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P4 down-regulates Jagged2 and Notch1 expression during primordial folliculogenesis

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
- *3.1. Animal treatment*
- *3.2 Real-time PCR*
- *3.3. Hormone measurement*
- *3.4. In vitro fetal ovary culture*
- *3.5. Western blot*
- *3.6. Immunohistochemistry*
- *3.7. Oocyte and follicle counting*
- *3.8. Hormone injection*
- *3.9. RNA Interference (RNAi) on cultured fetal mouse ovaries*
- *3.10. Statistical analysis*

4. Results
- *4.1. The expression of Notch pathway genes in perinatal ovaries*
- *4.2. Maternal P4 levels during midpregnancy inhibited Jagged2, Notch1 and Hey2 expression and primordial folliculogenesis*
- *4.3. Suppression of Jagged2 and Notch1 by antibody exposure, and RNAi inhibited nest breakdown and primordial folliculogenesis in vitro*
- *4.4. Localization of Jagged2 and Notch1 in the mouse ovary*
- *4.5. RU486 inhibited Jagged2, Notch1, and Hey2 expression, and primordial folliculogenesis in vitro*

5. Discussion
6. Acknowledgment
7. References

1. ABSTRACT

Nest breakdown and primordial folliculogenesis of the mouse ovary can be inhibited by progesterone (P4) and Notch signaling inhibitors. However, the relationship between these two signals during this process remains unknown. In the present study, transcript levels of Jagged2, Notch1, and their target, Hey2, increased markedly in ovaries during the beginning stage of folliculogenesis (17.5 days post coitus (dpc) to birth). Maternal P4 levels decreased simultaneously. We found that maternal midpregnancy P4 levels significantly inhibited Jagged2, Notch1, and Hey2 expression, and follicle formation *in vitro*. Maintaining high maternal P4 levels by daily injection also significantly suppressed the expression of Jagged2, Notch1, and Hey2, and follicle formation during late pregnancy. Based on immunohistochemistry, Jagged2 was localized in oocytes and Notch1 was strongly stained in pre-granulosa cells in 19.5 dpc ovaries. Suppression of their function by antibody addition and RNAi markedly inhibited nest breakdown and follicle formation. Taken together, these results demonstrate that maternal P4 levels during midpregnancy can inhibit the expression of Jagged2 and Notch1, which are involved in primordial folliculogenesis, in the mouse fetal ovary.

2. INTRODUCTION

The pool of primordial follicles established by primordial folliculogenesis endows nearly all oocytes of a mammalian female for use during her reproductive life (1). Prior to primordial follicle formation, primordial germ cells undergo mitosis with incomplete cytokinesis and form germline nests in the fetal mouse ovary (2).Subsequently, the germ cells enter meiosis and become oocytes. Beginning at 17.5 days post coitus (dpc), large nests decrease in size to form smaller nests. This process occurs through programmed cell death of two thirds of the oocytes and invasion of surrounding pre-granulosa cells; cytoplasmic bridges between oocytes in a nest break down and the primordial follicle (i.e., an oocyte enclosed by a single layer of flattened pre-granulosa cells) is formed (3-5). This process occurs primarily around the time of birth and lasts until 3 days post parturition (dpp) (4).

Sex hormones play an important role in primordial follicle formation. 17beta-estradiol (E2) dose-dependently inhibits nest breakdown and primordial follicle assembly in the neonatal mouse ovary (6-7). Progesterone (P4) also significantly suppresses nest breakdown and primordial follicle formation in mice and rats (6, 8). In

Table 1. Primers used in our study

Genes	Primer sequences (sense/antisense)
Jagged 1	5'-ACCACCACCTGCGAAGTGAT-3' 5'-GAGATATACCGCACCCCTTCAG-3'
Jagged 2	5'-CTATCACTCAGAGAGGAAATAGCTCACT-3' 5'-GGAAGAGCCACCCATAACAACT-3'
Notch 1	5'-CGTGGTCTTCAAGCGTGATG-3' 5'-GGTGCTTGCGCAGCTCTT-3'
Notch 2	5'-GCTGTCAATAATGTGGAGGCG-3' 5'-TTGGCCGCTTCATAACTTCC-3'
Notch 3	5'-CTTCCACTGTGAGATTGACTTGC-3' 5'-CTCGTATTGGCAGTGTGTGC-3'
Hes 1	5'-TCAACACGACACCGGACAAAC-3' 5'-CCCTTCGCCTCTTCTCCATG-3'
Hey 2	5'-ATGAGCATTGGATTCCGAGAGT-3' 5'-GGGTCCGACGGGTCAAG-3'
Beta-actin	5'-CACTATTGGCAACGAGCGGT-3' 5'-TGGATGCCACAGGATTCCAT-3'

murine species, maternal P4 levels drop precipitously during late pregnancy (9-10) and primordial follicle formation is initiated during this period (5). Based on these observations, it is possible that the maternal P4 milieu of the fetus is involved in inhibiting nest breakdown and primordial follicle formation in the prenatal ovary (6). Moreover, the molecular mechanism by which P4 exerts its role is not well understood.

The onset of nest breakdown and primordial follicle formation requires communication between select oocytes and surrounding pre-granulosa cells (11-12). As a critical cell-to-cell communicator, the Notch signaling pathway plays a pivotal role in primordial folliculogenesis of the mouse ovary. By eliminating the Notch modification protein Lunatic fringe or by administering gamma-secretase inhibitors that attenuate Notch signaling, germline nest separation and primordial folliculogenesis in the mouse ovary can be markedly suppressed (13-14). The Notch signaling pathway is evolutionarily conserved in both vertebrates and invertebrates and contributes to several developmental processes, such as cell differentiation and migration (15-16). Because Notch receptors and their ligands are single transmembrane proteins (17), the signaling event is mediated by direct cell-to-cell contact. After activation by ligand binding, Notch receptors are cleaved by the gamma-secretase complex and release Notch intracellular domains (NICD), which translocate into the nucleus to activate the transcription of target genes (18). In mammals, there are five Notch ligands, Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4, and four Notch receptors, Notch1–4 (19). Major target genes are in the hairy/enhancer of split (Hes) and hairy-related (Hrt; Hey, CHF, HESR) families (20). The transcripts of Jagged1, Jagged2, Notch1–3, and Notch downstream targets Hes1 and Hey2 are largely expressed in the neonatal mouse ovary (14). Yet, it remains unclear as to how Notch signaling is regulated and which Notch components function in primordial folliculogenesis.

In the present study, we investigated whether P4 can regulate Notch pathway components during primordial follicle formation. We indicate that maternal P4 levels during midpregnancy can inhibit the expression of the Notch pathway components Jagged2 and Notch1 in the mouse fetal ovary, which are involved in primordial folliculogenesis. P4 likely inhibits primordial follicle formation by suppressing Jagged2 and Notch1 expression.

3. MATERIALS AND METHODS

3.1. Animal treatment

All mice used in the study were Kunming white mice purchased from the Laboratory Animal Center at the Institute of Genetics in Beijing. The mice were maintained in the University Animal Care Facility with free access to food and water on a 12:12 h light–dark cycle. Adults were mated in the late afternoon to induce pregnancy. The morning after mating was defined as 0.5 dpc when a vaginal plug was detected, and 8.5 dpc to 15.5 dpc was defined as midpregnancy according to changes in maternal hormone levels (21).

3.2. Real-time PCR

The total RNA of the mouse perinatal ovaries was isolated with TRIZOL Reagent (Invitrogen Corporation, Carlsbad, CA, USA). Reverse transcription (RT) was conducted with oligo (dT) using Moloney Murine Leukemia Virus Reverse Transcriptase according to the manufacturer's instructions (Promega, Madison, WI, USA). Real-time PCR was then performed on an ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA) using QuantiTect® SYBR Green PCR Kits (Qiagen, Valencia, CA). The relative fold change of the selected genes was calculated by the 2-ΔΔCt method as described previously (22). In each experiment, levels of mRNA were presented as relative changes to a specific group (control) in which its expression level was set at 1. All primer sequences are shown in Tables 1. In the present study, we tested all the Notch pathway genes that are expressed largely in neonatal ovaries (14).

