BIOLOGY OF REPRODUCTION **84,** 859–865 (2011)
Published online before print 5 January 2011.
DOI 10.1095/biolreprod.110.088708

Role of the DLL4-NOTCH System in PGF2alpha-Induced Luteolysis in the Pregnant Rat[1]

Fatima Hernandez,[3] Marina C. Peluffo,[4] Richard L. Stouffer,[4] Griselda Irusta,[3] and Marta Tesone[2,3,5]

Instituto de Biología y Medicina Experimental (IBYME-CONICET),[3] Buenos Aires, Argentina
Oregon National Primate Research Center,[4] Oregon Health & Science University (ONPRC-OHSU), Beaverton, Oregon
Departamento de Química Biológica,[5] Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

ABSTRACT

We investigated the expression and cell localization of NOTCH1, NOTCH4, and the delta-like ligand DLL4 in corpus luteum (CL) from pregnant rats during prostaglandin F2alpha (PGF2alpha)-induced luteolysis. We also examined serum progesterone (P_4) and CL proteins related to apoptosis after local administration of the notch inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (DAPT). Specific staining for NOTCH1 and NOTCH4 receptors was detected predominantly in large and small luteal cells. Furthermore, in line with the fact that the notch intracellular domain is translocated to the nucleus, where it regulates gene expression, staining was evident in the nuclei of luteal cells. In addition, we detected diffuse cytoplasmic immunostaining for DLL4 in small and large luteal cells, in accordance with the fact that DLL4 undergoes proteolytic degradation after receptor binding. The mRNA expression of *Notch1*, *Notch4*, and *Dll4* in CL isolated on Day 19 of pregnancy decreased significantly after administration of PGF2alpha. Consistent with the mRNA results, administration of PGF2alpha to pregnant rats on Day 19 of pregnancy decreased the protein fragment corresponding to the cleaved forms of NOTCH1/4 CL receptors. In contrast, no significant changes were detected in protein levels for the ligand DLL4. The local intrabursal administration of DAPT decreased serum P_4 levels and increased luteal levels of active caspase 3 and the BAX:BCL2 ratio 24 h after the treatment. These results support a luteotropic role for notch signaling to promote luteal cell viability and steroidogenesis, and they suggest that the luteolytic hormone PGF2alpha may act in part by reducing the expression of some notch system members.

apoptosis, corpus luteum, ovary, pregnancy, progesterone/progesterone receptor

INTRODUCTION

The corpus luteum (CL) is a transient endocrine gland, the main function of which is to secrete the steroid hormone progesterone (P_4), essential for implantation of the blastocyst and maintenance of pregnancy in mammals [1–3]. If pregnancy does not occur, however, or when P_4 is no longer required for the maintenance of pregnancy, the CL ceases to produce this hormone and regresses in a process called luteolysis. The formation and regression of the CL require rapid remodeling, growth, differentiation, and death of its heterogeneous population of cells. During luteolysis, the death of luteal cells is preceded by a loss of their capacity to synthesize and secrete P_4 [4].

Prostaglandin F2alpha (PGF2α) is a uterine-derived factor that initiates luteolysis in many nonprimate species. Interestingly, the prostaglandin synthesis inhibitor indomethacin prevents luteolysis in pseudopregnant rodents [5]. The actions of PGF2α include a reduction in ovarian blood flow, uncoupling of the luteinizing hormone (LH) receptor from adenylate cyclase, reduction in steroidogenic enzyme activity and P_4 production, a decrease in LH receptor concentration, changes in membrane fluidity, changes in luteal cell population, and an increase in lysosomal enzyme activity [6–8]. Although PGF2α has been well established as the most important luteolytic hormone for more than 30 years, the cellular mechanisms by which PGF2α exerts its luteolytic effects remain incompletely understood.

Luteal regression has been related to apoptosis in many species [9–16]. Several genes belonging to the *Bcl2* family may function as intracellular mediators of cell survival. The protein products of the *Bcl2* and *Bax* genes have been described as anti- and proapoptotic factors, respectively [17–19]. Depending on the balance of these proteins, the initiator caspase 9 (CASP9) is activated, and several effectors caspases, such as CASP3 or CASP7, are sequentially activated and promote apoptosis in various systems [20]. In previous studies, we reported the expression and activation of proapoptotic caspase-mediated pathways during both spontaneous luteolysis in pregnancy and natural cycles as well as PGF2α-induced luteolysis [21, 22].

The notch pathway includes a conserved family of transmembrane receptors (NOTCH1, NOTCH2, NOTCH3, and NOTCH4) that interact with a number of specific ligands (the delta-like family, jagged [JAG] 1, and JAG2) to regulate cell fate [23]. Notch signaling plays a critical role in many developmental processes, influencing differentiation, proliferation, and apoptosis [24, 25]. In addition, notch family members, particularly delta-like ligand (DLL4) and its receptors NOTCH1 and NOTCH4, have been recently identified as novel factors involved in the regulation of angiogenesis [26, 27]. The interaction between notch receptors and their ligands leads to intracellular cleavage of notch receptors by the gamma-secretase complex [28]. The cleaved notch intracellular domain (NICD) traffics to the nucleus, where it interacts with transcriptional factors. This processing requires the activity of two proteases—namely, tumor necrosis factor α-converting enzyme and presenilin/gamma-secretase.

[1]Supported by ANPCYT (BID 1201 OC-AR PICT 99:05-06384), NIH-FIRCA RO3-TW007041, and NIH-NCCR RR00163.
[2]Correspondence: Marta Tesone, Instituto de Biologia y Medicina Experimental, Obligado 2490, Buenos Aires, 1428, Argentina.
FAX: 54 11 4786 2564; e-mail: mtesone@ibyme.conicet.gov.ar

Received: 21 September 2010.
First decision: 8 October 2010.
Accepted: 22 December 2010.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

Johnson et al. [29] have examined the expression patterns of notch receptor genes and their ligands by immunohistochemistry (IHC) in rodent mammalian ovary. Their data indicate that NOTCH2, NOTCH3, and JAG2 are expressed in an overlapping pattern in the granulosa cells of developing follicles. However, the expression and regulation of the notch ligand-DLL4 system in the structure-function of CL remain unknown. We thus hypothesized that if the notch system plays a key role in luteolysis during pregnancy in rats, then *Notch* mRNA and/or protein expression would change in PGF2α-induced CL regression. Therefore, studies were designed to analyze the expression and cell localization of NOTCH1, NOTCH4, and the ligand DLL4 in CL from pregnant rats during PGF2α-induced luteolysis. To elucidate whether the notch system has a direct effect in luteal function during pregnancy, we also examined serum P_4 and proteins related to apoptosis in rat CL after local administration of the gamma-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (DAPT).

