Notch1^{f/f}$ mice. Differentially expressed genes were selected using a two-sample comparison and 2-fold change. (B) Venn diagrams demonstrating the relationship between genes modulated in decidua in response to *Notch1* deletion in different pregnancies. The purple set indicates genes selected by comparison 1, the pink circle shows genes selected by comparison 2, the green circle by comparison 3, and the yellow circle by comparison 4. The numbers within the intersections of the circles indicate the common genes in two, three, or all four comparisons.

15 up-regulated and 46 down-regulated genes were identified in comparison 3, but up to 115 up-regulated and 177 down-regulated genes were observed in comparison 4.

Pathway analysis of mechanisms regulated by Notch1 during decidualization

Since decidualization impaired pregnancy loss occurred only in the first pregnancy of $Notch1^{d/d}$ mice, we surmised that genes that were significantly changed in the $Notch1^{d/d}$ mice in the first pregnancy but not in the subsequent pregnancies were responsible for this pregnancy loss. Therefore, we excluded the differentially expressed genes in comparison 2 from that in comparison 1. There were a total of 333 genes in this gene set, with 118 up-regulated and 215 down-regulated. A heatmap of these 333 genes expressed in all the groups is shown in Figure 4A. Additionally, as validation, the expression pattern of these genes determined by Microarray was highly accordant with RNA-seq data in both first and subsequent pregnancies (Figure 4B and C).

GO analysis of this set of 333 genes was performed using DAVID online tools, and GO terms were divided into three parts: biological process (BP), cellular component (CC), and molecular function (MF). There were a total of 28 significantly enriched GO terms ($P < 0.01$, FDR < 0.05). The top-ranked were plasma membrane, extracellular region, calcium ion binding, ion transport, cell adhesion, biological adhesion,alkali metal ion binding, cation transport, and metal ion transport (Table 1, Figure 5C). All the top-ranked enriched GO terms pointed to cell membrane functions such as cell–cell or cell–matrix adhesion and communication. This result indicated that adhesion and communication of decidual cells with each other, as well as with other cell types such as trophoblast cells, is likely the most critical process regulated by Notch1 in the first pregnancy. The KEGG pathway analysis was performed using the same tool, which showed that only one pathway, the cytokine–cytokine receptor interaction pathway, a pathway that is important for cell regulation and communication, was significantly enriched (Table 1, Figure 5A). In the network analysis, we identified nine hub genes. The genes that were highly connected with others include Matrix metallopeptidase 9 (*Mmp9*), Secreted phosphoprotein 1 (*Spp1*), Proto-oncogene tyrosine-protein kinase Kit (*Kit*), Prostaglandin-endoperoxide synthase 2 (*Ptgs2*), Insulin-like growth factor 2 (*Igf2*), Interleukin 2 receptor subunit alpha (*Il2ra*), Cytotoxic T-lymphocyte associated protein 4 (*Ctla4*), and Serine peptidase inhibitor, clade B, member 9c (*Serpinb9c*) (Figure 5B and C). All of them are downregulated in the *Pgr-Cre* driven conditional knockout of *Notch1*. Functions of these hub genes are mainly related to the cellular matrix (*Mmp9*), cell adhesion (*Spp1, Itga2*), cell proliferation (*Kit, Igf2*), and inflammation/immune cells, and many of these genes, such as *Mmp9*, *Spp1*, *Kit*, and *Ptgs2*, have been reported to play roles in uterine receptivity and decidualization. (*Ptgs2*, *Il2ra*, *Ctla4*) [1, 2, 21–25].