3.3. Hormone measurement

Mice were anaesthetized and exsanguinated through the orbital sinus. The blood was collected in heparinized disposable plastic syringes fitted with 27-gauge needles, centrifuged, and the serums obtained were frozen at -20ºC and assayed at the same assay. Serum P4 and E2 levels were measured by radioimmunoassay (RIA) using commercially available kits (Institute of Isotopes Research of Chinese Academy of Atomic Energy, Beijing, China).

3.4. In vitro fetal ovary culture

Mouse ovaries at 15.5 dpc were dissected as described previously (23). Five to seven isolated ovaries were placed in 1 mL DMEM/F-12 medium (GIBCO, Gaithersburg, MD, America) and cultured in 24-cell culture dishes (Nunclon, Nunc, Roskilde, Denmark) at 37ºC, 5% CO2, 95% air atmosphere, and saturated humidity. All of the media were equilibrated in an incubator for 1 h prior to use. The developmental status was observed every day under an inverted microscopy and the medium was changed every other day.

The chemicals used in the culture were P4 (0.04-1 μM), E2 (1 μM), nuclear P4 receptor steroid antagonist RU486 (mifeprostone; 1 μM) and cortisol receptor agonist dexamethasone (0.1-10 μM). They were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All of these chemicals were dissolved in ethanol, which was added to the media at the same percent as the compound treatment (≤0.1%) to serve as a vehicle control. Polyclonal rabbit anti-Jagged2 antibodies (0.5 μg/mL) and anti-

Notch1 antibodies (0.5 μg/mL; Santa Cruz Biotechnology, Inc) were used to block the function of the membrane receptor Jagged2 and Notch1, respectively. The product information shows that the two polyclonal antibodies react without cross-reactivity with other Notch ligands or receptors by Western blot. Rabbit serum immunoglobulin (IgG) was used as a negative homotype contrast at the same concentration. Primordial follicle formation is a gradual process which lasts for 6-7 days in perinatal ovaries, so 15.5 dpc ovaries were cultured with these chemicals for 4 days (equal to 19.5 dpc ovaries, where nests are breaking down largely) to detect the expression of Notch pathway genes and proteins, and cultured for 7 days (equal to 3 dpp ovaries, where most follicles have been formed) to analyze shape and counting of oocytes and follicles.

3.5. Western blot

Western blot analysis was performed as described in a previous report (24). In brief, perinatal ovary proteins were extracted with MEM-R according to the manufacturer's protocol (Pierce, Rockford, IL) and the concentrations were measured using a BCA procedure (CellChip.BJ Biotechnology Co., Ltd, Beijing, China). The lysates were heated to 100ºC for 5 min. After cooling down on ice for 20 min immediately, 50 μg protein complexes for each sample were separated on 10% SDS–PAGE and transferred to pieces of Protran nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membranes were saturated with 5% nonfat dry milk in TBST (20 mM Tris–HCl, 150 mM NaCl, and 0.5‰ Tween 20, pH 7.6) for 1 h and incubated overnight at 4ºC with 1:500 rabbit anti-Jagged2 antibody (150 kDa), 1:500 rabbit anti-Notch1 antibody (120 kDa), 1:100 rabbit anti-NICD1 antibody (80 kDa, anti-Notch1 intracellular domain, Abcam, Cambridge, UK) and 1:200 rabbit anti-Hey2 antibody (36 kDa, Abcam), respectively. After three washes in TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG) diluted 1:5,000 in the TBST. Following three washes in TBST, the proteins on the membranes were visualized using a SuperSignal West Pico (enhanced chemiluminescence) detection system (Pierce Chemical Co., Rockford, IL). The GAPDH levels were examined at the same time as the loading control.

3.6. Immunohistochemistry

Perinatal ovaries obtained from each treatment group were fixed in 4% polyformaldehyde (pH 7.2–7.4) at 4ºC and embedded in paraffin. Embedded ovaries were sectioned at 5 μm. Whole sections were immunostained as described previously (25). After dewaxing, rehydration, and antigen retrieval with a 0.01% sodium citrate buffer (pH 6.0), the primary antibody was incubated at a suitable dilution overnight at 4ºC. The sections were washed in PBS and then incubated with biotinylated goat anti-rabbit IgG (1:200, Zhongshan Company, Beijing, China) and ABC complex (Zhongshan Company). Peroxidase activity was detected by incubation for 1 min with diaminobenzidine (Zhongshan Company). Sections were counterstained with hematoxylin, dehydrated, and coverslipped with histomount reagent.

The antibody of the germline marker Mouse Vasa Homolog (MVH; a gift from Professor Toshiaki Noce) was incubated at a 1:200 dilution. Jagged2 and Notch1 antibodies were used at 1:200 and 1:100 dilutions, respectively. Nonimmunized rabbit serum was incubated in the same dilution as the control group.

3.7. Oocyte and follicle counting

Ovarian oocytes and follicles were counted according to a generally accepted approach where the ovary was fixed, paraffin-embedded, and serially sectioned at 5 μm widths. The serial sections were placed in order on microscope slides and stained with MVH and haematoxylin. Every fifth section was analyzed for the presence of oocytes and follicles. An oocyte was defined as MVH–positive. A primordial follicle was defined as an oocyte surrounded by a single layer of flattened pre-granulosa cells. There were no primary follicles observed in the ovaries after the 7-day culture, so all of the counted follicles were primordial follicles. The starting section was selected randomly. Finally, the cumulative oocyte and follicle counts for each ovary were multiplied by five, because four-fifths of the ovary was not analyzed. There were at least six ovaries analyzed from each treatment group.

3.8. Hormone injection

To maintain high levels of maternal P4 during late pregnancy, pregnant mice at 15.5 dpc were given a daily injection of P4 (3 mg /40 g body weight) or sesame oil (Sigma-Aldrich). Serum was collected at 19.5 dpc for RIA. The fetal ovaries were collected to analyze the mRNA and protein expression of Notch pathway components. Fetuses were normally delivered at 19.5 dpc after oil treatment, but not following P4 treatment. Fetal ovaries at 1 dpp (oil treatment) and 20.5 dpc (P4 treatment) were isolated to analyze follicle formation. To exclude the possible influence of E2 on follicle formation (6), the pure estrogen antagonist ICI 182780 (Tocris Cookson Inc., Ellisville, MO) was used concomitantly. The fetal ovaries after 5 days of treatment with oil, P4 alone, ICI 182780 (125 μg/40 g body weight) alone, or P4 plus ICI 182780 were isolated and immediately fixed for follicle formation analysis. The P4 injection doses were chosen based on our RIA and the ICI 182780 injection doses were based on a previous report (26).

3.9. RNA interference (RNAi) on cultured fetal mouse ovaries

siRNAs are difficult to transfect into the inner cells of an organ using traditional transfection methods. To assure that siRNA would be transfected into the inner cells of fetal ovaries, 0.5 μL siRNA (20 μM) were first injected into the isolated fetal ovaries at 17.5 dpc using glass pipettes with a stereo microscope. After the ovaries were full of the liquid, electrotransfection was accomplished by applying three 5-ms-long quasi-square pulses at a pulse-field strength of up to 40 V/cm. The ovaries were cultured as described above for 4 or 7 days to test transfection efficiency and follicle formation.

The sequence of Jagged2 siRNA was 5'-GGATCAACTGCCAAATCAAdTdT-3', which targeted 1377-1393 bps of Jagged2 mRNA, and the sequence of Notch1 siRNA was 5'-GGAACAACTCCTTCCACTTdTdT-3', which targeted 5021-5039 bps of Notch1 mRNA. The scrambled siRNA sequence was 5'-TTCTCCGAACGTGTCACGdTdT-3', which has no homology to any known mouse mRNA. All of the siRNAs were chemically synthesized by Invitrogen. The siRNAs were first transfected into mouse cell lines to check their effectiveness.