MATERIALS AND METHODS

Animal Model

Adult female Sprague-Dawley rats (body weight, 200–250 g; age, 8 wk) were housed at room temperature (21–23°C) with a 12L:12D photoperiod in an air-conditioned environment. The rats had ad libitum access to food and water. Animals showing three consecutive 4-day cycles were used for the experiment, and cycling stages were determined daily by vaginal cytology. Proestrous females were caged with fertile males overnight; after separation the next morning, vaginal smears were analyzed for the presence of spermatozoa. The day spermatozoa were detected was considered to be Day 1 of pregnancy.

For prostaglandin experiments, a group of rats was injected intraperitoneally with 400 μg of analogue (PGF2α group; Lutalyse; Pfizer) on Day 19 of pregnancy to induce luteolysis, and another group of animals was injected with saline solution (control group). The animals were euthanized by CO_2 aspiration, and ovaries were collected at 4 and 24 h after treatment. Day 19 of pregnancy was selected for PGF2α treatment, because this is when the CL becomes more sensitive to the action of PGF2α [30].

For the inhibition of the notch pathway, DAPT (Sigma-Aldrich), a chemical component that specifically inhibits the activity of the gamma-secretase complex, was used. This inhibitor has been extensively used for experimental studies of notch signaling, both in vitro and in vivo [31]. Pregnant rats were anesthetized on Day 19 of pregnancy with ketamine HCl (70 mg/kg; Holliday-Scott S.A.) and xylazine (5 mg/kg; König Laboratories). The ovaries were exteriorized through an incision made in the ventral region. One group of rats then received 10 μg of DAPT (1 μg/μl) under the bursa of both ovaries (DAPT group), and another group of animals received 10 μl of vehicle solution (6% dimethyl sulfoxide; control group). Twenty-four hours after the surgery, animals were euthanized by decapitation for ovary and blood collection. The experimental protocols were approved by the Animal Experimentation Committee of the Institute of Experimental Biology and Medicine (IBYME-CONICET).

Tissue Preparation

Corpora lutea were dissected from ovaries under a stereoscopic microscope as previously described [22, 32]. Pools of CL (n = 5–6 CL/rat) were frozen in liquid nitrogen and stored at −80°C for mRNA and protein extraction. Also, half of each ovary was fixed for IHC studies.

Immunohistochemistry

Ovaries from the different groups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 12 h. Tissues were dehydrated in a series of ethanol solutions (70%, 96%, and 100%) and embedded in paraffin. Then, sections (thickness, 4 μm) were deparaffinized and hydrated with xylene and a graded series of ethanol. Endogenous peroxidases were then quenched with 3% H_2O_2 in PBS, followed by successive PBS washes. To study the localization of CASP3 protein, the slides were heated in a microwave oven in a citric acid buffer (0.01 M, pH 6) for 9 min at 600 W, followed by 20 min of cooling at room temperature. For NOTCH1, NOTCH4, and DLL4 proteins, the slides were exposed to proteinase K in PBS (20 mg/ml) for 15 min, followed by two successive washes with distilled water. All primary antibodies were purchased from Santa Cruz Biotechnology. Sections were placed in a blocking buffer (2% bovine serum albumin in PBS) for 20 min, and then incubated with the primary antibody at a concentration of 1:100 in PBS buffer for 1 h at room temperature. Finally, all sections were incubated overnight at 4°C.

The primary antibody was detected using a biotinylated anti-rabbit immunoglobulin (Ig) G secondary antibody (1:400; Vector Laboratories) and the Vector ABC-Elite Kit. Positive-stained slides were visualized with 3,5-diaminobenzide tetrahydrochloride (0.05% vol/vol) and counterstained with hematoxylin. Negative controls were obtained in the absence of primary antibody or by preabsorbing the antibodies with blocking peptides (when commercially available). The images were digitized using a camera (Nikon) mounted on a conventional light microscope (Nikon), with magnifications of 100× and 400× used. Finally, the images were converted to TIFF format (bilevel scale) for analysis.

RNA Isolation and Quality Control

Total RNA was extracted from a pool of CL from each rat using TRIzol (Invitrogen Corp.) according to the manufacturer's instructions. The integrity of RNA samples was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies), and all samples were estimated to be of high quality.

Complementary DNA Synthesis and TaqMan Quantitative Real-Time PCR

Reverse transcription was carried out on 1 μg of DNase-treated RNA in a 20-μl reaction volume using Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 50 min at 37°C. Real-time PCR was performed using the TaqMan PCR Reagent Kit with the 7500 Fast Real Time PCR System (Applied Biosystems). Primers and TaqMan MGB Probe for DLL4 ligand and NOTCH1 and NOTCH4 receptors were predeveloped and purchased from Applied Biosystems (catalog no. Mm00444619_m1, Rn 01758633_m1, and Rn 01525743_g1, respectively). The *Rn18s* mRNA was used as an active endogenous control in each well. Forward and reverse primers, as well as MGB Probe sequences for *Rn18s*, were as follows: 5′-CGGCTACCACATC CAAGGAA-3′, 5′-GGGCCTCGAAAGAGTCCTGT-3′, and 5′-CAG CAGGCGCGCAAATTACCCA-3′, respectively. Amplifications were conducted in a 10-μl final volume containing the NOTCH1, NOTCH4, or DLL4 probe (labeled with the reporter dye carboxyfluorescein), the RN18S probe (labeled with the 50 reporter dye VIC), forward and reverse primers, and TaqMan Universal PCR master mix. The number of amplification cycles required for the fluorescence to reach a predetermined threshold level (CT) was recorded for each unknown sample and for an internal standard curve. The internal standard curve, used for relative mRNA quantification, was generated from five 10-fold dilutions of pooled samples. CT values for unknown samples were used to extrapolate the amount of RNA equivalents from the internal standard curve. Relative expression of the target genes was normalized to *RN18s* rRNA levels, and the ratios were log-transformed before statistical analysis.