Discussion

Notch1 has been reported to play a critical role during the artificial decidualization of stromal cells in mice [7]. However, there is still limited data regarding the role of *Notch1* in a natural pregnancy: $Notch1^{d/d}$ females are fertile, without significant differences in litter size or number compared with $Notch^{f/f}$ mice in a long-term 6-month fertility test [7]. But in the first 30 days of the fertility test reported in this manuscript, $Notch1^{d/d}$ mice showed impaired reproductive capacity, with only 14.3% of these females able to give birth to a litter of pups compared with 100% in $Notch^{f/f}$ mice [7]. Therefore, whether *Notch1* plays a role in the natural pregnancy of mouse becomes an interesting question to be investigated. In this study, we undertook a closer analysis of $Notch1^{d/d}$ mice in their first natural pregnancy. Our results showed that most of $Notch1^{d/d}$ mice lost their first pregnancy between E7.5 and 11.5 of gestation, which can explain the impaired reproductive capacity in $Notch1^{d/d}$ mice in the first 30 days of the fertility test. Furthermore, the loss of pregnancy in this period is a consequence of impaired decidualization evidenced by the significant decrease in the weight of the ISs on E7.5 of natural pregnancy.

Unlike artificial decidualization, which is initiated by mechanical stimulation, in a natural pregnancy, the decidualization of stromal cells is a consequence of the implanting embryo. Other investigators reported 5277 differentially expressed genes on E7.5 of natural pregnancy when they compared decidua to non-decidualized uterine tissue from inter-ISs, and 4294 differentially expressed genes in the artificial decidualization model. However, only 1114 down-regulated and 863 up-regulated genes are shared by the two models

