

Suppression of Notch Signaling in the Neonatal Mouse Ovary Decreases Primordial Follicle Formation

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Notch signaling directs cell fate during embryogenesis by influencing cell proliferation, differentiation, and apoptosis. Notch genes are expressed in the adult mouse ovary, and roles for Notch in regulating folliculogenesis are beginning to emerge from mouse genetic models. We investigated how Notch signaling might influence the formation of primordial follicles. Follicle assembly takes place when germ cell syncytia within the ovary break down and germ cells are encapsulated by pregranulosa cells. In the mouse, this occurs during the first 4–5 d of postnatal life. The expression of Notch family genes in the neonatal mouse ovary was determined through RT-PCR measurements. Jagged1, Notch2, and Hes1 transcripts were the most abundantly expressed ligand, receptor, and target gene, respectively. Jagged1 and Hey2 mRNAs were up-regulated over the period of follicle formation. Localization studies demonstrated that JAGGED1 is expressed in germ cells prior to follicle assembly and in the oocytes of primordial follicles. Pregranulosa cells that surround germ cell nests express HES1. In addition, pregranulosa cells of primordial follicles expressed NOTCH2 and Hey2 mRNA. We used an *ex vivo* ovary culture system to assess the requirement for Notch signaling during early follicle development. Newborn ovaries cultured in the presence of γ -secretase inhibitors, compounds that attenuate Notch signaling, had a marked reduction in primordial follicles compared with vehicle-treated ovaries, and there was a corresponding increase in germ cells that remained within nests. These data support a functional role for Notch signaling in regulating primordial follicle formation. (*Endocrinology* 150: 1014–1024, 2009)

Ovarian follicles are the functional units within the female gonad that nurture maturation of the oocyte and enable production of steroid hormones. Follicles are comprised of three cell types: oocytes, surrounding granulosa cells, and an external thecal cell layer. Select numbers of follicles mature in response to circulating gonadotropins and to the local actions of growth factors during the female reproductive cycle (1). Follicle maturation continues until ovulation, when an egg or eggs competent for fertilization are extruded from the ovary and the remaining somatic cells of the follicle luteinize. Although much is known about how secondary follicles progressively develop into pre-ovulatory follicles, the molecular events mediating primordial follicle formation and initial follicle growth are less clear.

In mice, primordial germ cells migrate to the urogenital ridge around embryonic d 11 (2). By embryonic d 13.5, synchronous

rounds of mitotic division in the female gonad yield clusters of oocytes arranged in syncytia commonly referred to as cysts or nests. (3). Syncytia persist until germ cells undergo a wave of apoptosis near the time of birth (4). During programmed nest breakdown, germ cells are encapsulated by squamous somatic cells (pregranulosa cells) to generate primordial follicles. The newborn mouse ovary contains few primordial follicles, whereas at postnatal d 2 approximately 40% of germ cells are within primordial follicles (4). This number increases to greater than 80% by postnatal d 6 (4, 5). Perturbations during the critical period of primordial follicle formation can significantly affect the size of the primordial follicle pool and follicular phenotypes. For example, administration of activin to neonatal mice increases the primordial follicle pool by 30% (5), whereas the ovaries of neonatal mice injected with estradiol, the synthetic

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Abbreviations: Ct, Cycle threshold; DAPT, *N*-(*N*-(3,5-difluorophenyl)-L-alanyl)-S-phenylglycine t-butyl ester; DMSO, dimethylsulfoxide; DSL, *Drosophila* homolog-8 and -Serate, and the *C. elegans* homolog Lag-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hes, hairy and enhancer of split; L-685,458, (5S)-(t-butoxycarbonylamino)-6-phenyl-(4R)hydroxy-(2R)benzylhexanoyl-L-leu-L-phe-amide; MOF, multiocytic follicle; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

estrogen diethylstilbestrol, or the phytoestrogen genistein develop multioocytic follicles (MOFs) (6–10). MOFs, which have two or more germ cells trapped within a follicle boundary (6, 7), are also observed in mouse ovary cultures treated with estradiol (11). These structures likely arise from incomplete breakdown of germ cell nests.

Contacts between germ cells and somatic cells are established as early as embryonic d 13.5 in the mouse ovary (4). Thus, communication between germ cells and pregranulosa cells is likely important for orchestrating follicle assembly. Given the many roles of the Notch signaling pathway in cell communication and morphogenesis, this pathway is a likely candidate for regulating early follicle development. Notch signaling affects cell fate during embryogenesis and in turn influences cell proliferation, differentiation, and apoptosis (12).

Originally characterized in *Drosophila* and *Caenorhabditis elegans*, components of the Notch pathway have several mammalian orthologues. These include four Notch receptors (Notch1–4), and five Notch ligands: Jagged1, Jagged2, Delta-like 1, Delta-like 3, and Delta-like 4 (13). Notch ligands belong to the *Drosophila* homolog Delta and Serrate, and the *C. elegans* homolog Lag-2 (DSL) family of proteins (14). Notch genes encode conserved transmembrane receptors, and the DSL ligands are also membrane bound. Signaling occurs between apposing cells that express Notch receptors and DSL ligands. After ligand binding, a cascade of proteolytic cleavages of the Notch receptor ensues (15). The active form of Notch, the Notch intracellular domain, is generated by cleavage at the receptor juxtamembrane region by the γ -secretase complex (16). Liberated Notch intracellular domain translocates into the nucleus in which it associates with the transcriptional regulator C-promoter binding factor 1/suppressor of hairless/Lag-1 (CSL) to promote Notch target gene transcription (13). Well-characterized Notch target genes include two families of basic-helix-loop-helix transcription factors: hairy and enhancer of split (Hes) and a related family (Hes related with YRPW motif, hairy related transcription factor) (17–20). Depending on the cellular context, Notch signaling is reduced or potentiated by Fringe proteins, a class of glycosyltransferases that modify the receptor (21). In mammals, the three Fringe proteins that modulate Notch signaling are Lunatic, Manic, and Radical Fringe (22). Interestingly, the Lunatic Fringe knockout mouse ovary exhibits meiotic defects and develops MOFs (23). An analogous phenotype occurs in the *Drosophila* gonad, in which the absence of Notch signaling during egg chamber formation results in fused egg chambers (24). Taken together, these data suggest that Notch signaling may have conserved roles in follicle development within the female gonad.

We tested the hypothesis that Notch signaling is required for follicle assembly in the mouse ovary. Our studies centered on the interval between birth and postnatal d 4, the time period when most primordial follicles form in the mouse. We found that Notch receptors and ligands were expressed in a complementary pattern in the neonatal mouse ovary, with Notch2 in granulosa cells and Jagged1 in germ cells. Germ cell nests were retained and primordial follicles were reduced in ovaries cultured with γ -secretase inhibitors that suppress Notch signaling. These data

support a functional role for Notch signaling in regulating primordial follicle formation.