Western Blot Analysis

Five or six CL per rat were resuspended in lysis buffer (20 mM Tris-HCl [pH 8], 137 mM NaCl, 1% NP-40, and 10% glycerol) supplemented with protease inhibitors (Sigma) and homogenized with an Ultra-Turrax homogenizer (IKA Werk). Samples were centrifuged at 4°C for 10 min at 10 000 × *g*, and the resulting pellets were discarded. Protein concentration in the supernatant was measured by the Bradford assay. After boiling, samples were loaded onto the polyacrylamide gel, separated by 10–15% SDS-PAGE, and subsequently transferred to nitrocellulose membranes. Nonspecific binding sites were blocked overnight in TBS (4 mM Tris–HCl [pH 7.5] and 100 mM NaCl) containing low-fat powdered milk (5%) and Tween-20 (0.05%) at 4°C. The membranes were then incubated overnight with the appropriate anti-rabbit or -mouse primary antibodies: anti-DLL4 (1:500; Abcam), anti-NOTCH1 (1:200; Cell Signaling Technology), anti-BCL2 (1:400; DAKO), anti-NOTCH4 (1:500; Santa Cruz Biotechnology), anti-BAX (1:200; Santa Cruz Biotechnology), and anti-CASP3 (1:200; Santa Cruz Biotechnology). Protein bands were visualized by incubating the blots for 1 h with peroxidase-conjugated secondary anti-rabbit IgG (1:1000; Sigma Chemical Co.). Finally, the membranes were incubated with Amersham ECL plus and exposed to radiographic film. Protein expression was quantified by densitometric analysis using Scion Image Software for Windows (Scion Corporation). The density in each specific band was normalized to the density of an internal control, glyceraldehyde phosphate dehydrogenase (1:10 000; Cell Signaling Technology).

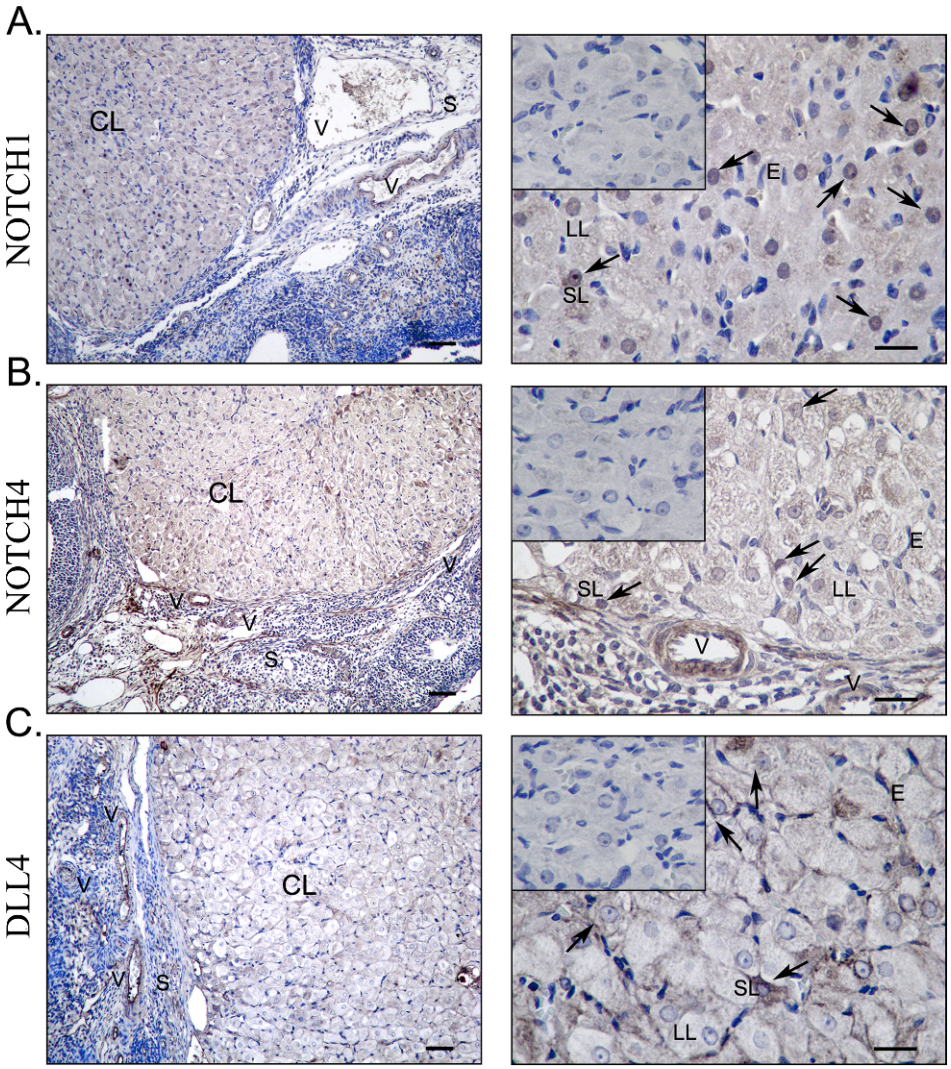

FIG. 1. Localization of NOTCH1 and NOTCH4 receptors and DLL4 ligand in pregnant rat CL. **A** and **B**) Immunostaining for NOTCH1 and NOTCH4 was detected in large luteal cells (LL), small luteal cells (SL), endothelial cells (E), and blood vessels (V). Arrows indicate luteal nuclear staining for these receptors. **C**) DLL4 specific immunoreactivity was also found in E, LL, and SL (arrows), and V. No immunoreactivity was detected in the stroma (S) for NOTCH1, NOTCH4, or DLL4. Insets show negative controls. Bar = 50 μm (left) or 20 μm (right).

Serum P_4 Levels

Following ether extraction, P_4 levels were measured by radioimmunoassay in serum from the control and DAPT groups, as described previously [33], using a specific antibody supplied by Dr. G.D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO). Under these conditions, the intra- and interassay variations were 8.0% and 14.2%, respectively.

Statistical Analysis

Data are expressed as the mean ± SEM. Experiments were repeated at least three times (n = 5–6 animals/group). Data were analyzed using unpaired Student *t*-test. Differences were considered to be significant at $P < 0.05$. All data were assessed for heterogeneity of variance and found to be nonsignificant.