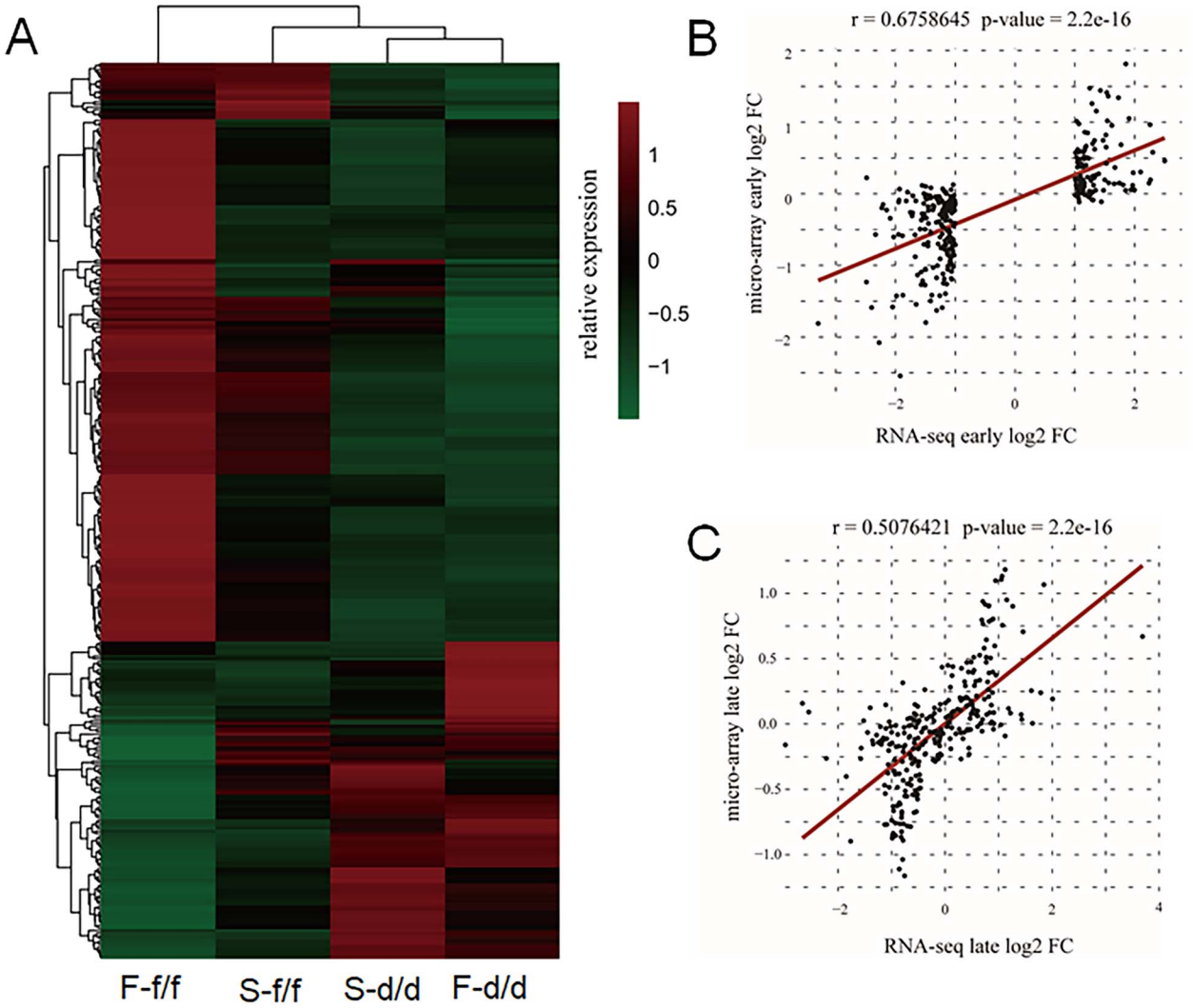

Figure 4. (A) Heatmap plot for differentially expressed genes. (F-f/f: first pregnancy of *Notch1*$^{f/f}$ mice, S-f/f: subsequent pregnancy of *Notch1*$^{f/f}$ mice, S-d/d: subsequent pregnancy of *Notch1*$^{d/d}$ mice, F-d/d: first pregnancy of *Notch1*$^{d/d}$ mice). (B) Linear regression for the correlation between microarray and RNA-seq data based on the first pregnancy group. The correlation coefficient and *P*-values were calculated using the Pearson correlation test. (C) Linear regression of the correlation between microarray and RNA-seq data based on multiple pregnancies group. The correlation coefficient and *P*-value were calculated using the Pearson correlation test.

[26]. Therefore, in addition to the previously reported artificial decidualization model [7], we further investigated the gene expression profiles of E7.5 natural pregnant decidua from *Notch1*$^{d/d}$ mice to uncover the underlying molecular mechanism of impaired decidualization and consequential pregnancy loss. In this study, after excluding the differentially expressed genes of the subsequent pregnancy from that of the first pregnancy, we had a list of 333 genes that were affected by *Notch1* and essential for decidualization. GO terms showed these genes played roles in the processes of cell–cell and cell–matrix interaction, which is different from the artificial decidualization model in which cell proliferation and apoptosis are the most enriched terms [7]. Previously, as reported by Kashiwagi and co-workers, more than 1500 genes were differentially expressed in decidual tissues between the natural pregnancy and artificial decidualization and the number of proliferating cells in the artificial decidualization model is significantly higher than that in the natural pregnancy model [27]. This study may explain the different enriched terms between these two models in *Notch1*$^{d/d}$ mice.

The decidua plays both a protective and supportive roles to control trophoblast invasion in the presence of an embryo [2]. The matrix metalloproteinases (MMPs) secreted by trophoblast cells can degrade the extracellular matrix of the decidua [28, 29]. In addition, other studies demonstrate that decidual cells also produce MMPs to help facilitate the invasion of trophoblast cells [30, 31]. However, the expression of *MMP9* and other genes involved in tissue remodeling is significantly higher in the uterus from a natural pregnancy compared with an artificially decidualized uterus [27]. In our study, genes that are associated with the extracellular matrix, including MMP9 as a hub gene, are decreased in *Notch1*$^{d/d}$ mice. Other enriched terms, such as plasma membrane function and cell adhesion, as well as hub genes *Spp1*, *Itga2*, which are involved in cell adhesion and also contribute to the remodeling of the extracellular matrix and are

Table 1. List of Enriched GO terms.