Materials and Methods

Animal treatment and tissue collection

CD-1 mice (Harlan, Indianapolis, IN) were maintained on a 12-h light, 12-h dark cycle (lights off at 1700 h) with food and water available *ad libitum*. Breeders (90–180 d old) were fed with a soy-free mouse chow (Harlan 7926) to limit exogenous phytoestrogen intake through food. Matings were timed, and vaginal plug detection was considered d 0.5 of pregnancy. Ovaries were collected from newborn mice (within 2 h of birth) or mice at different postnatal times. Day 0 marks the first 24 h after birth. Ovaries were either stored at -80°C for subsequent RNA isolation, immediately fixed for follicle counting and immunohistochemical studies, or prepared for *ex vivo* culture. Animals were cared for in accordance with all federal and institutional guidelines.

Antibodies and inhibitors

Jagged1 (SC-6011) and Notch2 (SC-5545) polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and used at a 1:50 and 1:100 dilution, respectively. A monoclonal Notch2 antibody (C651.6DbHN) was provided by the Iowa Developmental Studies Hybridoma Bank (Iowa City, IA) and used at 8 $\mu\text{g}/\text{ml}$. A Hes1 polyclonal antibody (25) was kindly provided by Dr. Tetsuo Sudo (Toray Industries Inc., Tokyo, Japan) and used at a 1:500 dilution. γ -Secretase inhibitors (5S)-(t-butoxycarbonylamino)-6-phenyl-(4R)-hydroxy-(2R)-benzylhexanoyl-L-leu-L-phe-amide (L-685,458; L1790) and N-(N-(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycine t-butyl ester (DAPT; D5942) were purchased from Sigma-Aldrich (St. Louis, MO).

Semiquantitative RT-PCR and real-time PCR

For semiquantitative PCR studies, dissected ovaries from postnatal d 3 CD-1 mice were stripped of the bursal sac and immediately frozen. An RNeasy kit (QIAGEN, Valencia, CA) was used to isolate RNA from pooled ovaries ($n = 12$), and 500 ng RNA was reverse transcribed using avian myeloma virus reverse transcriptase. One quarter of the resulting cDNA was used in PCRs. Primers for ribosomal protein RPL19 were used as a control, and PCRs were run for 26 cycles. ^{32}P -dCTP was incorporated during PCR, and products were visualized after exposing dried gels to Hyperfilm (GE Healthcare, Piscataway, NJ). (See supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>, for primer sequences and amplicon sizes). For real-time PCR experiments, ovaries were dissected from d 0, 2, 6, and 18 CD-1 mice. One microgram of RNA from each set of pooled ovaries ($n = 12$ –14 for d 0–6; $n = 2$ for day 18) was used for each reverse transcription reaction, and 100 ng of cDNA served as the template for PCR. Real-time PCR assays were performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), and mouse RPL19 served as the internal control (26). The comparative cycle threshold (Ct) method ($\Delta\Delta\text{Ct}$) was used for relative quantification (27). To examine mRNA expression in cultured ovaries, two ovaries were pooled, and eluates from RNeasy columns were precipitated. RNA samples were resuspended in a small volume of diethylpyrocabonate water, and real-time PCR experiments were performed using 50 ng of cDNA. Relative mRNA values were normalized to RPL19.

In situ hybridization

Ovarian sections from CD-1 mice were processed for *in situ* hybridization as previously described (28), with modifications. A 630 bp Hey2 fragment was amplified from d 18 mouse cDNA and cloned into the pcDNA3 vector (Invitrogen Corp., Carlsbad, CA). Primers used to generate the Hey2 amplicon were: forward 5'-TGAAGATGCTCCAGGC-

TACAGG-3' and reverse 5'-ATACCGACAAGGGTGGGCTGAT-3'. A Hey2 riboprobe was prepared using digoxigenin-11-UTP (Roche Applied Science, Indianapolis, IN) and the Riboprobe combination system (Promega Corp., Madison, WI). Detection of mRNA was achieved using a digoxigenin-AP antibody (Roche Applied Science, Indianapolis, IN) and the nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt substrate (Sigma-Aldrich).

Immunohistochemistry

Ovaries harvested from CD-1 mice were fixed in 4% paraformaldehyde overnight at 4 C. Before tissue processing, samples were dehydrated with 50 and then 70% ethanol. Ovaries were further dehydrated through a graded series of ethanols and infiltrated with paraffin. Embedded samples were sectioned at 5 μ m. Tissue sections were dewaxed and then rehydrated. Antigen retrieval was performed by microwaving samples on high power for 2 min and on low power for 7 min in 0.01 M sodium citrate (pH 6). After incubating in 3% H₂O₂/PBS for 15 min to block endogenous peroxidase activity, tissue sections were blocked with avidin/biotin reagents (Vector Laboratories, Inc., Burlingame, CA). Blocking was achieved by immersing slides in a solution containing 10% serum for 1 h at room temperature. Samples were incubated with primary antibodies diluted in blocking serum overnight at 4 C and were then exposed to biotin-conjugated secondary antibodies (1:200 dilution; Vector Laboratories) for 30 min at room temperature. The ABC reagent (Vectastain Elite ABC kits; Vector Laboratories) was used according to the manufacturer's instructions. All washes were performed in PBS-Tween 20 after incubations with antibody and ABC reagent. The 3,3'-diaminobenzidine substrate (Vector Laboratories) was used for colorimetric detection, and samples were counterstained with hematoxylin. A proliferating cell nuclear antigen staining kit (Zymed Laboratories, South San Francisco, CA) was used to identify proliferating cells in tissue sections. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining was performed using the DeadEnd fluorometric TUNEL system kit (Promega) to assess cellular apoptosis. All sections processed for fluorescence detection were mounted in medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories). Immunohistochemical images were acquired on a Nikon E600 microscope using a Spot Insight Mosaic 11.2 color digital camera (Diagnostic Instruments, Sterling Heights, MI) and Advanced Spot Imaging software (version 4.6; Universal Imaging, Downingtown, PA). Immunofluorescent images were generated using a Leica DM5000B fluorescence microscope and OpenLab 4.0 software (Improvision, Lexington, MA).