RESULTS

IHC of NOTCH1, NOTCH4, and DLL4 in CL from Pregnant Rats

Figure 1 illustrates the presence and cell distribution of NOTCH1, NOTCH4, and DLL4 investigated in sections of rat ovaries obtained on Day 19 of pregnancy. Panels on the right in Fig. 1 illustrate higher magnification for each protein.

Specific staining for NOTCH1 and NOTCH4 receptors was detected predominantly in large and small luteal cells as well as in endothelial cells in the CL. In addition, positive staining was detected in ovarian blood vessels. No immunoreactivity was observed in the surrounding stroma (Fig. 1, A and B). Furthermore, we detected positive staining for both receptors in the nuclei of luteal cells, in line with the fact that the NICD is translocated to the nucleus, where it regulates gene expression [34].

Specific staining for the DLL4 ligand was also detected in large and small luteal cells as well as in endothelial cells. The ovarian blood vessels were also immunoreactive (Fig. 1C). Notably, DLL4 staining was associated mainly with the cellular membrane defining the shape of the luteal cell. In addition, we detected a diffuse cytoplasmic immunostaining for DLL4 in luteal cells, in accordance with the fact that DLL4 undergoes proteolytic degradation after receptor binding [35].

Messenger RNA Levels of Notch1, Notch4, *and* Dll4 *During Luteolysis Induced by PGF2α*

To determine whether expression of members of the notch pathway changes during luteolysis, we studied the mRNA expression of *Notch1*, *Notch4*, and *Dll4* by real-time PCR in CL isolated on Day 19 of pregnancy, both 4 and 24 h after the administration of a luteolytic dose of PGF2α (Fig. 2). The mRNA levels of both *Notch1* and *Notch4* receptors decreased 4 h after PGF2α administration compared with the control group

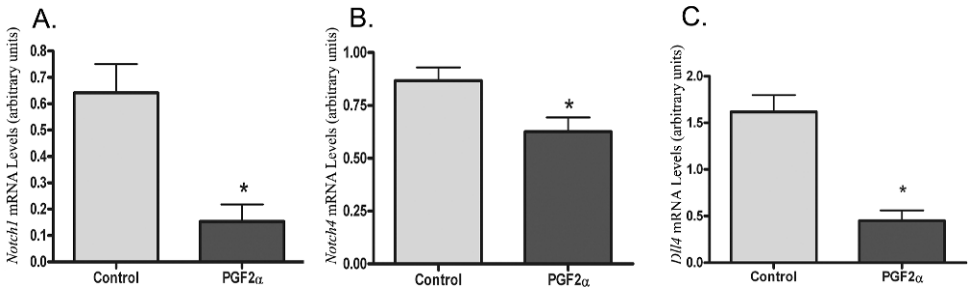

FIG. 2. *Notch1*, *Notch4*, and *Dll4* mRNA levels in CL at 4 h after PGF2α administration on Day 19 of pregnancy. CL were obtained from animals of the control and PGF2α groups (n = 6 per group). *Notch1* (**A**), *Notch4* (**B**), and *Dll4* (**C**) mRNA levels were determined by quantitative PCR and normalized to *Rn18s* rRNA. Bars represent the mean ± SEM. *$P < 0.05$.

(4.30-fold and 1.38-fold, respectively; $P < 0.05$, n = 6) (Fig. 2, A and B). Similarly, the levels of *Dll4* mRNA were significantly lower in the PGF2α group than in the control group (3.6-fold; $P < 0.001$, n = 6) (Fig. 2C). However, 24 h after the PGF2α administration, no differences were found in the mRNA levels of *Notch1*, *Notch4*, and *Dll4* between control and PGF2α groups (data not shown).

Protein Levels of NOTCH1, NOTCH4, and DLL4 During Luteolysis Induced by PGF2α

Based on the results obtained for mRNA, the protein levels of the notch members were determined by Western blot analysis in CL obtained 4 h after PGF2α administration (Fig. 3). Analogous to the results obtained by quantitative PCR, administration of PGF2α to pregnant rats on Day 19 of pregnancy decreased the fragment corresponding to the cleaved forms of NOTCH1 and NOTCH4 receptors compared to the control group (2.4-fold and 1.5-fold, respectively; $P < 0.05$, n = 6) (Fig. 3, A and B). In contrast, no significant changes were detected in protein levels for the ligand DLL4 between groups (Fig. 3C).

Involvement of Notch Signaling in CL Function and Apoptosis During Rat Pregnancy

To examine whether notch signaling influences the function and survival of luteal cells during pregnancy, we administered DAPT intrabursally to the ovary of rats on Day 19 of pregnancy. We first studied the cleavage of NOTCH1 in CL of pregnant rats 24 h after DAPT administration to verify if DAPT treatment was able to inhibit the notch signaling. By Western blot analyses of CL proteins, we detected a significant decrease of NICD in the DAPT group when compared to the control group (control, 1.00 ± 0.10; DAPT, 0.75 ± 0.04; $P < 0.05$, n = 5–6). The local injection of DAPT produced a 1.9-fold decreased in serum P_4 levels 24 h after the treatment (control, 78.99 ± 13.33 ng/ml; DAPT, 42.40 ± 8.34 ng/ml; $P < 0.05$, n = 5–6).

Western blot analysis of CL proteins using an anti-CASP3-specific antibody detected a significant increase, approximately 1.6-fold higher ($P < 0.05$, n = 5), in the level of the active p17 form of CASP3 in the DAPT group when compared to the control group (Fig. 4A). Nevertheless, no significant differences were found in the relative pro-CASP3 levels after the treatment (data not shown).

We next analyzed CASP3 protein expression by IHC studies in ovarian sections from DAPT-treated and control rats. Luteal cells in the DAPT group exhibited a high signal for CASP3 (Fig. 4C). In contrast, luteal cells from the control group (Fig. 4B) showed low immunoreactivity for this protein. Interestingly, CASP3 staining was more evident in the nuclei of the CL of the sections obtained from DAPT-treated ovaries than in those of the control group, in line with the fact that once activated, CASP3 translocates to the nucleus [36, 37].

In light of these results, we examined the luteal content of the proapoptotic protein BAX and the antiapoptotic protein BCL2, two known regulators of ovarian apoptosis [38, 39]. Intrabursal injection of DAPT to pregnant rats significantly increased BAX protein expression (1.5-fold; $P < 0.05$, n = 5–6) (Fig. 5A) and considerably decreased the levels of BCL2 protein (1.6-fold; $P < 0.05$, n = 5–6) (Fig. 5B) relative to control levels. Consequently, the BAX:BCL2 ratio was significantly increased in CL obtained from ovaries treated with DAPT ($P < 0.05$) (Fig. 5C).