To detect if an interferon-like response was invoked by siRNA transfection in cultured ovaries, Real-

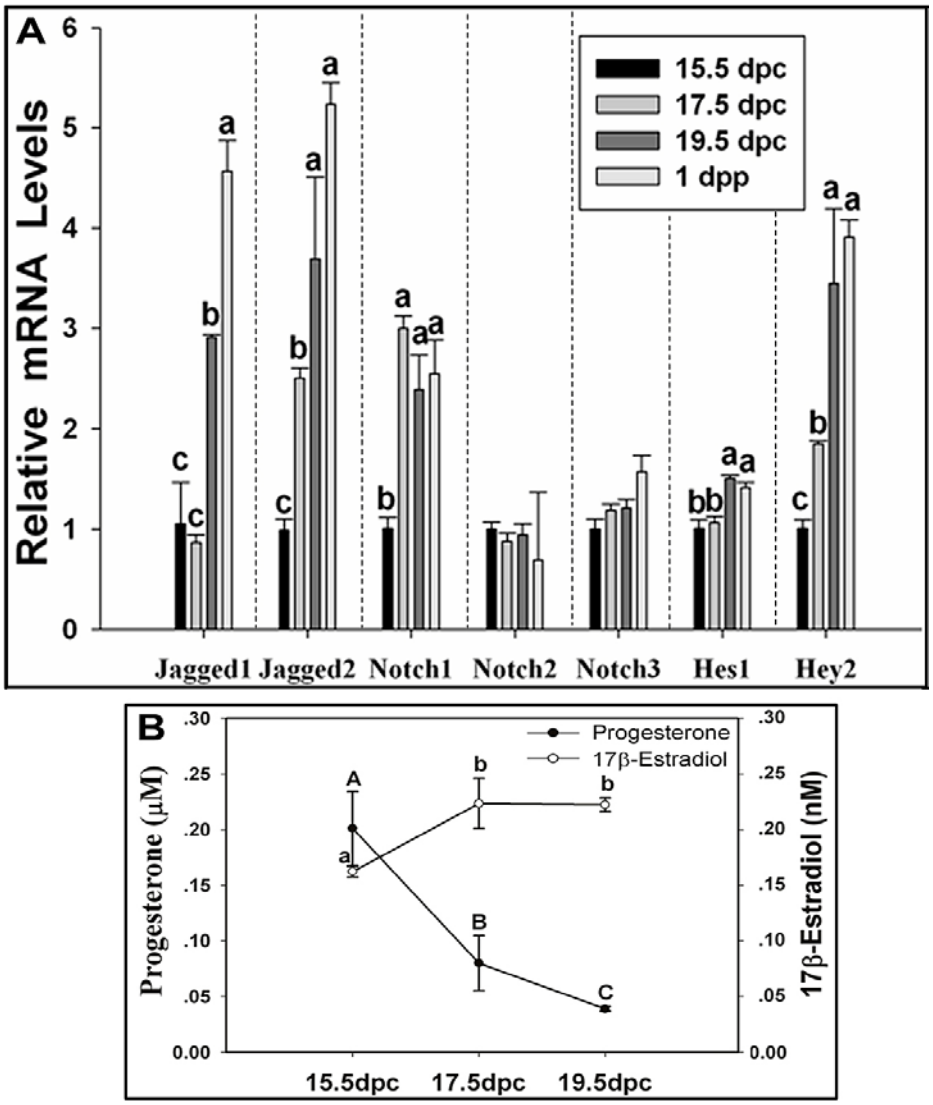

Figure 1. The expression characteristics of Notch pathway genes in perinatal ovaries and the levels of maternal steroids during mid-late pregnancy. The mRNA levels of Notch signaling genes in the mouse ovary from 15.5 dpc to 1 dpp were measured using Real-time PCR. Letters denote statistical significance at $P<0.01$ (A). The P4 and E2 levels in maternal serum taken at 15.5 dpc to 19.5 dpc were assayed by RIA. Letters denote statistical significance at $P<0.05$ (B). dpc: days post coitus; dpp: days post parturition.

time PCR was used to measure mRNA content of Oas1, a classical interferon target gene (27-28), in ovaries transfected with siRNAs targeting Jagged2 or Notch1 mRNA.

3.10. Statistical analysis

All experiments were performed at least three times, and the values are presented as mean ± SEM. Statistical analysis by ANOVA was performed using StatView software (SAS Institute, Inc., Cary, NC, USA). When a significant F ratio was defined by ANOVA, groups were further compared by using Fisher's protected least significant difference post hoc test. $P<0.05$ was considered statistically significant.

4. RESULTS

4.1. The expression of Notch pathway genes in perinatal ovaries

The expression of Notch pathway genes in perinatal ovaries during primordial folliculogenesis was studied using Real-time PCR. As shown in Figure 1A, the mRNA levels of Jagged2, Notch1, and the Notch signaling target Hey2 increased significantly from 17.5 dpc to 1 dpp. However, the transcripts of Jagged1 and another Notch signaling target Hes1 did not increase until 19.5 dpc. Notch2 and Notch3 mRNA levels showed no obvious changes during the test stages. Meanwhile, we examined the P4 and E2 levels of maternal serum during mid-late pregnancy by RIA. P4 levels were relatively high at 15.5