Organ culture and morphometric analysis

Ovaries from newborn CD-1 mice were dissected and the bursal sac removed in PBS. Ovaries were placed in 5- μ l drops of media and cultured for 4 d on 0.4- μ m floating filters (Millicell-CM; Millipore Corp., Billerica, MA) at 37 C in a chamber containing 5% CO₂. Filters were placed in 14-mm culture wells and rested on top of 0.4 ml DMEM-F12 media supplemented with 0.1% Albumax (Invitrogen), penicillin-streptomycin (Invitrogen), 0.1% BSA (Sigma), 27.5 μ g/ml transferrin (Sigma), and 0.05 mg/ml L-ascorbic acid (Sigma). Culture medium contained 1, 10, or 50 μ g/ml insulin (Sigma) and was changed daily. The media formulation was adapted from Kezele and Skinner (29). In studies using γ -secretase inhibitors, ovaries were cultured for 1, 2, or 4 d in media containing either 10 μ M L-685,458 or 20 μ M DAPT. Vehicle-treated ovaries were cultured in media containing 0.2% dimethylsulfoxide (DMSO). Cultured ovaries were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin. To compare follicle formation between *in vivo* and cultured ovaries, ovaries were isolated from littermate animals the same day cultured ovaries were fixed. Follicle populations among isolated and cultured ovaries were quantified. Ovaries were sectioned at 5 μ m, and follicles were counted in every fifth section as described elsewhere (5) to avoid duplicate counts. Images of ovarian sections were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD), and follicles were manually counted by two individuals. Germ cells not surrounded by pregranulosa cells were scored as unassembled (remaining in nests).

Oocytes surrounded by pregranulosa cells or a mixture of squamous and cuboidal somatic cells were scored as primordial follicles. Primary and secondary follicles were scored when oocytes were surrounded by a single or double layer of cuboidal granulosa cells, respectively. Primary and secondary follicle counts were added together (developing follicles). Follicle populations were expressed as a percentage of the total number of germ cells counted.

Statistics

Data are presented as means \pm SEM. One-way ANOVA followed by a Tukey-Kramer *post hoc* analysis was used for statistical comparisons among multiple groups. ANOVA was performed using GraphPad PRISM 4.0 (GraphPad Software, Inc., San Diego, CA). For statistical comparisons between two groups, the Student's two-tailed *t* test was used. *P* < 0.05 was considered significant.

Results

Notch family genes are expressed in the neonatal mouse ovary

Johnson *et al.* (30) described the expression patterns for several Notch pathway genes in the adult mouse ovary. We investigated Notch gene expression in the neonatal mouse ovary during the period when primordial follicles form. The d 0 mouse ovary chiefly contains germ cells that are arranged in nests (supplemental Fig. 1A). The ovarian architecture changes dramatically 3 d into postnatal development as germ cell nests breakdown. Ovaries at this stage contain a heterogeneous population of germ cell nests, primordial and primary follicles (supplemental Fig. 1B). Because Notch signaling may affect processes mediating germ cell nest breakdown and/or initial follicle growth, we used neonatal d 3 ovaries for semiquantitative RT-PCR assays to determine the expression profiles for Notch family genes. Transcripts for all Notch receptors were expressed in the d 3 mouse ovary, with Notch2 showing the highest expression (Fig. 1A). Jagged1 and Jagged2 mRNAs were more abundant than the Delta-like ligands in the neonatal ovary. Hes1 and Hey2 transcripts showed the highest abundance of the eight Notch target genes tested. We also examined the expression of the three Fringe molecules, and Radical Fringe showed the strongest expression (data not shown). We performed real-time PCR experiments to determine whether Notch family genes, those strongly expressed by d 3, were regulated during postnatal development (Fig. 1B). There was no change in Notch2, Notch3, Jagged2, and Hes1 mRNA expression between d 0 and 6, but a significant decrease was observed in d 18 ovaries compared with earlier ovaries (Fig. 1B). In contrast, Jagged1 mRNA increased 2.5-fold during the peak period of follicle formation, between d 0 and 6, and then declined such that by d 18 (prepubertal), the level of Jagged1 transcript was similar to that found in d 0 ovaries (Fig. 1B). Hey2 mRNA increased 5-fold between d 0 and 6 and then increased further, 10-fold, in d 18 ovaries compared with d 0 ovaries (Fig. 1B). These data show that multiple Notch genes are expressed and dynamically regulated during the time of follicle formation.

Immunohistochemical experiments were used to determine the cellular localization of NOTCH2, JAGGED1, and HES1 proteins (Fig. 2). NOTCH2 was expressed in pregranulosa cells of the newborn mouse ovary (Fig. 2A). Light NOTCH2 staining

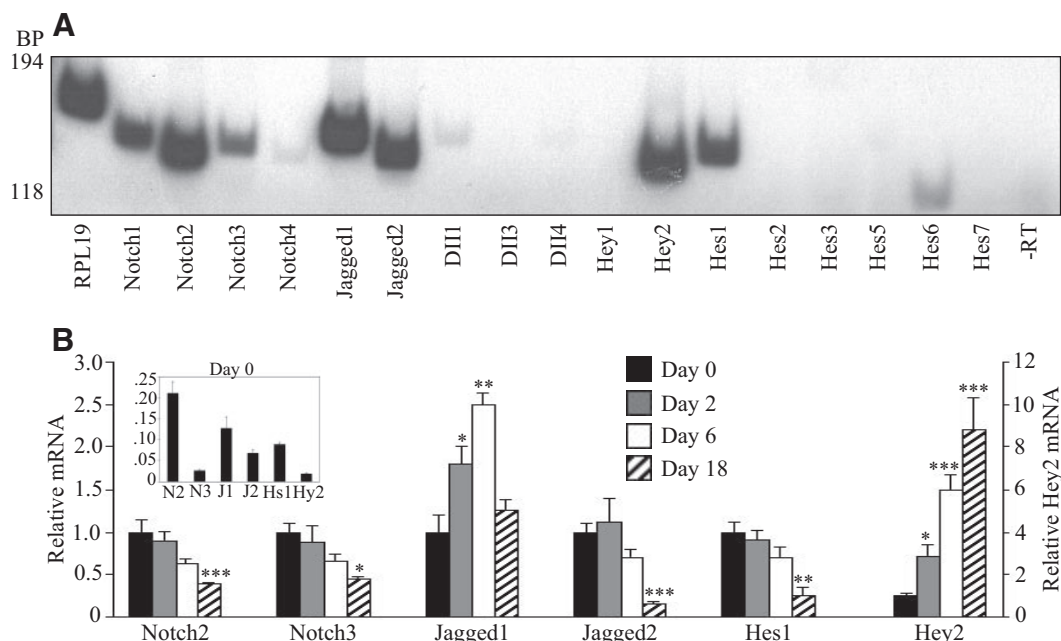


FIG. 1. Notch family gene expression in the neonatal mouse ovary. **A**, RT-PCR was performed to detect Notch receptor, ligand, and target gene transcripts in ovaries from d 3 CD-1 mice. Primers for ribosomal protein RPL19 were used as a control. A complete list of primer sequences can be found in supplemental Table 1. Notch2 primers were used for the negative control reverse transcription reaction. **B**, Real-time PCR was used to examine the regulation of selected Notch family transcripts during postnatal ovarian development. The second y-axis represents relative mRNA values for Hey2. The comparative Ct method ($\Delta\Delta Ct$) was used for relative quantification. Genes were normalized to RPL19, and the graph shows changes for each gene relative to d 0. The inset shows mRNA values for Notch2 (N2), Notch3 (N3), Jagged1 (J1), Jagged2 (J2), Hes1 (Hs1), and Hey2 (Hy2) at d 0, normalized to RPL19. The graph represents the average of samples from three to five experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