DISCUSSION

The present study was designed to determine whether the notch pathway has a critical role in the luteolytic progression of CL from pregnant rats. We report here, to our knowledge for the first time, that 1) NOTCH1, NOTCH4, and the ligand DLL4 are expressed and localized in small and large luteal cells of CL from pregnant rats; 2) administration of the luteolytic hormone PGF2α decreased CL mRNA and protein expression of these notch family members; and 3) blocking the notch cleavage at the level of the gamma-secretase induced a decrease in circulating P_4 levels and an increase in the

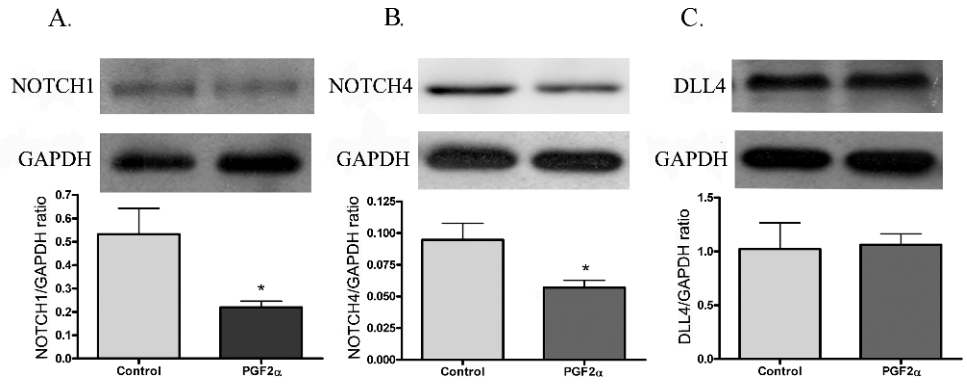

FIG. 3. NOTCH1, NOTCH4, and DLL4 proteins levels in rat CL at 4 h after PGF2α administration on Day 19 of pregnancy. CL were obtained from animals of the control and PGF2α groups (n = 6 per group). NOTCH1 (**A**), NOTCH4 (**B**), and DLL4 (**C**) protein levels were determined by Western blot analysis and normalized to glyceraldehyde phosphate dehydrogenase (GAPDH). Bars represent the mean ± SEM. *$P < 0.05$.

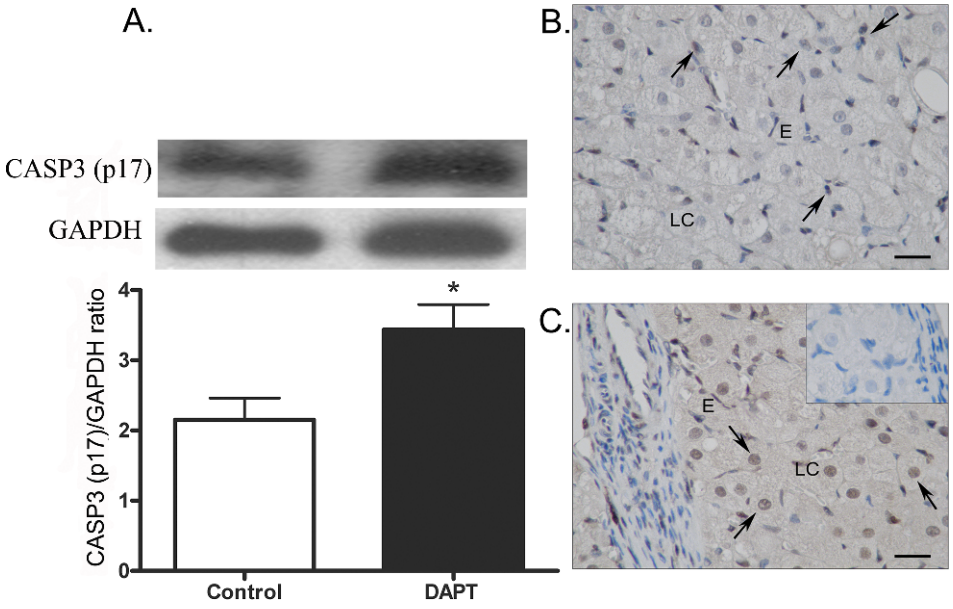

FIG. 4. Effect of DAPT treatment on the cleavage of CASP3 protein in rat CL on Day 19 of pregnancy. **A**) Representative Western blots of CASP3 protein visualized using an anti-CASP3 antibody that recognizes its cleaved form (top) and densitometric quantification of CL CASP3 active fragment (bottom). Bars represent the mean ± SEM. *$P < 0.05$. **B** and **C**) Representative photomicrographs of CASP3 in both the control (**B**) and DAPT (**C**) groups. The inset shows the negative control. Arrows indicate nuclear staining of activated CASP3. E, endothelial cells; LC, luteal cells. Bar = 20 μm.

activation of CASP3 and in the proapoptotic ratio of BAX to BCL2 proteins in CL. These results suggest that notch signaling plays a role in regulating the fate of luteal cells.

Previously, NOTCH2, NOTCH3, and JAG2 were localized by IHC to granulosa cells in developing follicles of mammalian ovaries, thus suggesting that the notch signaling pathway is involved in the regulation of folliculogenesis [29]. In addition, the suppression of notch signaling by gamma-secretase inhibitors in the neonatal mouse ovary decreases primordial follicle formation [40]. The expression patterns of NOTCH1, NOTCH4, and JAG1 proteins were also characterized during the process of folliculogenesis and CL formation in rodent ovaries, and it was shown that they are expressed in a subset of ovarian vessels, including mature ovarian vasculature and angiogenic neovessels, in both endothelial and vascular-associated cells [41]. Given the implications for cell growth, differentiation, and death in the CL life span, we investigated the role of the notch pathway in regression of CL during pregnancy in rats. It is known that CL development and luteolysis require dynamic changes in the ovarian vascular network; thus, treatments that disrupt the vasculature can affect luteal function in rodents [42]. In addition, among the notch family members, DLL4 and its receptors NOTCH1 and NOTCH4 have been recently identified as novel factors involved in the regulation of angiogenesis [26, 27, 43]. These observations have been the rationale for the present study about the notch system in CL function by analyzing DLL4 and NOTCH1 and NOTCH4 receptors.