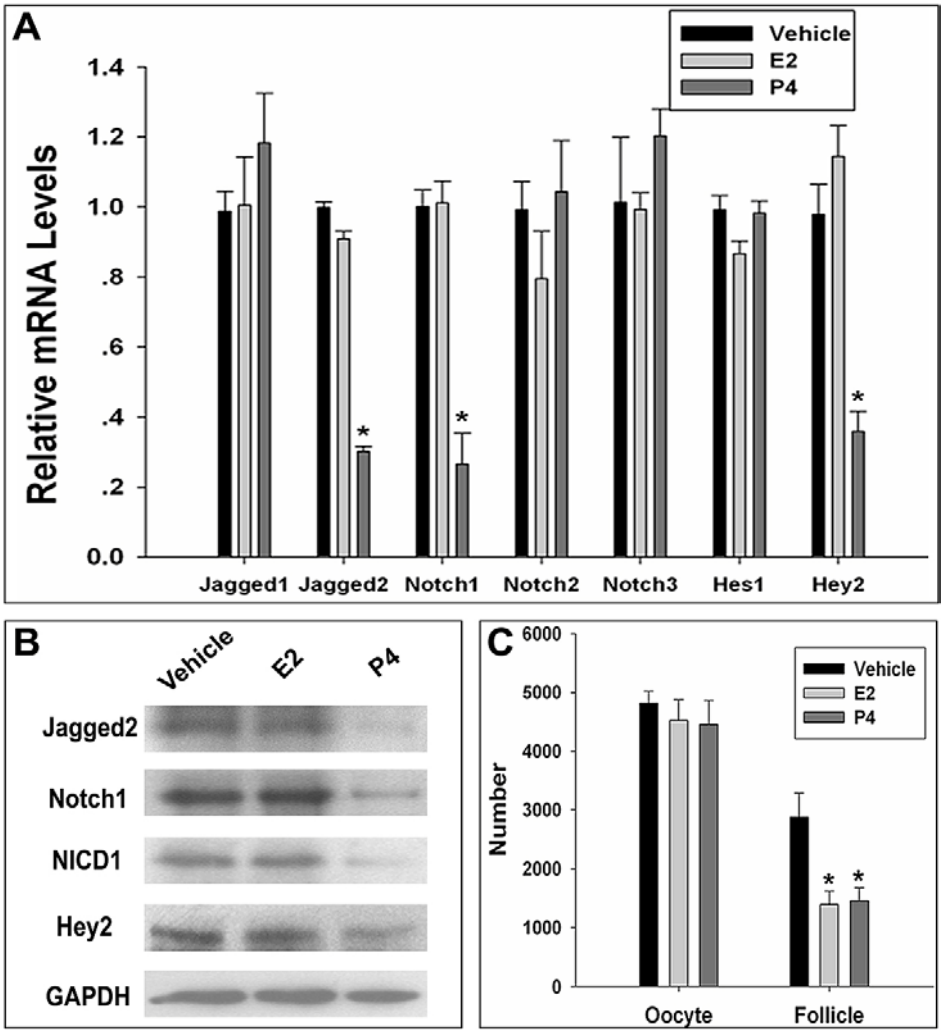

Figure 2. The Notch pathway components Jagged2, Notch1, and Hey2 were down-regulated by P4 *in vitro*. 15.5 dpc ovaries were cultured with vehicle, 1 μM E2, or 1 μM P4 for 4 days. The mRNA levels of Notch pathway gene components were measured using Real-time PCR (A) and changes in the expression of Jagged2, Notch1, NICD1, and Hey2 at the protein level were measured by Western blot (B). After the same treatments for 7 days, paraffin ovary sections were stained with the germline marker MVH and hematoxylin for counting oocytes and primordial follicles (C). * denotes statistical significance at $P<0.001$

dpc (0.20 ± 0.03 μM), decreased significantly at 17.5 dpc (0.08 ± 0.02 μM), and then dropped further at 19.5 dpc (0.04 ± 0.01 μM). In contrast, E2 levels increased from 15.5 dpc (0.16 ± 0.01 nM) to 17.5 dpc (0.22 ± 0.02 nM), and remained high at 19.5 dpc (Figure 1B).

4.2. Maternal P4 levels during midpregnancy inhibited Jagged2, Notch1, and Hey2 expression, and primordial folliculogenesis

To investigate the possible relationship between P4/E2 and Notch signaling during primordial follicle formation, 15.5 dpc ovaries, in which there was no nest dissociation (5), were cultured *in vitro* with different treatments for 4 days and the gene and protein expression level of the Notch pathway were analyzed via Real-time PCR and Western blot, respectively. As shown in Figure 2A, 1 μM P4 (6) significantly decreased Jagged2, Notch1, and Hey2 mRNA levels as compared with vehicle, whereas the levels of Jagged1, Notch2, Notch3, and Hes1 mRNA showed no obvious changes. P4 also markedly reduced the protein levels of Jagged2, Notch1, NICD1 (active Notch1), and Hey2 (Figure 2B). However, E2 had no effect on the expression of any tested gene or protein (Figure 2A and B). After a 7-day culture, both P4 and E2 significantly inhibited primordial follicle formation (P4, 1449 ± 113; E2, 1380 ± 110) as compared with the vehicle (2875 ± 184, Figure 2C).

Interestingly, maternal P4 levels during midpregnancy (15.5 dpc, 0.2 μM) significantly down-regulated the mRNA and protein levels of Jagged2, Notch1, and Hey2 (Figure 3A and B), and inhibited nest breakdown and primordial follicle formation (Figure 3 C and E) *in vitro*. In contrast, pre-labor P4 levels (19.5 dpc, 0.04 μM) had no remarkable effect on mRNA and protein expression (Figure 3A and B), nest dissociation, and primordial

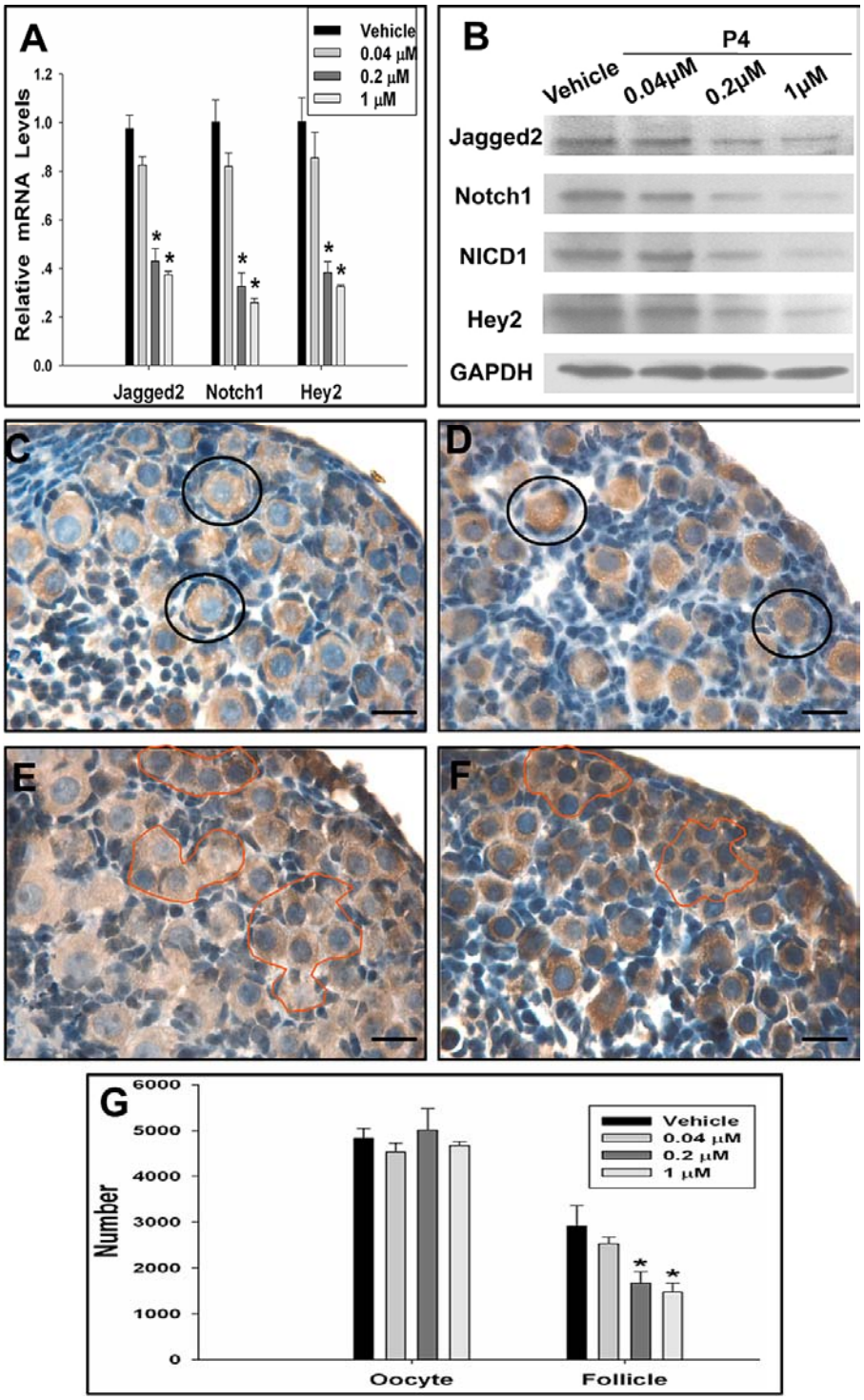

Figure 3. Maternal midpregnancy P4 levels significantly suppressed Jagged2, Notch1, and Hey2 expression, and primordial follicle formation in vitro. Ovaries at 15.5 dpc were exposed to vehicle (C) or P4 at doses of 0.04 μM (pre-labor maternal P4 levels, D), 0.2 μM (maternal midpregnancy P4 levels, E), or 1 μM P4 (F) for 4 and 7 days. After a 4-day culture, Real-time PCR (A) and Western blot (B) were used to measure changes in the mRNA and protein abundance of Jagged2, Notch1, and Hey2, respectively. After a 7-day culture, paraffin sections of the ovaries were prepared and stained with MVH and hematoxylin for shape (C-F) and counting (G) analysis of oocytes and primordial follicles. Red circles indicate germline nests in which the Mvh-stained cytoplasm of oocytes is still connected and black rings indicate primordial follicles. Bar 20 μm * denotes statistical significance at $P<0.01$.