was also observed in germ cells of the newborn mouse ovary. NOTCH2 was localized predominantly in the pregranulosa cells of primordial follicles and granulosa cells of primary follicles in the d 3 ovary (Fig. 2B and supplemental Fig. 2A). In contrast, JAGGED1 was expressed in germ cells of germ cell nests in the newborn ovary (Fig. 2C). At d 3, JAGGED1 continued to be expressed in oocytes of primordial and primary follicles (Fig. 2D and supplemental Fig. 2B). HES1 was expressed in pregranulosa cells that surround germ cell nests (Fig. 2E) in the newborn ovary. HES1 expression was maintained in pregranulosa cells surrounding primordial follicles in the d 3 ovary, whereas oocytes of early primary follicles also stained positively for HES1 (Fig. 2F). Due to the lack of a reliable Hey2 antibody, we examined Hey2 mRNA localization via *in situ* hybridization. Hey2 transcripts were expressed in the pregranulosa cells and oocytes of follicles in the d 3 ovary (Fig. 3A and supplemental Fig. 2C). RT-PCR experiments verified that Hey2 transcripts are expressed in purified preparations of primary granulosa cells (data not shown). Incubation of d 3 ovary sections with a sense Hey2 probe revealed negligible staining (Fig. 3B). Thus, at a time when follicle formation is occurring, JAGGED1 and NOTCH2 are expressed in germ cells and pregranulosa cells respectively, consistent with reports in *Drosophila* (24) in which Notch ligand-receptor pairs mediate germ cell-somatic cell interactions. That NOTCH2 and HES1 are expressed in early granulosa cells suggests that the Notch pathway is active at the time of follicle assembly.

Ex vivo ovary culture

Collectively, our gene expression and immunohistochemistry data support the hypothesis that Notch signaling occurs during early folliculogenesis, but how Notch functions during this period of ovary development is unknown. Although Notch3 and Notch4 knockout animals are viable and fertile (31, 32), targeted disruption of other Notch receptor or ligand genes results in either embryonic or perinatal lethality (33). An alternative method for disrupting the function of proteins is through the use of small molecule inhibitors, a strategy that has been successfully performed in *ex vivo* organ culture experiments (34). Thus, we used an *ex vivo* ovary culture system to address functional roles for Notch signaling during follicle assembly. In this system, newborn mouse ovaries are maintained in culture for 4 d, a time span that allows for follicle formation. To validate the organ culture system, we compared follicle formation and initial growth between cultured and *in vivo* ovaries. Cultured ovaries and ovaries isolated from littermate animals were fixed and stained for histological analysis at the end of the 4-d culture period. Isolated and cultured ovaries contained primary follicles in the medulla of the ovary and primordial follicles in the ovarian cortex (supplemental Fig. 3, A and B). Although primordial follicles formed in this *ex vivo* culture system, isolated ovaries contained 10% more primordial follicles than cultured ovaries (supplemental Fig. 3C). In addition, isolated ovaries had significantly more developing follicles (primary and secondary) compared with cultured ovaries (supplemental Fig. 3C). The ovary culture duration and media formulation may account for the lower percentage of

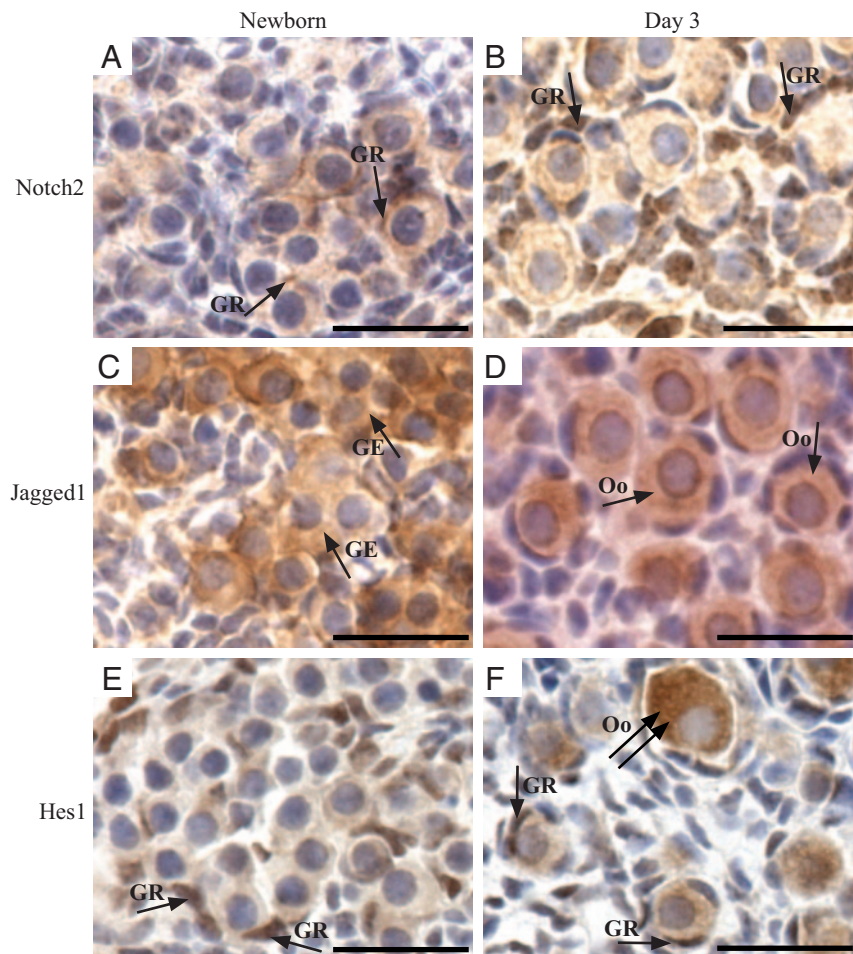


FIG. 2. NOTCH2, JAGGED1, and HES1 localization in the neonatal mouse ovary. In the newborn ovary, NOTCH2 and HES1 were expressed in pregranulosa cells of germ cell nests (A and E, arrows), whereas the Notch ligand JAGGED1 was expressed in germ cells of germ cell nests (C, arrows). Three days into postnatal development, the pregranulosa cells of primordial follicles stained positively for NOTCH2 and HES1 (B and F, arrows), and oocytes surrounded by a mixture of squamous and cuboidal somatic cells (F, double arrow) also expressed HES1. By d 3, JAGGED1 expression was maintained in oocytes of primordial follicles (D, arrows). Experiments were repeated three times and representative images are shown. Scale bars, 25 μ m. GR, Pregranulosa cells; GE, germ cells; Oo, oocytes.