Our data demonstrate both that notch pathway components are present in CL from pregnant rats and that the expression of the notch genes analyzed is dynamically regulated during the PGF2α-induced luteolysis. The diffuse staining of DLL4 and the localized immunoreactivity for NOTCH1 and NOTCH4 in the nuclei of luteal cells may be a result of the activation of the notch system in situ. Diffuse DLL4 staining appears to be common in the developing vascular system and in cancerous lesions when the cell contains both the ligand and notch receptors [44–46]. PGF2α treatment decreased mRNA and protein levels of NOTCH1, NOTCH4, and DLL4 at 4 h after administration. However, the decrease in mRNA levels was reversed at 24 h. This time difference could be explained by the classic signaling pathway of PGF2α, because this hormone interacts with its receptor and activates the Ca^{2+}-dependent protein kinase C pathway, which is a rapid signal transduction mechanism [6]. Thus, the transient change in DLL4/NOTCH expression may be related to acute PGF receptor signaling at the onset of luteolytic events. Furthermore, we have examined DLL4 ligand as well as NOTCH1 and NOTCH4 receptor expression in other key stages of the CL life span (Days 12 and 21 of pregnancy and Day 4 postpartum). In our preliminary results, no significant differences were observed among groups (data not shown), suggesting that in the CL, these notch members are modulated by acute stimulation of luteolysis (e.g., PGF2α administration).

We further investigated whether notch signaling has a direct effect on luteal cell survival. To this purpose, a gamma-secretase inhibitor, DAPT, was intrabursally injected into

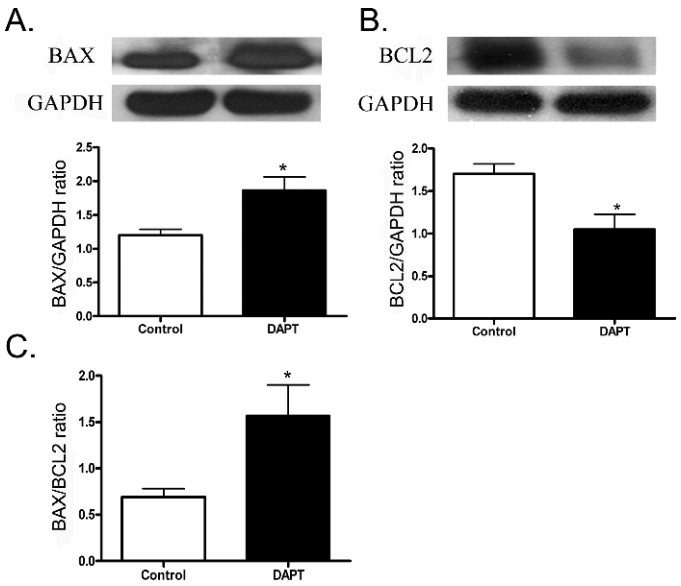

FIG. 5. Effect of DAPT treatment on BAX and BCL2 protein content in CL of pregnant rats. **A** and **B**) Western blots of BAX and BCL2 proteins visualized with an anti-BAX and BCL2 antibody (top) and densitometric quantification of BAX and BCL2 content (bottom). Bars represent the mean ± SEM *$P < 0.05$. **C**) BAX:BCL-2 ratio in the control and DAPT groups. *$P < 0.05$.

pregnant rats. A number of reports have indicated that notch can suppress apoptotic cell death [24, 47, 48]. In addition, it has been demonstrated that notch activation prevents apoptosis in breast cancer cell lines through activation of the PIK3/AKT pathway [49]. Another study reported that DAPT led to an increase in active CASP3 in both mouse insulinoma MIN6 cells and human pancreatic islets and, conversely, that gamma-secretase overactivity resulted in protection from apoptosis [50]. However, most of these studies have been performed using freshly isolated cells and/or cell lines. One novel aspect of our present work is the attempt to study the role of the notch pathway in the regulation of luteal apoptosis in vivo. The fact that circulating P_4 levels decreased after local DAPT treatment confirms that NOTCH has a direct action on luteal function. We also found that the active CASP3 and the BAX:BCL2 ratio were significantly higher after DAPT treatment, suggesting that notch signaling is involved in the apoptosis of luteal cells associated with CL regression. Taking into account that the endothelial cells compromise approximately 50% of the CL cell population, we are performing studies to determine whether DAPT is able to affect CL vasculature. In previous studies, we demonstrated the association between an increase of CASP2, CASP8, CASP9, and CASP3 activity and a decrease of luteal P_4 content in pregnant rats treated with PGF2α [51]. We also observed that intrabursal administration of the antiapoptotic agent S1P to PGF2α-treated pregnant rats prevents caspase activation associated with luteolysis and causes an increase in the vascular area density of CL [22]. In addition, we demonstrated that follicular atresia is associated with an imbalance among the BCL2 family members [52]. These data, together with those from other studies [38, 53], demonstrate that the intrinsic pathway of apoptosis is involved in follicular atresia. A similar mechanism could take part in the effect of notch on CL survival. However, a role of the apoptotic extrinsic pathway in this process cannot be discarded, because data also support the involvement of this pathway in luteal cell death [16, 54–56].

Our results suggest that notch signaling has an important role in the survival of luteal cells. It is worth pointing out that alterations of NOTCH have been associated with different human cancers, including ovarian cancer [57–59], implying that malfunction of this pathway can have significant consequences in the ovary.

In summary, we demonstrated that NOTCH1, NOTCH4, and the ligand DLL4 are expressed in small and large luteal cells of CL from pregnant rats and that the luteolytic hormone PGF2α decreases the CL mRNA and protein expression of these notch family members. In addition, NOTCH inhibition decreases circulating P_4 levels and increases proapoptotic markers, such as active CASP3 expression and the BAX:BCL2 ratio in CL from pregnant rats. These results support a luteotropic role for notch signaling to promote both luteal cell viability and steroidogenesis, and they suggest that the luteolytic hormone PGF2α might act in part by reducing the expression of some notch system components.

ACKNOWLEDGMENTS

We thank Diana Bas (IBYME-CONICET) for technical assistance and the animal care staff of the Division of Animal Resources at IBYME-CONICET, especially Victor Fernandez and Natalia Vasta, for their contributions.