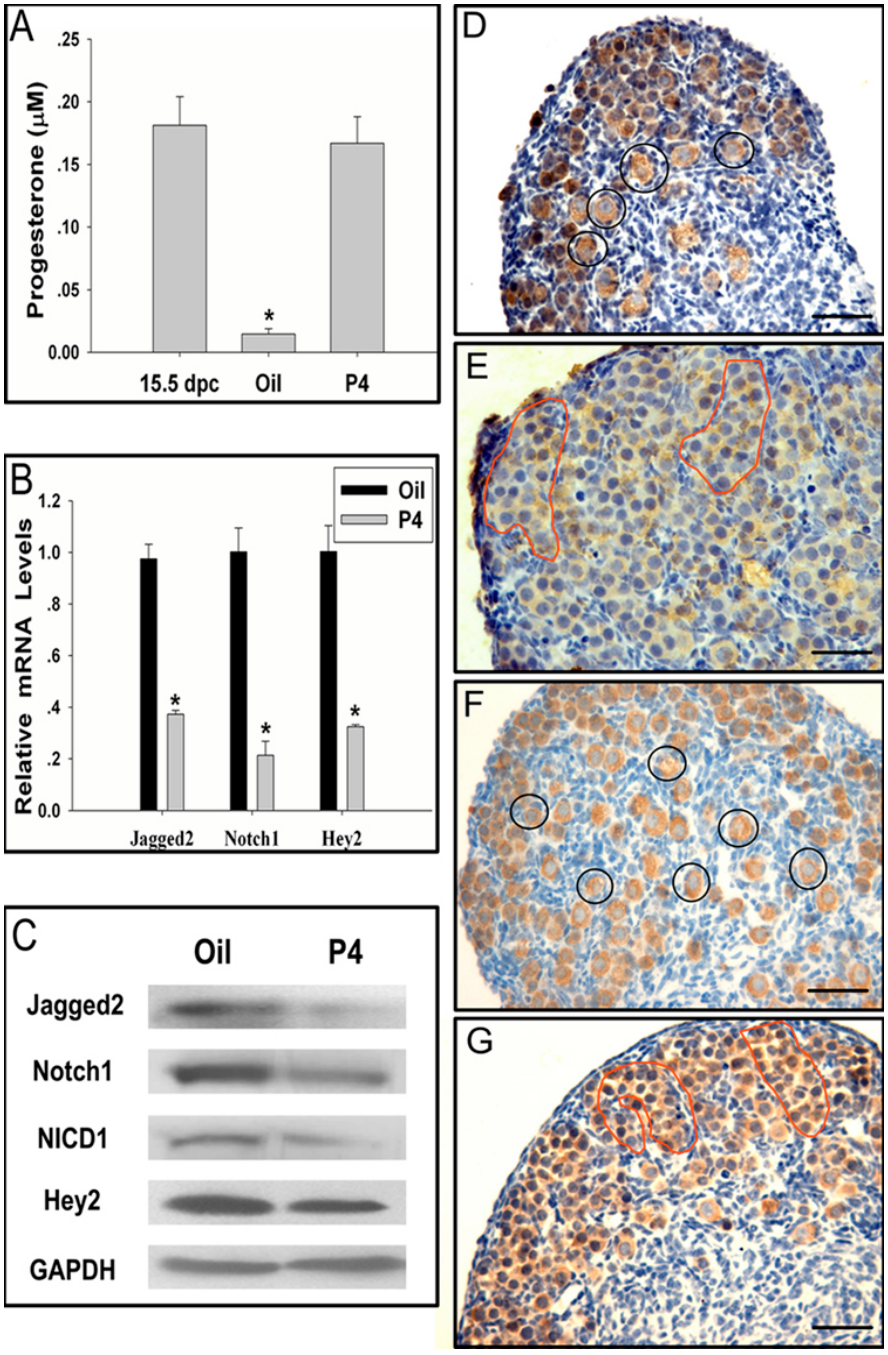

Figure 4. Maintenance of high levels of maternal P4 by injection significantly inhibited Jagged2, Notch1, and Hey2 expression, and primordial follicle formation. Pregnant mice at 15.5 dpc were injected with sesame oil (oil) or P4 (3 mg /40 g body weight) daily for 4 days, and then the serum of treated and pregnant mice on 15.5 dpc were collected to measure P4 levels by RIA (A). Fetal ovaries at 19.5 dpc from the treated pregnant mice were isolated, and Real-time PCR (B) and Western blot (C) were used to detect changes in the mRNA and protein abundance of Jagged2, Notch1, and Hey2, respectively. After 5 days of treatment with oil (D), P4 alone (E), ICI 182780 alone (F), or P4 plus ICI 182780 (G), fetal ovaries at 1 dpp (oil treatment) and 20.5 dpc (P4, ICI182780 and P4 plus ICI182780 treatment) were collected, and paraffin sections of the ovaries were prepared and stained with MVH and hematoxylin for shape and counting analysis of oocytes and primordial follicles. Red circles indicate germline nests and black rings indicate primordial follicles. Bar 50 μm * denotes statistical significance at $P<0.001$.

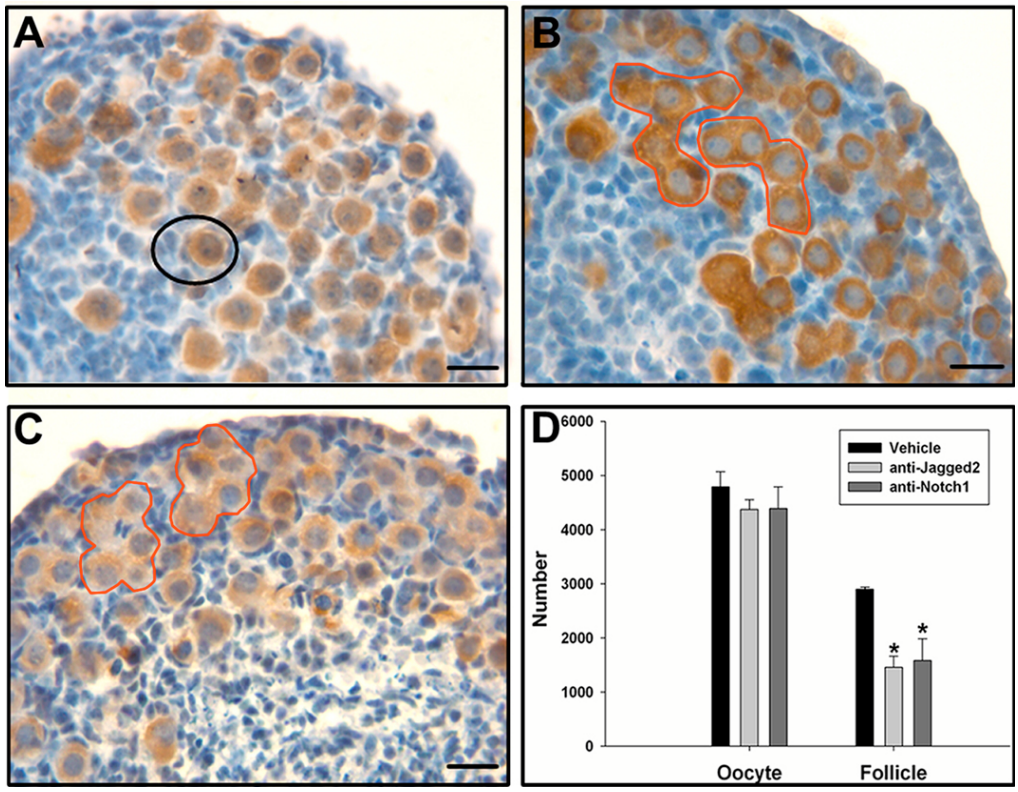

Figure 5. Anti-Jagged2 and anti-Notch1 antibody addition suppressed nest breakdown and primordial follicle formation *in vitro*. Ovaries at 15.5 dpc were treated with 0.5 μg/mL rabbit IgG as a homotype control (A), 0.5 μg/mL anti-Jagged2 antibody (B), or 0.5 μg/mL anti-Notch1 antibody (C). After a 7-day culture, the paraffin sections of the ovaries were prepared and stained with MVH and hematoxylin for shape (A-C) and counting analysis of oocytes and primordial follicles (D). Red circles indicate germline nests and black rings indicate primordial follicles. Bar 20 μm * denotes statistical significance at $P<0.001$.

folliculogenesis (Figure 3C and D) (primordial follicle numbers: vehicle, 2914 ± 445; 0.04 μM P4, 2381 ± 314; 0.2 μM P4, 1673 ± 250; 1 μM P4, 1475 ± 198). There was no difference in the total oocyte population among all groups (Figure 3G).