developing follicles in cultured ovaries. Immunolocalization experiments revealed that JAGGED1 and HES1 proteins were expressed in the same cell types as observed in isolated ovaries (supplemental Fig. 3, D and E). Isolated and cultured ovaries displayed a similar distribution and number of apoptotic (Fig. 4, A and B) and proliferating cells (Fig. 4, C and D). Thus, the culture conditions do not appear to cause cell death, and somatic cells continue to proliferate normally. These findings suggest that follicle dynamics and Notch family protein expression in *ex vivo* cultured ovaries are similar to those of *in vivo* ovaries.

Attenuating Notch signaling decreases primordial follicle formation

Because primordial follicles assemble in this culture system, we used this *ex vivo* approach in subsequent studies to investigate follicle formation. We tested the ability of two chemically distinct γ -secretase inhibitors, DAPT and L-685,458 (35, 36), to block Notch signaling in ovary cultures. We first validated the efficacy of these compounds in cell lines known to express Notch

signaling components and demonstrated that both inhibitors strongly suppress Notch signaling without affecting cell viability (supplemental Fig. 4). *Hes1* and *Hey2* are Notch target genes and thus provide a measure of Notch signaling activity (37). Therefore, we examined the mRNA expression of these genes in cultured ovaries treated with vehicle or DAPT. Day 0 ovaries cultured for 1 d with 20 μ M DAPT displayed a 35% decrease in *Hes1* mRNA levels and a 90% decrease in *Hey2* mRNA compared with controls (Fig. 5A). *Hey2* mRNA down-regulation persisted when ovaries were cultured for 4 d with DAPT (Fig. 5B). Similarly, *Notch2* mRNA decreased 30% upon DAPT treatment for 1 d, and this level of receptor down-regulation was maintained when ovaries were treated for 4 d with the inhibitor (Fig. 5, A and B). In contrast, *Jagged1* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were not changed by DAPT (Fig. 5). These studies reveal that Notch signaling can be suppressed by culturing ovaries with a γ -secretase inhibitor.

To examine the actions of Notch during early follicle development, ovary cultures were treated with DAPT or L-685,458 for 4 d, and the ovarian histology was analyzed. Ovaries treated with DMSO for 4 d were chiefly composed of primordial follicles. Some small germ cells nests persisted in these ovaries and were found near the ovarian cortex (Fig. 6A). Conversely, DAPT-treated ovaries showed expanded tracts of germ cells not assembled into follicles (Fig. 6B). Follicle counting data revealed that

DAPT-treated ovaries had a significantly reduced percentage of primordial follicles, 35 vs. 58% for controls, and a correspondingly significant increase in the percentage of germ cells remaining in nests, 64 vs. 42% for controls (Fig. 6E). There was no significant difference in the percentage of more advanced, developing follicles between vehicle and inhibitor-treated ovaries (Fig. 6E), although the number of these follicles is low. Similar to DAPT, L-685,458-treated ovaries displayed reduced germ cell nest breakdown compared with controls (Fig. 7, A and B). L-685,458-treated ovaries had a lower percentage of primordial follicles, 48 vs. 64% for controls, and this was accompanied by a rise in the percentage of germ cells not encapsulated by somatic cells, 50 vs. 34% for controls (Fig. 7E). Vehicle and L-685,458-treated ovaries had comparable percentages of developing follicles (Fig. 7E). Ovaries exposed to L-685,458 and cultured in media containing 50 μ g/ml insulin had a higher percentage of developing follicles than that found in DAPT-treated ovaries, which were cultured in media containing 1 μ g/ml insulin. This is expected because insulin has been shown promote the primordial

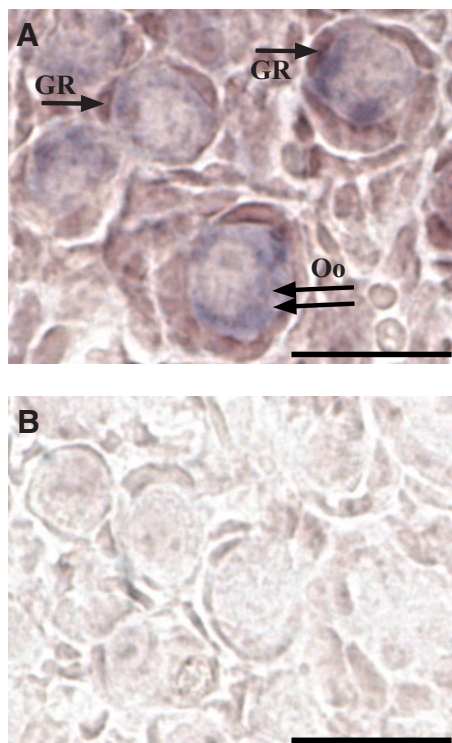


FIG. 3. *In situ* hybridization for Hey2 mRNA in the d 3 ovary. Ovary sections were incubated with either an antisense Hey2 (A) or sense Hey2 (B) probe. Hey2 transcripts were expressed in pregranulosa cells (A, arrows) and oocytes (A, double arrow) of primordial follicles. Scale bars, 25 μ m. GR, Pregranulosa cells; Oo, oocytes.

to primary follicle transition (38). Regardless of the insulin concentration used in the *ex vivo* culture studies, we observed a marked affect on follicle formation upon γ -secretase inhibitor treatment.

Our inhibitor experiments suggest that Notch signaling promotes primordial follicle formation, but the mechanism for how this occurs is unknown. Notch signaling is a regulator of apo-

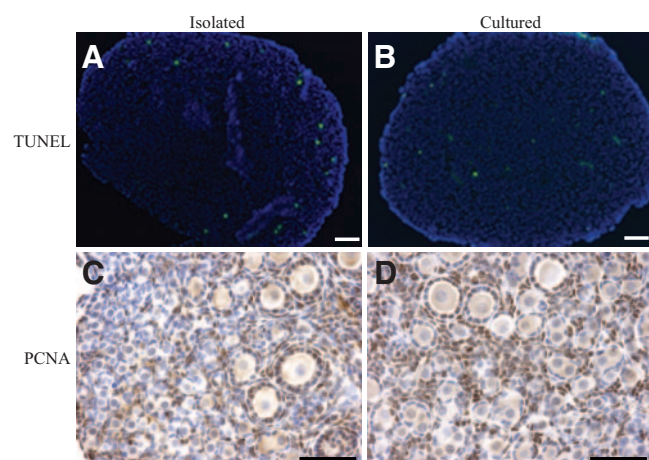


FIG. 4. Cellular dynamics in cultured and *in vivo* ovaries. The TUNEL assay was used to determine the distribution of apoptotic cells between isolated (A) and cultured ovaries (B). Newborn mouse ovaries were cultured for 4 d. Immunohistochemistry for proliferating cell nuclear antigen (PCNA) was used to assess cell proliferation in isolated (C) and cultured ovaries (D). The concentration of insulin used in these ovary culture studies was 50 μ g/ml. Scale bars, 40 μ m (A and B); 50 μ m (C and D).