REFERENCES

1. Bowen-Shauver JM, Gibori G. The corpus luteum of pregnancy. In: Leung PCK, Adashi EY (eds.), The Ovary. San Diego: Elsevier, Academic Press; 2004:201–230.
2. Stocco C, Telleria C, Gibori G. The molecular control of corpus luteum formation, function, and regression. Endocr Rev 2007; 28:117–149.
3. Stouffer RL. The function and regulation of cell populations comprising the corpus luteum during the ovarian cycle. In: Leung PCK, Adashi EY (eds.), The Ovary. San Diego: Elsevier, Academic Press; 2004:169–184.
4. Patel T, Gores GJ, Kaufmann SH. The role of proteases during apoptosis. FASEB J 1996; 10:587–597.
5. Lau IF, Saksena SK, Chang MC. Effect of indomethacin, an inhibitor of prostaglandin biosynthesis on the length of pseudopregnancy in rats and hamsters. Acta Endocrinol (Copenh) 1975; 78:343–348.
6. Niswender GD, Nett TM. Corpus luteum and its control in infraprimate species. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, vol. 1. New York: Raven Press; 1994:781–816.
7. Hansel W, Blair RM. Bovine corpus luteum: a historic overview and implications for future research. Theriogenology 1996; 45:1267–1294.
8. Milvae RA, Hinckley ST, Carlson JC. Luteotropic and luteolytic mechanisms in the bovine corpus luteum. Theriogenology 1996; 45: 1327–1349.
9. Bacci ML, Barazzoni AM, Forni M, Costerbosa GL. In situ detection of apoptosis in regressing corpus luteum of pregnant sow: evidence of an early presence of DNA fragmentation. Domest Anim Endocrinol 1996; 13: 361–372.
10. Bowen J, Keyes P, Warren J, Townson D. Prolactin-induced regression of the rat corpus luteum: expression of monocyte chemoattractant protein-1 and invasion of macrophages. Biol Reprod 1996; 54:1120–1127.
11. Gaytan F, Bellido C, Morales C, Sanchez-Criado JE. Both prolactin and progesterone in proestrus are necessary for the induction of apoptosis in the regressing corpus luteum of the rat. Biol Reprod 1998; 59:1200–1206.
12. Goodman SB, Kugu K, Chen SH, Preutthipan S, Tilly KI, Tilly JL, Dharmarajan AM. Estradiol-mediated suppression of apoptosis in the rabbit corpus luteum is associated with a shift in expression of bcl-2 family members favoring cellular survival. Biol Reprod 1998; 59:820–827.
13. Guo K, Wolf V, Dharmarajan AM, Feng Z, Bielke W, Saurer S, Friis R. Apoptosis-associated gene expression in the corpus luteum of the rat. Biol Reprod 1998; 58:739–746.
14. Hasumoto K, Sugimoto Y, Yamasaki A, Morimoto K, Kakizuka A, Negishi M, Ichikawa A. Association of expression of mRNA encoding the PGF2alpha receptor with luteal cell apoptosis in ovaries of pseudopregnant mice. J Reprod Fertil 1997; 109:45–51.
15. Telleria CM, Goyeneche AA, Cavicchia JC, Stati AO, Deis RP. Apoptosis induced by antigestagen RU486 in rat corpus luteum of pregnancy. Endocrine 2001; 15:147–155.
16. Yadav VK, Lakshmi G, Medhamurthy R. Prostaglandin F2alpha-mediated activation of apoptotic signaling cascades in the corpus luteum during apoptosis: involvement of caspase-activated DNase. J Biol Chem 2005; 280:10357–10367.
17. Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. Blood 1992; 80:879–886.
18. Reed JC. Bcl-2 and the regulation of programmed cell death. J Cell Biol 1994; 124:1–6.
19. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB. bcl-x, A bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993; 74: 597–608.
20. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999; 15: 269–290.
21. Peluffo MC, Bussmann L, Stouffer RL, Tesone M. Expression of caspase-2, −3, −8, and −9 proteins and enzyme activity in the corpus luteum of the rat at different stages during the natural estrous cycle. Reproduction 2006; 132:465–475.
22. Hernandez F, Peluffo MC, Bas D, Stouffer RL, Tesone M. Local effects of the sphingosine 1-phosphate on prostaglandin F2alpha-induced luteolysis in the pregnant rat. Mol Reprod Dev 2009; 76:1153–1164.
23. Beatus P, Lendahl U. Notch and neurogenesis. J Neurosci Res 1998; 54: 125–136.
24. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999; 284:770–776.
25. Greenwald I. LIN-12/Notch signaling: lessons from worms and flies. Genes Dev 1998; 12:1751–1762.
26. Gridley T. Notch signaling in vascular development and physiology. Development 2007; 134:2709–2718.
27. Siekmann AF, Lawson ND. Notch signaling limits angiogenic cell behavior in developing zebrafish arteries. Nature 2007; 445:781–784.
28. Hardy J, Israel A. Alzheimer's disease. In search of gamma-secretase. Nature 1999; 398:466–467.

