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Progesterone down-regulates SLIT/ROBO expression in mouse corpus luteum

Xuejing Zhang[a,1], Meiyan Mi[b,1], Weili Hao[a], Qiongying Fan[a], Bulang Gao[a,*]

[a] Department of Scientific Research Centre, Shijiazhuang First Hospital, HeBei Medical University, Hebei, China
[b] Department of Gynecology, Shijiazhuang Fourth Hospital, Hebei Medical University, Hebei, China

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ABSTRACT

Background: Progesterone produced by the corpus luteum (CL) is essential for preparation, implantation and maintenance of gestation. Furthermore, progesterone plays a protective role against luteolysis in rodents. It has been reported that Slit/Robo family members expressed in the CL and involved in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induced luteolysis. However, the interactions between progesterone and Slits/Robos in CL are not clear. This study was designed to examine whether or not luteolysis is regulated by the interaction of progesterone and Slits/Robos in mouse CL.

Methods: In the current study, we used Real-time PCR to identify the effect of progesterone on Slit2/Robo1 expression in cultured luteal cells *in vitro*, and the exogenous progesterone injection on mouse luteolysis and Slit/Robo expression *in vivo* was studied via Real-time PCR and Western bolt.

Results: Our *in vitro* experiment revealed that 1 μM progesterone significantly decreased Slit2/Robo1 mRNA levels at 6 h, 12 h and 24 h. Our *in vivo* experiment showed that the mRNA and protein levels of Slit2 and Robo1 decreased significantly 7 days after progesterone supplement.

Conclusion: These findings indicate that progesterone maintains CL function and resists luteolysis possibly through down-regulating Slit/Robo signaling pathway in the CL.

1. Background

The corpus luteum (CL) is a transient endocrine gland to produce progesterone, and the function of CL, including regulation of estrous cycle and maintenance of pregnancy, is largely carried out by progesterone. If pregnancy does not occur, the CL will regress to allow a new cycle to begin (Stocco et al., 2007). The CL regression includes functional regression and structural regression that is associated with luteal cells death through programmed cell death. Previous studies have shown that $PGF_{2\alpha}$ induced luteal cells apoptosis through up-regulation of the activities of caspase-9 and −3 together with DNA fragmentation in the CL (Yadav et al., 2005). Furthermore, prolactin (PRL) also as an important physiological molecule that involved in the induction of structural luteal regression (Vallcaneras et al., 2016).

Different from the promoting apoptosis function of $PGF_{2\alpha}$ and PRL on luteolysis, the progesterone plays a protective role against structural luteolysis in rodents (Stocco et al., 2007). Kuranaga et al. have shown that progesterone suppressed luteal cells apoptosis induced by PRL (Kuranaga et al., 2000). In another study, progesterone suppressed PRL-induced luteal cells apoptosis by inhibiting the expression of Fas, which is a cell surface molecule that mediates cell apoptosis by stimulation of the Fas ligand (FasL) (Nagata and Golstein, 1995). Moreover, *in vivo* administration of progesterone delayed the occurrence of DNA fragmentation in CL (Goyeneche et al., 2003).

Several studies have investigated the relationship between $PGF_{2\alpha}$ and progesterone in structural luteal regression. $PGF_{2\alpha}$ has no impact on the secretion of progesterone but stimulates the expression of 20α hydroxysteroid dehydrogenase (20αHSD) that catabolizes progesterone into the inactive progestin, 20α-dihydroprogesterone (20α-DHP) (Stocco et al., 2007). Once 20αHSD is expressed in the CL, progesterone secretion drops while the Fas expression level increases. The immune cells that express FasL thus invade the CL and activate the caspase apoptosis pathway.