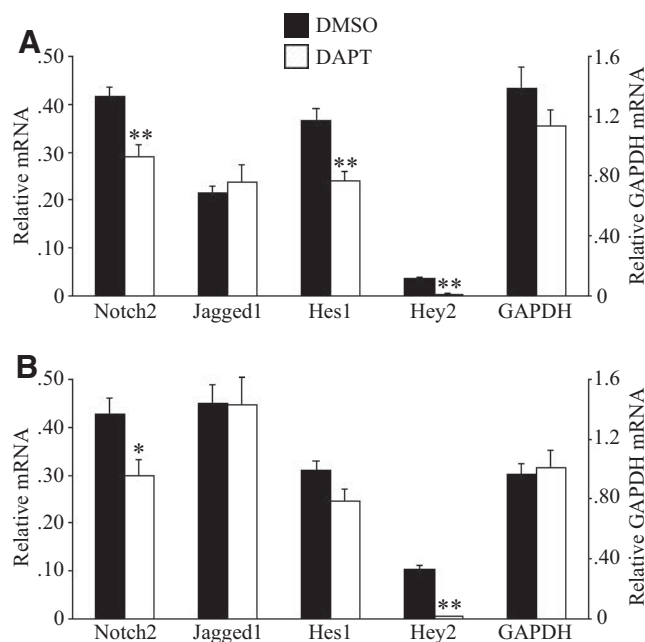


FIG. 5. γ -Secretase inhibitor efficacy in ovary cultures. Real-time PCR was used to measure mRNA expression of selected genes in d 0 ovaries treated with DMSO or DAPT for 1 d (A) or 4 d (B) ($n = 6$ –8/group). The concentration of insulin used in these ovary culture experiments was 10 μ g/ml. Relative mRNA values in these studies were normalized to RPL19. The second y-axis corresponds to relative mRNA values for GAPDH. The graph represents the average of samples from three experiments. *, $P < 0.05$; **, $P < 0.005$.

ptosis and has been shown to have antiapoptotic properties in different contexts (39). Therefore, we examined cell death in newborn mouse ovaries cultured for 1, 2, or 4 d with DAPT, the inhibitor most potent in preventing primordial follicle formation. We observed apoptosis in both germ and somatic cell compartments (Fig. 8B). Ovaries treated with DAPT for 1 and 2 d contained more apoptotic cells than controls, whereas the number of TUNEL-positive cells was comparable between vehicle and inhibitor-treated ovaries after 4 d of culture (Fig. 8C). The small but significant changes we observe in ovaries treated with DAPT for 1 and 2 d may reflect a role for Notch signaling in promoting cell survival.

Discussion

Abscissions in ovarian follicle maturation can inhibit the proper development of the oocyte and thus impact fertility. This is best evidenced by a variety of human diseases (40, 41) and mouse mutations (42, 43) that affect folliculogenesis. The first follicles that form, primordial follicles, establish a resting pool from which a small number of selected follicles will develop throughout the reproductive lifespan. Bidirectional communication between oocytes and granulosa cells is crucial for this maturation process (44, 45). Communication between germ cells and pregranulosa cells likely occurs during early folliculogenesis, and this is highlighted by the finding that somatic cells fail to form follicular structures in ovaries devoid of germ cells (46). Disruption of either germ cell- or somatic cell-derived factors, *i.e.* *FIG α* and *Wnt4*, respectively (47, 48), can lead to defects in follicle