Category	Term	*P*-value	FDR
GOTERM_CC_FAT	GO:0005886 ~ plasma membrane	1.91E−08	2.42E−07
GOTERM_CC_FAT	GO:0044459 ~ plasma membrane part	7.63E−07	9.68E−06
GOTERM_CC_FAT	GO:0005576 ~ extracellular region	1.95E−06	2.48E−05
GOTERM_MF_FAT	GO:0005509 ~ calcium ion binding	8.33E−05	0.001179
GOTERM_BP_FAT	GO:0006811 ~ ion transport	1.05E−04	0.001702
GOTERM_BP_FAT	GO:0007155 ~ cell adhesion	1.13E−04	0.001843
GOTERM_BP_FAT	GO:0022610 ~ biological adhesion	1.15E−04	0.001873
GOTERM_MF_FAT	GO:0031420 ~ alkali metal ion binding	2.03E−04	0.002866
GOTERM_BP_FAT	GO:0006812 ~ cation transport	2.42E−04	0.003923
GOTERM_BP_FAT	GO:0030001 ~ metal ion transport	2.48E−04	0.004027
GOTERM_CC_FAT	GO:0009986 ~ cell surface	8.14E−04	0.010281
GOTERM_BP_FAT	GO:0007610 ~ behavior	6.89E−04	0.011145
GOTERM_BP_FAT	GO:0015672 ~ monovalent inorganic cation transport	8.16E−04	0.013188
GOTERM_BP_FAT	GO:0008217 ~ regulation of blood pressure	8.85E−04	0.014286
GOTERM_CC_FAT	GO:0005887 ~ integral to plasma membrane	0.001377	0.017323
GOTERM_MF_FAT	GO:0043167 ~ ion binding	0.001313	0.018427
GOTERM_BP_FAT	GO:0006814 ~ sodium ion transport	0.001473	0.023673
GOTERM_MF_FAT	GO:0031402 ~ sodium ion binding	0.00182	0.025452
GOTERM_CC_FAT	GO:0031226 ~ intrinsic to plasma membrane	0.002207	0.027635
GOTERM_CC_FAT	GO:0043005 ~ neuron projection	0.002756	0.034392
GOTERM_MF_FAT	GO:0046872 ~ metal ion binding	0.002545	0.035422
GOTERM_MF_FAT	GO:0005179 ~ hormone activity	0.003078	0.042702
GOTERM_MF_FAT	GO:0070011 ~ peptidase activity, acting on L-amino acid peptides	0.003191	0.044234
GOTERM_MF_FAT	GO:0043169 ~ cation binding	0.003352	0.046411
GOTERM_MF_FAT	GO:0022838 ~ substrate specific channel activity	0.003388	0.046904
GOTERM_BP_FAT	GO:0007626 ~ locomotory behavior	0.003	0.047673
GOTERM_BP_FAT	GO:0003013 ~ circulatory system process	0.003135	0.049771
GOTERM_BP_FAT	GO:0008015 ~ blood circulation	0.003135	0.049771