The Slit/Robo family includes four transmembrane Robo (Robo1, Robo2, Robo3 and Robo4) receptors that interact with their Slit (Slit1, Slit2 and Slit3) ligands, and the Slit/Robo family plays important roles

Abbreviations: CL, corpus luteum; PGF2α, prostaglandin F2α; 20αHSD, 20α hydroxysteroid dehydrogenase; 20α-DHP, 20α-dihydroprogesterone; FasL, Fas ligand; PRL, prolactin; mPRs, progesterone membrane receptors

* Corresponding author.
E-mail address: browngao@163.com (B. Gao).
[1] These authors contributed equally to this article.

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in regulating cell fate, including migration, death, angiogenesis and organogenesis (Dickinson et al., 2008). Previous studies about Slit/Robo signal pathway mainly focus on its function on nerve cells migration and cancer cells migration/apoptosis (Andrews et al., 2008; Blockus and Chedotal, 2016). The mechanism by which the Slit/Robo signal pathway induced apoptosis have not clear, Dickinson et al. have reported that Slit binding to Robo possible also relieves inhibition of DCC by netrin-1, DCC then transmit pro-apoptosis signal in the absence of netrin-1 through activating caspase-3 and -9 (Dickinson and Duncan, 2010).

The function of Slit/Robo family in reproductive system has been investigated. Slit/Robo family members are expressed in the ovary and associated with follicle formation, oocyte survival (Dickinson et al., 2010), menstrual cycle (Duncan et al., 2010) and luteolysis by promoting apoptosis of the luteal cells (Dickinson et al., 2008). Furthermore, Zhang et al. have revealed that Slit/Robo signaling plays an important role in $PGF_{2\alpha}$-induced luteolysis by mediating the $PGF_{2\alpha}$ signaling pathway in CL (Zhang et al., 2013). However, the interaction of progesterone and Slit/Robo in luteolysis requires further investigation. Since Slit/Robo family plays an important role in luteolysis, we thus hypothesized that progesterone might decrease Slit/Robo expression to protect luteal cells from apoptosis. Classic membrane progesterone receptor (mPR) is not expressed in the CL of rodent (Park-Sarge et al., 1995), progesterone play a role in CL through glucocorticoid receptor (GR). A PR/GR antagonist, RU486 (Sugino et al., 1997), was used to examine the relationship between progesterone and Slit/Robo. Our results indicate that progesterone possibly protects CL against regression via down regulating Slit/Robo expression in cultured isolated luteal cells *in vitro* and CL tissues in *vivo*.

2. Methods

2.1. Reagents

Rabbit IgG anti-Slit2 polyclonal antibody, mouse IgM anti-Robo1 MABs and rabbit IgG anti-DCC polyclonal antibody were purchased from Abcam, Inc. (Cambridge, MA, USA), Developmental Studies Hybridoma Bank (DSHB, Iowa City, IO, USA) and bioss Inc. (Beijing, China), respectively. Progesterone and RU486 were obtained from Sigma-Aldrich (St. Louis, MO. USA). M-MLV (Moloney murine Leukemia Virus) was purchased from Promega (Mannheim, Germany). DMED/F12 and Fetal bovine serum (FBS) were obtained from GIBICO (Carlsbad, CA, USA), 0.25% pancreatin was from Amresco Inc. (Ohio, USA). Percoll was from GE Healthcare Lifesciences (Castle Hill, Australia). RIPA Lysis buffer and BCA assay reagent were purchased from Biotech Corporation (Beijing, China). PVDF (polyvinylidene difluoride) membranes were from Bio-Rad Laboratories (Hercules, CA, USA). SuperSignal West Pico kit was from Thermo Scientific (Rockford, IL, USA). In situ apoptosis analysis kit was purchased from Roche Diagnostics (Mannheim, Germany). All other reagents were purchased from Takara or TianGen Biotech CO., LTD.