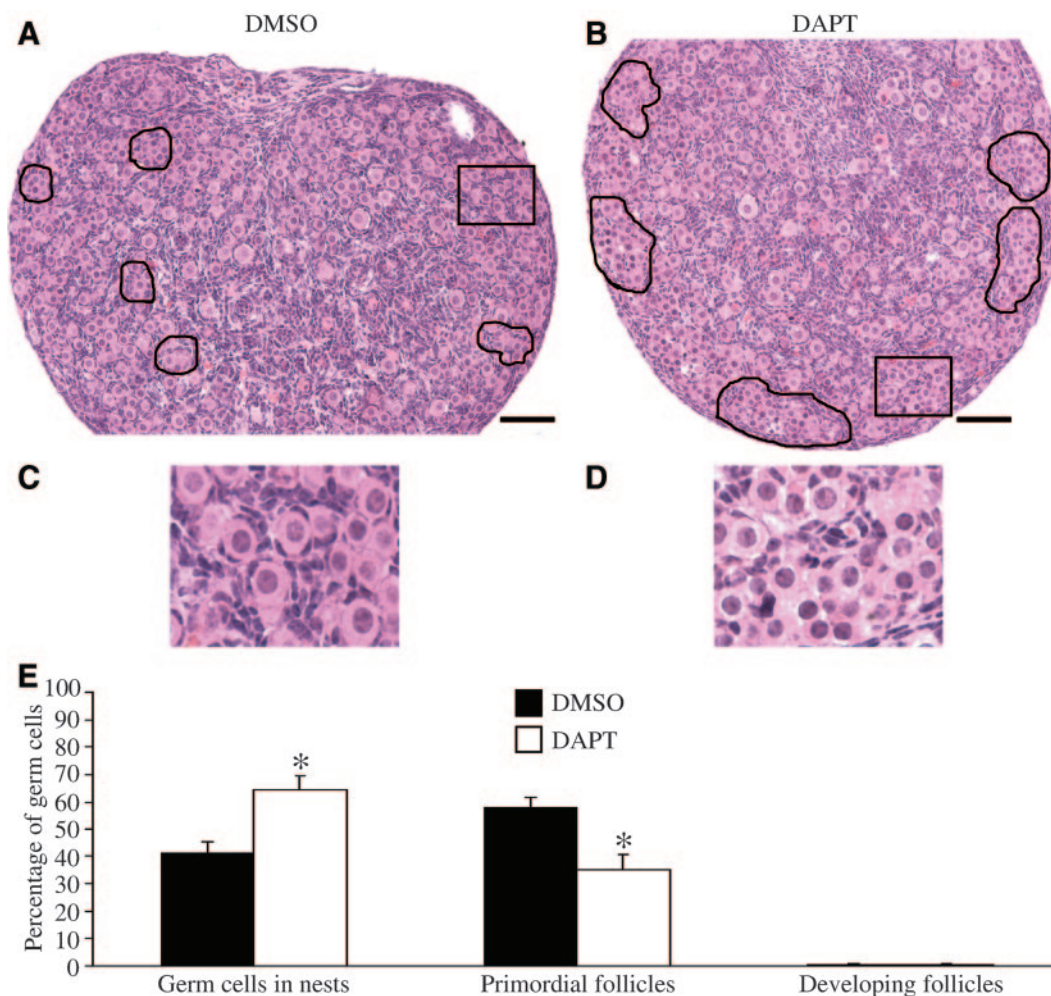


FIG. 6. Phenotypes of ovary cultures treated with vehicle and DAPT. Newborn mouse ovaries cultured for 4 d in media containing 0.2% DMSO (A) or 20 μ M DAPT (B) were fixed and then H+E stained. Germ cell nests are indicated by black boundaries. C and D, Enlarged images of the black rectangles in A and B. E, Follicle populations in DMSO- ($n = 4$) and DAPT ($n = 5$)-treated ovaries were quantified. The concentration of insulin used in these studies was 1 μ g/ml. The graph represents average follicle counts from ovaries cultured in three independent experiments. Scale bars, 50 μ m. *, $P < 0.05$.

formation. Insights into how primordial follicles assemble may provide an avenue to better treat reproductive disorders that negatively affect fertility.

We provide evidence for the expression of Notch pathway genes in the neonatal mouse ovary and propose a novel role for Notch signaling in regulating primordial follicle formation. JAGGED1 and NOTCH2 are expressed in oocytes and granulosa cells, respectively, expression patterns that are consistent with what has been reported in the adult mouse ovary (30). The complementary expression pattern of JAGGED1 and NOTCH2 provides a potential role for these molecules in mediating interactions between the germ and somatic cell compartments during early follicle development. The total number of germ cells decreases between d 0 and 6 (4), so increased Jagged1 mRNA may reflect enhanced production by the remaining germ cells and relate to the initial growth of primordial follicles. In the neonatal mouse ovary, the expression of Notch receptor genes are not coordinated with Jagged1, rather the mRNA levels are maximal compared with the prepubertal ovary. We cannot exclude more localized changes in Notch gene expression at the cellular level. HES1 is expressed in both pregranulosa cells surrounding germ

cell nests and also in oocytes of early primary follicles. That HES1, a transcription factor, is not expressed in oocyte nuclei but rather in the cytoplasm raises the possibility that it is not active in germ cells. Interestingly, Hey2 mRNA is up-regulated during follicle formation and initial growth. The expression pattern of Hey2 mRNA in the neonatal ovary is similar to HES1 protein: pregranulosa cells of primordial follicles and oocytes of primary follicles. The low levels of Hey2 mRNA in the d 0 ovary indicate that Hey2 may be expressed later than Hes1 during early folliculogenesis. Indeed, microarray studies point toward Hes1 and Hey2 being enriched in somatic cells at 18 d post coitum and 2 d postnatal, respectively (49). Future experiments are required to determine whether HEY2 is expressed in pregranulosa cells surrounding germ cell nests.

NOTCH2/HES1 and JAGGED1 are expressed in distinct cells before follicle formation, pregranulosa cells, and germ cells, respectively, suggesting that germ cell nest breakdown is in part coordinated through cellular interactions via Notch signaling. Notch activation would then appear to directly impact pregranulosa cells and may promote the proliferation of pregranulosa cells during follicle assembly. Enhanced somatic cell prolifera-

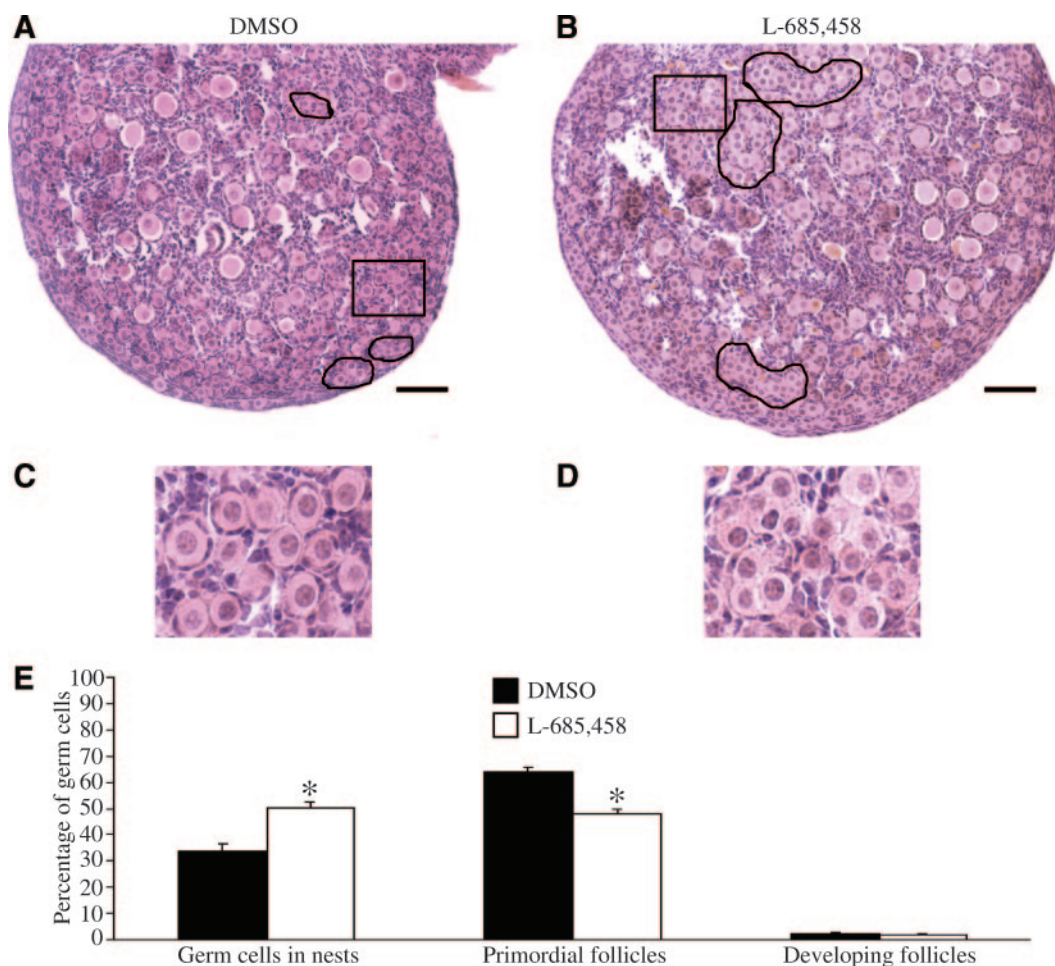


FIG. 7. Ovary cultures treated with a second γ -secretase inhibitor, L-685,458. Ovaries cultured for 4 d in media containing 0.2% DMSO (A) or 10 μ M L-685,458 (B) were fixed and then H+E stained. Germ cell nests are indicated by black boundaries. C and D, Enlarged images of the black rectangles in A and B. E, Follicle populations in ovaries treated with DMSO ($n = 5$) and L-685,458 ($n = 7$) were counted. The concentration of insulin used in these studies was 50 μ g/ml. The graph represents average follicle counts from ovaries cultured in four independent experiments. Scale bars, 50 μ m. *, $P < 0.005$.