29. Johnson J, Espinoza T, McGaughey RW, Rawls A, Wilson-Rawls J. Notch pathway genes are expressed in mammalian ovarian follicles. Mech Dev 2001; 109:355–361.
30. Bussmann LE. Prostaglandin F-2 alpha receptors in corpora lutea of pregnant rats and relationship with induction of 20 alpha-hydroxysteroid dehydrogenase. J Reprod Fertil 1989; 85:331–341.
31. Gordon WR, Arnett KL, Blacklow SC. The molecular logic of Notch signaling—a structural and biochemical perspective. J Cell Sci 2008; 121: 3109–3119.
32. Andreu C, Parborell F, Vanzulli S, Chemes H, Tesone M. Regulation of follicular luteinization by a gonadotropin-releasing hormone agonist: relationship between steroidogenesis and apoptosis. Mol Reprod Dev 1998; 51:287–294.
33. Irusta G, Parborell F, Peluffo M, Manna PR, Gonzalez-Calvar SI, Calandra R, Stocco DM, Tesone M. Steroidogenic acute regulatory protein in ovarian follicles of gonadotropin-stimulated rats is regulated by a gonadotropin-releasing hormone agonist. Biol Reprod 2003; 68:1577–1583.
34. Fischer A, Gessler M. Delta-Notch—and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. Nucleic Acids Res 2007; 35:4583–4596.
35. Mazella J, Liang S, Tseng L. Expression of Delta-like protein 4 in the human endometrium. Endocrinology 2008; 149:15–19.
36. Irusta G, Parborell F, Tesone M. Inhibition of cytochrome P-450 C17 enzyme by a GnRH agonist in ovarian follicles from gonadotropin-stimulated rats. Am J Physiol Endocrinol Metab 2007; 292:E1456–E1464.
37. Parborell F, Irusta G, Rodriguez CA, Tesone M. Regulation of ovarian angiogenesis and apoptosis by GnRH-I analogs. Mol Reprod Dev 2008; 75:623–631.
38. Tilly JL, Tilly KI, Kenton ML, Johnson AL. Expression of members of the bcl-2 gene family in the immature rat ovary: equine chorionic gonadotropin-mediated inhibition of granulosa cell apoptosis is associated with decreased bax and constitutive bcl-2 and bcl-xlong messenger ribonucleic acid levels. Endocrinology 1995; 136:232–241.
39. Flaws JA, Kugu K, Trbovich AM, DeSanti A, Tilly KI, Hirshfield AN, Tilly JL. Interleukin-1 beta-converting enzyme-related proteases (IRPs) and mammalian cell death: dissociation of IRP-induced oligonucleosomal endonuclease activity from morphological apoptosis in granulosa cells of the ovarian follicle. Endocrinology 1995; 136:5042–5053.
40. Trombly DJ, Woodruff TK, Mayo KE. Suppression of Notch signaling in the neonatal mouse ovary decreases primordial follicle formation. Endocrinology 2009; 150:1014–1024.
41. Vorontchikhina MA, Zimmermann RC, Shawber CJ, Tang H, Kitajewski J. Unique patterns of Notch1, Notch4 and Jagged1 expression in ovarian vessels during folliculogenesis and corpus luteum formation. Gene Expr Patterns 2005; 5:701–709.
42. Henkes LE, Sullivan BT, Lynch MP, Kolesnick R, Arsenault D, Puder M, Davis JS, Rueda BR. Acid sphingomyelinase involvement in tumor necrosis factor alpha-regulated vascular and steroid disruption during luteolysis in vivo. Proc Natl Acad Sci U S A 2008; 105:7670–7675.
43. Caolo V, van den Akker NM, Verbruggen S, Donners MM, Swennen G, Schulten H, Waltenberger J, Post MJ, Molin DG. Feed-forward signaling by membrane-bound ligand receptor circuit: the case of NOTCH-Delta-like 4 in endothelial cells. J Biol Chem 2010; 285:40681–40689.
44. Williams CK, Li JL, Murga M, Harris AL, Tosato G. Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. Blood 2006; 107:931–939.
45. Patel NS, Li JL, Generali D, Poulsom R, Cranston DW, Harris AL. Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. Cancer Res 2005; 65: 8690–8697.
46. Shutter JR, Scully S, Fan W, Richards WG, Kitajewski J, Deblandre GA, Kintner CR, Stark KL. Dll4, a novel Notch ligand expressed in arterial endothelium. Genes Dev 2000; 14:1313–1318.
47. Jang MS, Miao H, Carlesso N, Shelly L, Zlobin A, Darack N, Qin JZ, Nickoloff BJ, Miele L. Notch-1 regulates cell death independently of differentiation in murine erythroleukemia cells through multiple apoptosis and cell cycle pathways. J Cell Physiol 2004; 199:418–433.
48. Miele L, Osborne B. Arbiter of differentiation and death: notch signaling meets apoptosis. J Cell Physiol 1999; 181:393–409.
49. Meurette O, Stylianou S, Rock R, Collu GM, Gilmore AP, Brennan K. Notch activation induces Akt signaling via an autocrine loop to prevent apoptosis in breast epithelial cells. Cancer Res 2009; 69:5015–5022.
50. Dror V, Nguyen V, Walia P, Kalynyak TB, Hill JA, Johnson JD. Notch signaling suppresses apoptosis in adult human and mouse pancreatic islet cells. Diabetologia 2007; 50:2504–2515.
51. Peluffo MC, Stouffer RL, Tesone M. Activity and expression of different members of the caspase family in the rat corpus luteum during pregnancy and postpartum. Am J Physiol Endocrinol Metab 2007; 293:E1215–E1223.
52. Abramovich D, Parborell F, Tesone M. Effect of a vascular endothelial growth factor (VEGF) inhibitory treatment on the folliculogenesis and ovarian apoptosis in gonadotropin-treated prepubertal rats. Biol Reprod 2006; 75:434–441.
53. Parborell F, Pecci A, Gonzalez O, Vitale A, Tesone M. Effects of a gonadotropin-releasing hormone agonist on rat ovarian follicle apoptosis: regulation by EGF and the expression of Bcl-2-related genes. Biol Reprod 2002; 67:481–486.
54. Abdo M, Hisheh S, Dharmarajan A. Role of tumor necrosis factor-alpha and the modulating effect of the caspases in rat corpus luteum apoptosis. Biol Reprod 2003; 68:1241–1248.
55. Carambula SF, Pru JK, Lynch MP, Matikainen T, Goncalves PB, Flavell RA, Tilly JL, Rueda BR. Prostaglandin F2alpha- and FAS-activating antibody-induced regression of the corpus luteum involves caspase-8 and is defective in caspase-3 deficient mice. Reprod Biol Endocrinol 2003; 1: 15.
56. Kuranaga E, Kanuka H, Bannai M, Suzuki M, Nishihara M, Takahashi M. Fas/Fas ligand system in prolactin-induced apoptosis in rat corpus luteum: possible role of luteal immune cells. Biochem Biophys Res Commun 1999; 260:167–173.
57. Hopfer O, Zwahlen D, Fey MF, Aebi S. The Notch pathway in ovarian carcinomas and adenomas. Br J Cancer 2005; 93:709–718.
58. Park JT, Chen X, Trope CG, Davidson B, Shih IM, Wang TL. Notch3 overexpression is related to the recurrence of ovarian cancer and confers resistance to carboplatin. Am J Pathol 2010; 177:1087–1094.
59. Park JT, Li M, Nakayama K, Mao TL, Davidson B, Zhang Z, Kurman RJ, Eberhart CG, Shih I, Wang TL. Notch3 gene amplification in ovarian cancer. Cancer Res 2006; 66:6312–6318.