To further elucidate whether P4 decreased Jagged2, Notch1, and Hey2 expression, and primordial follicle formation, pregnant mice at 15.5 dpc were given a daily injection of P4 to maintain the maternal P4 levels of midpregnancy. As shown in Figure 4A, the 4-day injection resulted in a higher level of P4 (0.17 ± 0.02 μM) in 19.5 dpc pregnant mice, similar to that observed during midpregnancy. Meanwhile, P4 injection significantly down-regulated the expression of Jagged2, Notch1, and Hey2 at the mRNA and protein levels in fetal ovaries as compared with the vehicle (Figure 4B-C). Fetal ovaries at 1 dpp (oil treatment) and 20.5 dpc (P4, ICI182780 and P4 plus ICI182780 treatment) after 5 days of injections were isolated to analyze primordial follicle formation. The pure estrogen antagonist ICI 182780 (29) was used to exclude the possible influence of E2 on follicle formation. In oil or ICI182780 treatment groups, all large nests broke into small nests, and some oocytes were surrounded by somatic cells (Figure 4D, F); however, in P4 alone and P4 plus ICI 182780 treatment groups, nearly all the oocytes were still in the nests, and no follicles were observed (Figure 4E, G).

4.3. Suppression of Jagged2 and Notch1 by antibody exposure, and RNAi inhibited nest breakdown and primordial folliculogenesis *in vitro*

Ovaries at 15.5 dpc were cultured with rabbit immunoglobulin (Ig)G (as a negative homotype contrast), anti-Jagged2, or anti-Notch1 antibody to explore the effects of Jagged2 and Notch1 on primordial follicle formation. After a 7-day culture, nests were barely observable and oocytes were predominantly encapsulated by pre-granulosa cells in ovaries exposed to IgG (Figure 5A). However, in the ovaries that were treated with antibodies, most oocytes remained in the nests (Figure 5B and C). As quantified in Figure 5D, the follicle populations in the anti-Jagged2 (1465 ± 207) and anti-Notch1 antibody (1584 ± 397) groups were only half that of the IgG group (2894 ± 136). The total oocyte number was not significantly different among these treatments.

Further, we employed a siRNA-mediated gene knock-down approach. After 5 days of transfection of 17.5 dpc ovaries with scrambled, Jagged2, or Notch1 siRNA, Jagged2 and Notch1 mRNA were respectively reduced to 26% and 34% of the levels in scramble RNAi ovaries (Figure 6A and B). Jagged2 and Notch1 proteins were noticeably reduced (Figure 6C). In addition, NICD1 protein

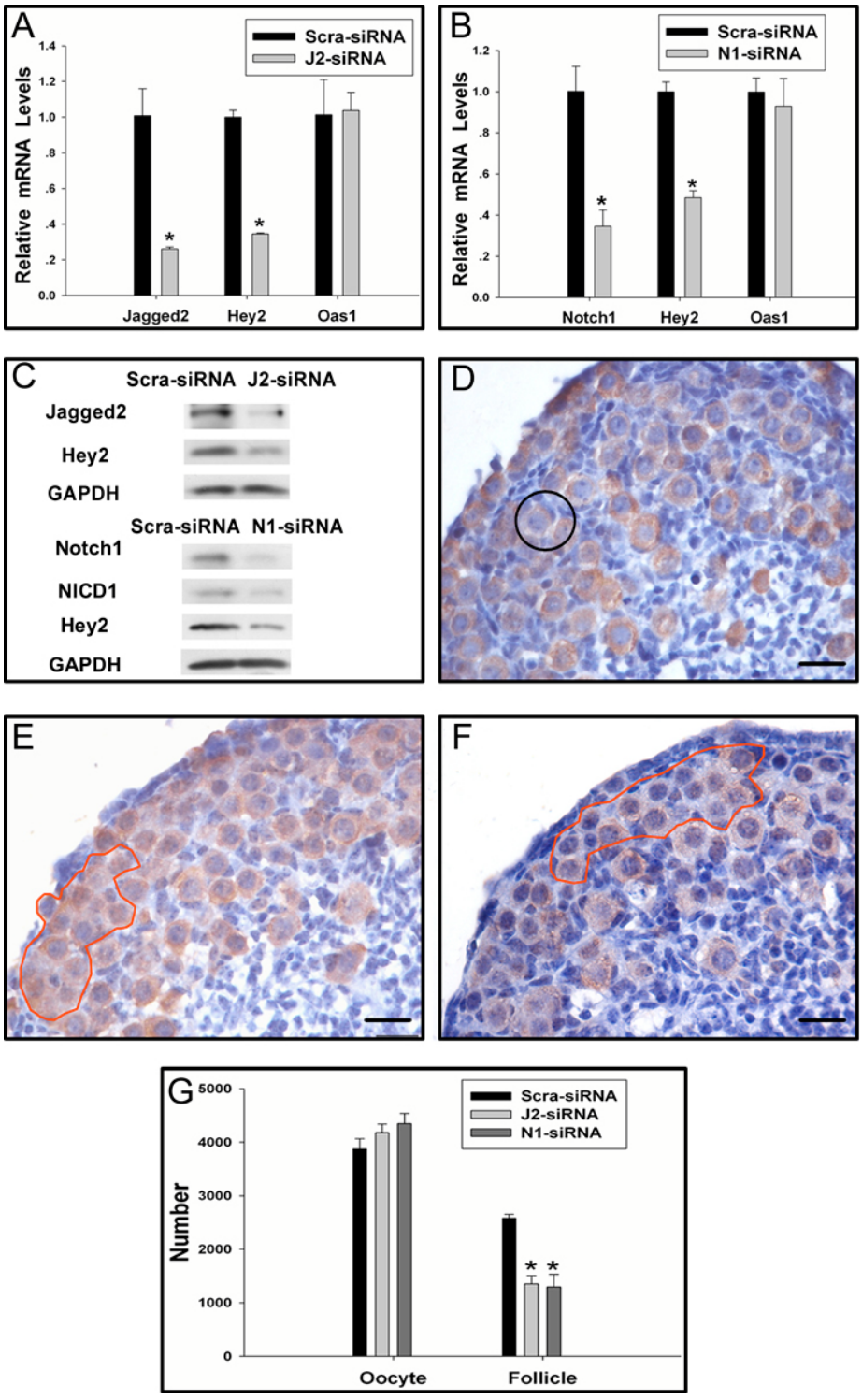

Figure 6. Downregulation of Jagged2 and Notch1 by RNAi inhibited primordial follicle formation *in vitro*. Jagged2 and Oas1, and Notch1 and Oas1 mRNA levels were determined by Real-time PCR in 17.5 dpc ovaries transfected with scrambled siRNA (scra-siRNA), siRNA against Jagged2 (siRNA-J2), or siRNA against Notch1 (siRNA-N1) for 5 days (A, B). The changes in Jagged2, Notch1, and Hey2 protein levels were determined by Western blot (C). After a 5-day culture, the paraffin sections of the scra-siRNA (D), siRNA-J2 (E), and siRNA-N1 (F) ovaries were prepared and stained with MVH and hematoxylin for shape (D-F) and counting analysis of oocytes and primordial follicles (G). Red circles indicate germline nests and black rings indicate primordial follicles. Bar 20 μm * denotes statistical significance at $P<0.001$.