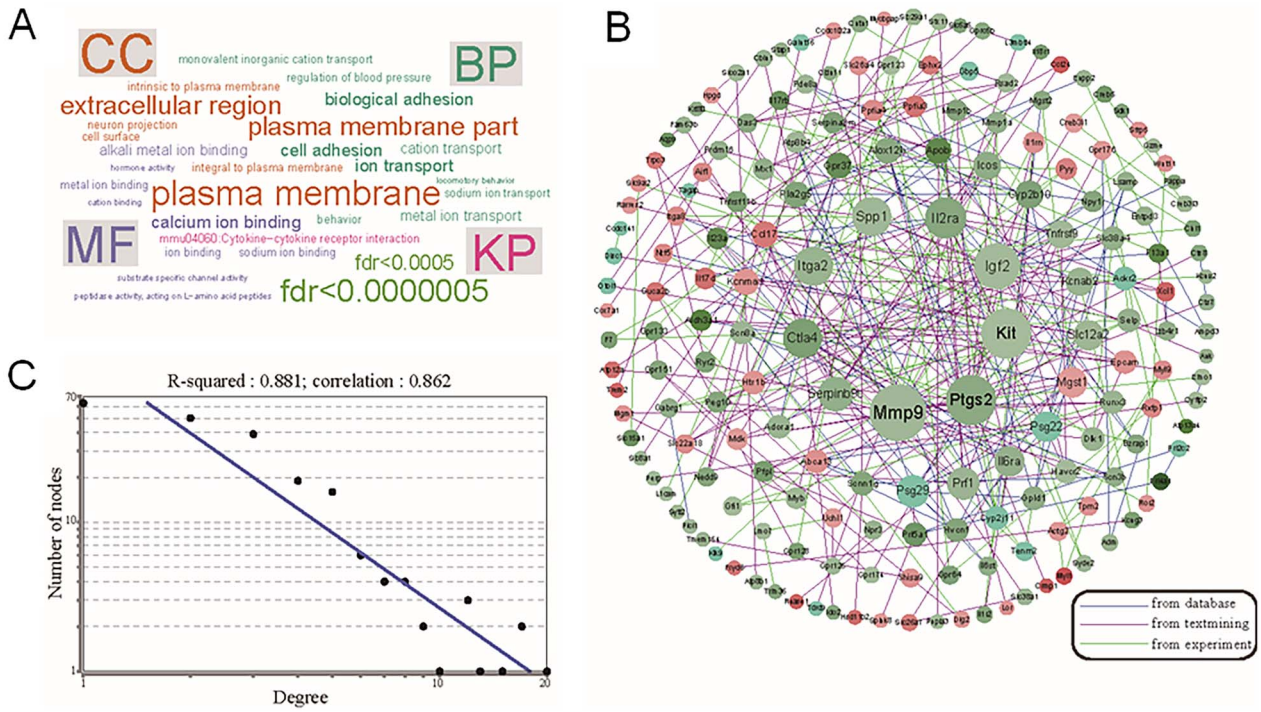

Figure 5. Network analysis of differentially expressed genes. (A) Wordcloud plot for presenting significantly enriched terms in GO and Kegg Pathway (KP) analysis with the cutoff for FDR set at 0.05. (B) Gene–gene interaction network with hub genes placed centrally. Up/down-regulated genes were colored in red and green, respectively. (C) The degree distribution of the network.

essential for the invasion of trophoblast cells, were also decreased. Inhibition of these BPs in *Notch1*$^{d/d}$ mice may limit trophoblast invasion and further lead to consequent pregnancy loss.

Immune cells, especially uNK and Treg cells, contribute positively to pregnancy by producing growth factors and cytokines that promote trophoblast invasion during early pregnancy [32]. The number of NK cells present in the natural pregnant decidua is much higher than the artificially induced decidua [27]. Factors produced by decidual cells increase the expression of the IL2Ralpha on uNK cells and further contribute to IL-2 mediated proliferation of NK cells [23, 33]. A high level of CTLA-4 produced by Treg cells has been reported associated with better pregnancy outcomes [34]. The percentage of Treg cells and the level of CTLA-4 are significantly lower compared with controls [25]. Both *IL2ra* and *Ctla4* are significantly down-regulated hub genes in our study, indicating that the number and functions of uNK and Treg cells are suppressed in *Notch1*$^{d/d}$ mice in the first pregnancy, which also contributes to pregnancy loss in later stages of gestation.

A study performed by Liu and co-workers has reported 58 down-regulated and 73 up-regulated genes in the decidua on E7.5 between the first and the second pregnancies [35]. In the present study, we observed 115 up-regulated and 177 down-regulated genes in subsequent pregnancies compared with the first pregnancy. These results indicate the possibility that the decidualization associated gene regulatory network of decidualization in subsequent pregnancies may differ from that of the first pregnancy in *Notch1*$_{d/d}$ mice and may provide an explanation for our findings in this study.

In conclusion, our study provides additional information for understanding the role of Notch1 signaling during decidualization and early pregnancy. We have shown that Notch1 plays an essential role during decidualization in natural pregnancy but more profoundly in the first pregnancy. The impaired decidualization response during the first pregnancy further results in pregnancy loss, which is supported by the significantly lower number of litters produced by *Notch1*$^{d/d}$ mice in the first month of the fertility test. We further show that *Notch1* mediates decidualization mainly through regulating the communication of decidua cells with other cell types, such as trophoblast cells and immune cells, as well as the extracellular matrix.

Author contributions

ATF and RWS conceived the study. ATF, RWS, and YW wrote the paper. RWS, YW, JX, KZW, and JWK performed experiments. RWS, JPH, and YW analyzed data.

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Conflict of interest

The authors declare no competing financial interests.

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