tion has been reported to be a potential mechanism for driving increased follicle formation (5). Alternatively, Notch signaling may serve to establish granulosa cell identity, a property that may be essential for subsequent primordial follicle assembly. Pregranulosa cells fail to differentiate and transition to cuboidal granulosa cells in *Foxl2* null mouse ovaries (43). Ovaries from these animals exhibit early follicular depletion (43) and potential defects in follicle formation (50), demonstrating the potential ties between granulosa cell differentiation and follicle assembly. Notch signaling has also been shown to support cell migration (51). During germ cell nest breakdown, Notch signaling may mediate pregranulosa cell migration within germ cell syncytia to facilitate primordial follicle assembly.

Rodent ovary culture has been successfully performed by other investigators (29, 52–55) to ascertain roles for signaling molecules during early follicle development. We observe a recapitulation of *in vivo* follicle formation in this system, arguing that factors important for follicle assembly are intrinsic to the ovary. Newborn mouse ovaries maintained in culture were treated with γ -secretase inhibitors to address functional roles for Notch in the neonatal mouse ovary. We used small-molecule inhibitors to maximize the chance of attenuating Notch signaling because all

four Notch receptors are processed by γ -secretase. γ -Secretase belongs to a class of aspartyl proteases that have multiple substrates (56). Although other targets may be affected by these inhibitors, we observed no decrease in GAPDH or Jagged1 mRNA expression. Our apoptosis studies show that cell death is limited in DAPT-treated ovaries. The viability of cells cultured with DAPT argues against a toxic effect of the inhibitor on *ex vivo* cultured ovaries. A more likely possibility is that the increase in apoptotic cell numbers in ovaries treated with DAPT for 1 and 2 d reflects a potential role for Notch signaling in promoting cell survival.

Treatment of ovary cultures with DAPT resulted in decreased Notch target gene (*Hes1*, *Hey2*) expression. In addition, similar ovarian phenotypes, namely germ cell nest retention, were observed in ovaries treated with chemically distinct γ -secretase inhibitors (57, 58). Therefore, it is likely that the effects on follicle formation are indeed mediated through Notch signaling. The higher suppression of *Hey2* mRNA compared with *Hes1* in inhibitor-treated ovaries supports the notion that Notch signals chiefly through *Hey2* during follicle formation. *Hes1* is expressed in the ovaries of late-stage embryos. Therefore, we cannot rule out the possibility that Notch signals through *Hes1*

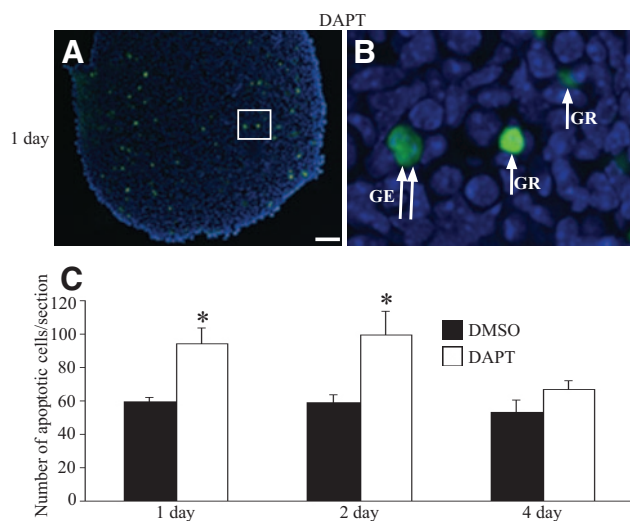


FIG. 8. Examination of apoptosis in vehicle and DAPT-treated ovaries. Ovaries were cultured for 1, 2, or 4 d with DMSO or DAPT. A, A TUNEL-stained tissue section from an ovary treated with DAPT for 1 d. B, Enlarged image of the white rectangle in A showing apoptotic pregranulosa cells (arrows) and an apoptotic germ cell (double arrow). C, The graph represents the average number of TUNEL-positive cells in one tissue section from three different control and inhibitor-treated ovaries at each time point. The concentration of insulin used in these studies was 10 μ g/ml. Scale bars, 40 μ m. *, $P < 0.05$. GR, Pregranulosa cells; GE, germ cells.

during the earliest stages of follicle formation. Given that Hey2 is expressed in pregranulosa cells and oocytes, it is unclear within which cellular compartment Hey2 mRNAs are decreased in response to DAPT treatment. Decreased Hey2 mRNA expression in pregranulosa cells would be considered a direct effect because Notch2 is expressed in pregranulosa cells. Alternatively, the decrease in Hey2 mRNA expression may reflect the lower percentages of Hey2-expressing germ cells formed into primordial follicles in DAPT-treated ovaries.

The early stages of follicle assembly appear to be the most sensitive to Notch signaling, as there were no significant differences in the percentages of later-stage follicles between the control and inhibitor-treated ovaries. Notch signaling may therefore direct the early stages of germ cell nest breakdown and primordial follicle maintenance. It is unknown whether the efficiency of nest breakdown is reduced and/or the kinetics of follicle formation delayed in the inhibitor-treated ovaries. Future experiments in which ovaries are cultured for longer times in the presence of γ -secretase inhibitors may address this question. It is unclear which receptors are required for promoting follicle formation, and this poses a challenge to using such inhibitors. Genetic studies using RNA interference knockdown in ovary culture or conditional gene disruption in mice should eventually shed light on this issue. Although several key players that mediate primordial follicle formation and initial growth have been identified (59–62), these processes likely require the coordinated actions of multiple cell signaling pathways. We previously described the interplay of estrogen and activin signaling pathways in the early mouse ovary (10), and this has been supported by *in vitro* experiments (63). Interactions between Notch and the activin/TGF β signaling pathways have also been described (64–67). Therefore, it will be important to investigate how Notch, estro-

gen, and activin signaling pathways interact in the context of early follicle development in the mammalian ovary.

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