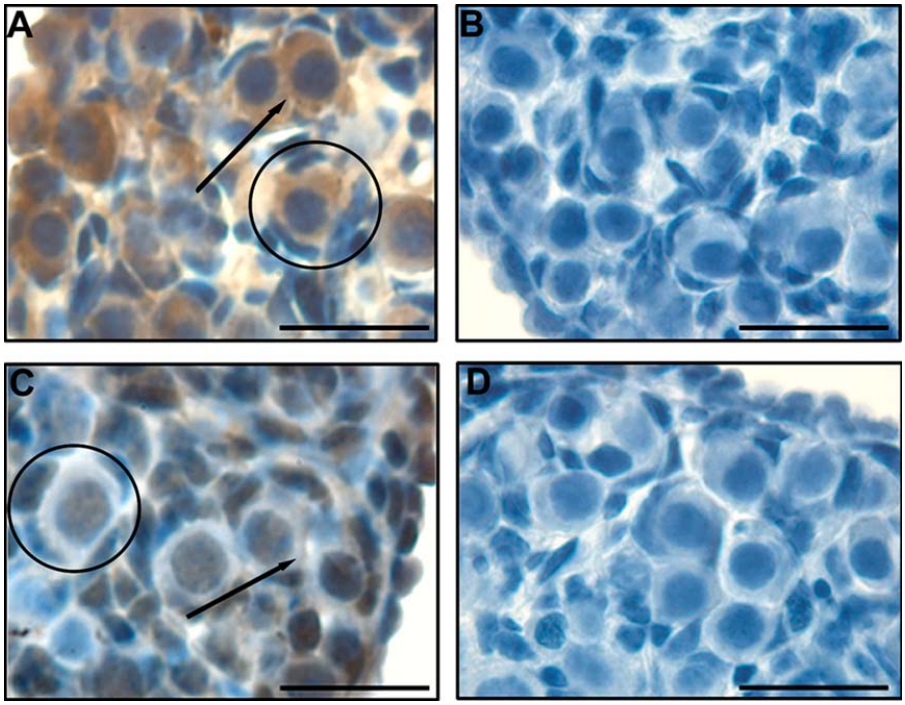

Figure 7. Localization of Jagged2 and Notch1 in the ovary at 19.5 dpc. At 19.5 dpc (the day of labor), Jagged2 was expressed in the cytoplasm of oocytes (A), whereas Notch1 was strongly expressed in the pre-granulosa cells surrounding oocytes and weakly expressed in the oocytes (C). No reaction was observed in control sections after replacing anti-Jagged2 or anti-Notch1 antibody with non-immune rabbit serum (B, D). Arrows indicate germline nests and rings indicate primordial follicles. Bar 25 μm.

levels, and Hey2 mRNA and protein levels also declined significantly after transfection with Jagged2 or Notch1 siRNAs (Figure 6A-C). To detect whether interferon responses were invoked after transfection, a classical interferon target gene, Oas1, was also measured by Real-time PCR. Oas1 mRNA levels in Jagged2 or Notch1 RNAi ovaries were not significantly different from that in scramble RNAi ovaries (Figure 6A and B).

As shown in Figure 6D-F, all scramble, Jagged2, and Notch1 RNAi ovaries were very healthy and had similar oocyte populations. However, the ovaries transfected with Jagged2 siRNA (1348 ± 156) or Notch1 siRNA (1293 ± 237) exhibited significantly less primordial follicles than the scrambled siRNA-treated ovaries (2585 ± 69, Figure 6G).

4.4. Localization of Jagged2 and Notch1 in the mouse ovary

The immunohistochemical results showed that in 19.5 dpc ovaries, many nests were breaking down and some primordial follicles were assembled (Figure 7A-D). Jagged2 was expressed in the oocytes of nests and primordial follicles (Figure 7A). Notch1 was strongly expressed in pre-granulosa cells, but weakly stained in oocytes (Figure 7C). No reaction was observed in control sections (Figure 7B and D).

4.5. RU486 inhibited Jagged2, Notch1, and Hey2 expression, and primordial folliculogenesis *in vitro*

We used the classical antagonist of nPRs, RU486 (30), to determine whether nPRs were responsible for the effect of P4 on Jagged2, Notch1, and Hey expression, and primordial follicle formation. Interestingly, similar to P4, RU486 also significantly reduced Jagged2, Notch1, and Hey2 mRNA and protein levels (Figure 8A-B), and primordial follicle numbers as compared with the vehicle (Figure 8C). The mRNA and protein levels of Jagged2, Notch1, and Hey2, and the populations of primordial follicles further decreased when RU486 was combined with P4 (Figure 8A-C; primordial follicle numbers: vehicle, 2866 ± 475; P4, 1448 ± 195; RU486, 1946 ± 326; P4 + RU486, 1136 ± 144). There was no difference in the oocyte population among groups (Figure 8C).

5. DISCUSSION

Both P4 and Notch signaling pathways can impact germline nest breakdown and primordial folliculogenesis in the mouse ovary (6, 14). In the present study, Jagged2, Notch1, and their target Hey2 transcripts increased in ovaries during the beginning stage of folliculogenesis (17.5 dpc to birth); simultaneously, maternal P4 levels decreased markedly. In vitro, maternal midpregnancy P4 levels can significantly inhibit the expression of Jagged2, Notch1, and Hey2, and primordial follicle formation, whereas the pre-labor levels cannot. Maintaining high maternal P4 levels by daily injection also significantly suppressed the expression of Jagged2, Notch1, and Hey2, as well as primordial follicle formation during late pregnancy. In addition, the administration of anti-