2.2. Animals

21-d-old female Kunming white mouse were used in this study. These mouse were raised under standard conditions of temperature (25 ± 1 °C) and light (12-h light and 12-h dark cycle) and had free access to water and mouse chow. These animals were injected intraperitoneally with 10 IU pregnant mare serum gonadotropin (PMSG) to stimulate follicular development, which was followed 48 h later by an injection of 10 IU human chorionic gonadotropin (HCG) to promote ovulation and to obtain luteinized ovaries. These animals were then mated with castrated male mouse and examined the vaginal smears daily. Day 0 was taken as the day of HCG injection. According to previous studies (Hasumoto et al., 1997; Olofsson and Selstam, 1988), the ovaries were categorized as early (D0 to D5), mid- (D6 to D10), and late (D11 to D15) luteal phase, and the $PGF_{2\alpha}$ content and DNA fragmentation in the CL were increased at D11 of this animal model. All animal procedures were approved by the Chinese Association for Laboratory Animal Sciences.

2.3. Mouse CL collection

PMSG-HCG-synchronized ovulation and luteinization were induced and the ovaries were collected from the mouse at mid-luteal stages. The ovaries were collected and washed in phosphate buffered saline (PBS). Under sterile conditions, the CL tissues were enucleated from ovaries under a microscope with the aid of fine forceps. CL tissues were stored at −80 °C until analysis.

2.4. CL tissues isolation and luteal cells culture

Dissociation of the CL tissues and culture of luteal cells were performed as described previously (Zhang et al., 2013). Briefly, the CL tissues obtained from mouse on D6 after HCG injection were transferred to a centrifuge tube, which contained 0.1% collagenaseII. The enzyme digestion was carried out in a shaking bath (130 rpm/min) at 37 °C for 1 h. In order to obtain individual cells, the tissue pieces were further dispersed by withdrawing and expelling at the end of the digestion. The supernatant, containing individual cells, was removed to another centrifuge tube. Undissociated clumps were further incubated in 0.25% pancreatin in a shaking bath at 37 °C for 10 min. After the digestion was terminated, the cell suspension was filtered, and layered onto a 2 ml cushion of 44% percoll in a centrifuge tube and centrifuged at 400 × *g* for 30 min. The luteal cells that banded at the interface between the percoll and the medium were harvested, washed, and resuspended in DMEM/F12 media containing 10% FBS. The cells were then counted and the viability assessed using trypan blue exclusion, viability being varied from 85 to 95% in cell preparations used for the further study. For the assay, cells were plated (1.0×10^5 cells/well) onto 6-well plates for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The cells were then serum-starved for an additional 24 h and then incubated with different treatments, and the concentration of progesterone and RU486 used in luteal cells was according to the previous report (Engmann et al., 2006). Luteal cells from 20 to 25 animals were collected for each culture.

2.5. Immunohistochemistry

Frozen ovaries sections were fixed for 10 min in cold methanol, antigen retrieval was performed by microwaving the sections in 0.01 M citric acid (pH 6.0), and left to cool at room temperature (RT). All sections were then blocked with 10% normal goat serum at RT for 1 h and incubated with rabbit IgG anti-Slit2 (1:100) and mouse IgM anti-Robo1 (1:100) antibodies at 4 °C overnight. The sections were then incubated with appropriate secondary antibodies for 2 h at room temperature. Subsequently, the slides were incubated with FITC-conjugated streptavidin (1:25) for 3 h at RT. The sections were counterstained with PI (1:1000). As negative controls, the sections were processed as described above, except that the primary antibody was replaced with blocking serum containing nonspecific immunoglobulins at the same concentration. The slides were imaged using a fluorescence microscope (Leica Microsystems, Cambridge, UK).

2.6. Progesterone injection

In order to maintain the progesterone level, the animals on D6 after HCG injection were injected intraperitoneally with 2 mg progesterone and the control subjects intraperitoneally with equal volume corn oil for consecutive 7 days (n ≥ 3 animals/group). The dose of progesterone was according to the previous report (Kaufman and Rothchild, 1966). The CLs were collected for Slit2/Robo1 expression level