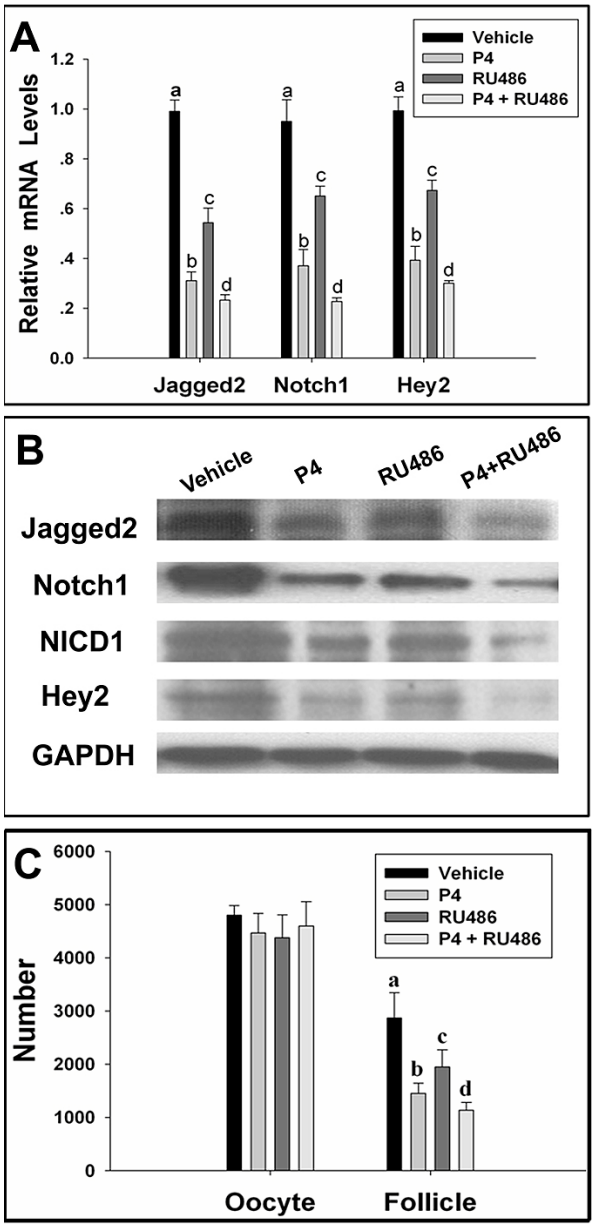

Figure 8. The effects of RU486 on Jagged2, Notch1, and Hey2 expression, and primordial follicle formation *in vitro*. To determine whether P4-mediated actions in the present study were via nPRs, 15.5 dpc ovaries were cultured with vehicle, 1 μM P4 alone, 1 μM RU486 alone, or both P4 and RU486 for 4 days. Changes in the expression of Jagged2, Notch1, and Hey2 were detected by Real-time PCR (A) and Western blot (B). And after the same treatments for 7 days, paraffin sections of the ovaries were stained with MVH for number analysis of oocytes and primordial follicles (C). Letters denote statistical significance at $P<0.05$.

Jagged2 and anti-Notch1 antibodies and Jagged2 and Notch1 siRNAs all inhibited nest breakdown and primordial follicle formation. However, RU486 did not abolish, but had a similar inhibitory effect with P4. Overall, these results indicate that maternal midpregnancy P4 levels inhibit primordial follicle formation and the expression of Jagged2 and Notch1, which participate in nest breakdown and primordial follicle formation.

Relative to 19.5 dpc ovaries, 50% of follicles are formed ahead when 16.5 dpc fetal mouse ovaries were cultured for 3 days, which could be partially rescued by 1 μM P4 (6). The decrease in maternal P4 before labor corresponds to the period when nests begin to break (5). As shown in an *in vitro* culture system, maternal P4 levels of midpregnancy can inhibit primordial follicle formation, but the levels before labor (when multiple nests dissociate) cannot. In addition, we maintained high maternal P4 levels by daily injection of P4 during late

pregnancy. To exclude the possible E2 interruption of follicle formation, the pure estrogen antagonist ICI 182780 was also used. Relative to treatment with ICI 182780 alone, both P4 alone and P4 plus ICI 182780 treatments can inhibit primordial follicle formation. These data collectively suggest that the maternal P4 milieu of the fetus during midpregnancy acts as a physiological inhibitor in nest breakdown and primordial follicle formation in the mouse ovary.

The Notch signaling pathway participates in the formation of primordial follicles (14). We demonstrated that *in vivo*, maternal P4 levels declined significantly from 17.5 dpc to 19.5 dpc, but the Notch pathway Jagged2 and Notch1 mRNA significantly increased in fetal ovaries. By *in vitro* culture or *in vivo* injection, maternal midpregnancy P4 levels significantly down-regulated the expression of Jagged2 and Notch1 in fetal ovaries, suggesting suppression of Jagged2 and Notch1 expression by maternal midpregnancy P4 in the fetal mouse ovary. Recent work also elucidated a negative modulation between progestin and Notch ligand gene expression in the human endometrium (31). Our antibody-inactivation and RNAi culture studies revealed the crucial roles of the Notch pathway components Jagged2 and Notch1 in nest breakdown and primordial folliculogenesis. Taken together, these results imply that maternal midpregnancy P4 levels inhibit nest breakdown and primordial follicle formation in the fetal mouse ovary, likely by suppressing the expression of Jagged2 and Notch1. With maternal P4 levels decreasing at 17.5 dpc, the inhibitory effect of P4 on Jagged2 and Notch1 expression gradually fails, resulting in nest breakdown. Although the expression of Jagged1, Notch2, and Hes1 was not affected by P4 in the present study, they are also expressed largely in perinatal ovaries (14). The role of these components in early ovarian development requires further investigation.

In the present study, the Notch pathway ligand Jagged2 was localized in oocytes and receptor Notch1 was strongly stained in the adjacent pre-granulosa cells of the 19.5 dpc ovary. Hey2 was chiefly expressed in pre-granulosa cells of perinatal ovaries (14). Furthermore, Hey2 expression was reduced significantly by P4 and Jagged2 and Notch1 RNAi treatments, and the progressive increase of Hey2 mRNA coincided with the upregulation of Jagged2 and Notch1 mRNA, revealing that Hey2 may be a direct target of the Jagged2-Notch1 pathway during primordial follicle formation. Together, it is likely that the reduced expression of Jagged2 in oocytes and Notch1 in pre-granulosa cells by P4 may hinder oocyte–somatic cell communication and the transcription of their target gene Hey2. This may disturb the migration or differentiation events of pre-granulosa cells (32) and finally result in the failure of pre-granulosa cell incursion and primordial follicle formation. However, the localization of Jagged2 in fetal ovaries is inconsistent with that in adult ovaries, where Jagged2 is expressed in granulosa cells of developing follicles (33). The reason for the localized change remains to be determined.

In our study, the well known nPRs antagonist RU486 did not abolish, but rather had similar effects to P4 on the expression of Jagged2, Notch1, and Hey2, and the formation of primordial follicles. Kezele *et al.* (8) also found that RU486 administration alone was able to reduce assembled follicle numbers by 10% in a rat model. RU486 can be presented as a partial agonist of nPRs (34). Previous reports demonstrated that in human breast cancer cells and HeLa cells, the agonistic action of RU486 was mediated by PR-B but no activating effect by PR-A (35-36). In addition to nPRs, RU486 also has high affinity for the glucocorticoid receptor (GR) (37). Thus, to exclude the possibility that RU486 plays a role in this model by binding GR, we also treated fetal ovaries with the GR agonist dexamethasone. We found that 0.1-10 μM dexamethasone was not able to alter the expression of Jagged2, Notch1, NICD1, and Hey2, or the proportion of formed follicles (Figure S1). These data suggest that PRB is possibly involved in mediating the functions of P4 and RU486 in the Notch pathway expression and follicle formation. However, future research on the respective roles of PR-A, PR-B, and P4 membrane receptors in primordial folliculogenesis is still needed.

The inhibitory role of E2 in primordial follicle formation was shown by Pepling ME *et al.* (6); however, in the present study E2 did not impact the expression of Notch pathway components in the mouse ovary. The effect of E2 and P4 on primordial follicle formation may be via different pathways, or E2 may influence the downstream events of Notch signaling. Based on recently reported studies, several identified key players in primordial folliculogenesis such as the Activin, Foxl2, and Cadherin families (38-40), may mediate the actions of E2 in primordial follicle formation (41-43). After a decrease in maternal P4 levels, not all nests begin to dissociate around the time of birth. It is possible that the remaining high E2 levels may partially compensate for the inhibitory effect of P4 on nest breakdown and follicle formation in the mouse ovary. This hypothesis requires further research.

In conclusion, we demonstrated the negative link between P4 and the Notch signaling pathway, which are two factors that are important in primordial folliculogenesis in the fetal mouse ovary. Maternal midpregnancy P4 levels inhibit primordial follicle formation, likely by suppressing the expression of Jagged2 and Notch1, which are involved in primordial follicle formation. Our data help elucidate the molecular endocrine regulation of primordial follicle formation in the mouse ovary.

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Abbreviations: DPC: Days Post Coitus, DPP: Days Post Parturition, P4: Progesterone, E2: 17 Beta-Estradiol, Ria: Radioimmunoassay, MVH: Mouse Vasa Homolog, NICD1: Notch1 Intracellular Domain, IG G: Immunoglobulin G, RNAI: Rna Interference, NPR: Nuclear Progesterone Receptor, RU486: Mifeprostone, GR: Glucocorticoid Receptor

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