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ORIGINAL PAPER

The effect of GnRH antagonist cetrorelix on Wnt signaling members in pubertal and adult mouse ovaries

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Abstract

Wide application of gonadotropin-releasing hormone (GnRH) agonists and antagonists for clinical purposes determines their effects on ovarian signaling pathways. Our study aimed to determine the localization, expression levels of Wnt signaling members in the pubertal and adult mouse ovary and the impact of GnRH antagonist cetrorelix on these signaling members. 0.5 mg/kg of cetrorelix was injected to 3-and 6-week-old mice for 2 weeks. At the end of injection, ovaries from 5 (5Ce)- to 8-week (8Ce)-old mice were embedded in paraffin for immunohistochemistry and homogenized for western blot to compare with control (5C–8C) and sham groups (5S–8S). WNT2 and WNT4 showed higher expression in thecal and stromal cells in adult mouse ovaries and only WNT4 expression was affected by cetrorelix. FZD1 was localized mainly in oocytes of pubertal ovaries and granulosa cells and oocytes of adult ovaries. FZD1 was reduced by cetrorelix in pubertal ovaries. FZD4 was abundantly localized in thecal and stromal cells of all groups and protein level was not affected by cetrorelix. LRP-6 was expressed mainly in oocytes and stromal cells of pubertal, oocytes of adult ovaries and its expression was reduced by cetrorelix in adult ovaries. CTNNB1 intensity in granulosa cells was the lowest in pubertal and the highest in adult ovaries and its expression was decreased by cetrorelix in adult ovaries. Cetrorelix affected the expression of specific members of the Wnt signaling depending on the developmental stage of mice, pointing out its possible interaction with gonadotropins during pubertal and adult stages.

Keywords WNT · FZD · LRP · CTNNB1 · Ovary

Introduction

Wnt signaling is a critical regulator of cell fate determination (Lee et al. 2004), tissue differentiation, proliferation and apoptosis during embryonic development (Nusse and Varmus 1992). The members of the Wnt signaling include Wnt ligands, G protein-coupled transmembrane frizzled receptors (FZD) and low-density lipoprotein receptor-related protein (LRP) co-receptors (Tepekoy et al. 2015a). Wnt receptor activation stimulates three different pathways: the canonical Wnt/β-catenin cascade, the non-canonical planar cell polarity pathway and the Wnt/Ca^{2+} pathway (Clevers 2006). The canonical Wnt signaling cascade which signals through β-catenin (CTNNB1) to regulate target gene expression is the most well-studied Wnt pathway (Roose et al. 1998). This signal becomes activated after the interaction between a member of FZD or LRP family and a Wnt ligand (Pinson et al. 2000). As a result of this interaction, the multiprotein complex of adenomatous polyposis coli (APC), glycogen synthase kinase (GSK-3B) and axin is disrupted. This multiprotein complex is normally responsible for phosphorylation and degradation of cytoplasmic CTNNB1 via disheveled (Dsh) and disruption of this complex avoids degradation of CTNNB1 (Stapp et al. 2014). Stabilized cytoplasmic CTNNB1 accumulates and is translocated to the nucleus and it binds T cell factor/lymphoid enhancer-binding protein (TCF/LEF) to regulate transcriptional regulation (Bienz and Clevers 2003).

Wnt signaling members take part in female reproduction as from the regulation of female gonad formation during

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embryogenesis (Vainio et al. 1999). Wnt signaling has also been found to be remarkable for proper functioning of adult ovaries (Boyer et al. 2010). Characterization of specific Wnt molecules during folliculogenesis has been focused primarily on WNT2 and WNT4 in mice, rats, and humans (Ricken et al. 2002; Wang et al. 2009). As follicles grow to the preovulatory stage, the expression of negative regulators of Wnt signaling such as sFRP4 decreases (Maman et al. 2011), while Wnt receptors such as FZD1 become more significant (Hsieh et al. 2002). Conditionally, knock out studies reveal that *WNT4* is required for antral follicle development and is associated with steroidogenic genes such as *Star*, *Cyp11a1*, and *Cyp19* (Boyer et al. 2010). Meiotic resumption of the oocytes is also suggested to be controlled by Wnt signaling (Spate et al. 2014). Wnt receptor FZD1 is involved in oocyte maturation process as well as cumulus cell function (Lapointe et al. 2012). Ovulatory capability is controlled by CTNNB1, the effector of Wnt signaling (Usongo et al. 2012).

Besides regulating follicular growth, ovulation, and steroidogenesis, Wnt signaling is also involved in the preservation of follicular reserve in the ovary. *WNT4* deficiency causes premature ovarian failure related to cell polarity defects in the follicle and reduced expression of the CX43 and antimullerian hormone (*Amh*) (Prunskaite-Hyyrylainen et al. 2014).

Since the events regarding ovarian function are under the control of gonadotropins, the effect of Wnt signaling in ovarian function is linked to the actions of these hormones (Parakh et al. 2006; Fan et al. 2010). Wnt effector CTNNB1 is an inducer of follicle-stimulating hormone (FSH)-regulated processes such as aromatase expression in cultured rat granulosa cells (Parakh et al. 2006), stimulation of *CYP19A1* in human granulosa cell lines (Parakh et al. 2006) as well as in primary mouse granulosa cell cultures (Hernandez Gifford et al. 2009). CTNNB1 is also associated with FSH-induced follicular growth and decreases the follicle atresia (Fan et al. 2010). In addition to folliculogenesis, Wnt signaling was reported to have roles in dominant follicle selection under the control of FSH (Gupta et al. 2014).

Gonadotropin-releasing hormone (GnRH) agonists and antagonists have been widely used in the treatment of gynecologic diseases, such as endometriosis, uterine fibroids, dysmenorrhea, and hormone-dependent tumors as well as in assisted reproductive technology (ART) in adults (Saatli et al. 2009), in addition to their usage in individuals in puberty for the treatment of delayed or precocious puberty (Wu et al. 2005). Depending on their wide application for clinical purposes, it is critical to determine the effects of GnRH agonists and antagonists on signaling pathways related to ovarian function. Though Wnt signaling is associated with the functional processes in the ovary (Hsieh et al. 2002; Parakh et al. 2006; Prunskaite-Hyyrylainen et al. 2014); the direct effects of gonadotropin inhibitors on Wnt signaling members are not very well defined, especially by in vivo experiments. The current study aimed to determine the distribution and expression levels of Wnt ligands WNT2 and WNT4, Wnt receptors FZD1, FZD4 and LRP-6, and Wnt effector CTNNB1 in pubertal and adult mouse ovaries before and after GnRH antagonist cetrorelix injection.

Materials and methods

Animals and tissue processing

Female ($n = 36$, 6 for each group) Balb/C intact mice supplied by Animal Care and Usage Committee of Akdeniz University were maintained under standard laboratory conditions (21 ± 1 °C; ambient temperature; controlled light/dark conditions, 14L:10D) and were given food and water ad libitum. The experimental protocol was approved by the Animal Ethics Committee of Akdeniz University, Turkey (2011.11.01).

Experimental procedures were conducted on six different animal groups including pubertal and adult mice. 0.5 mg/kg of intravenous cetrorelix (Sigma-Aldrich Co. LLC, Steinheim, Germany) injection was applied to pubertal groups of 3-week-old female mice that are at the beginning of the puberty. The injections were made every 24 h for 2 weeks. At the end of the injections, the mice became 5 weeks old and regarded as the 5-week cetrorelix group (5Ce). The second group of 3-week-old mice was injected 5% methanol as sham group for 2 weeks and regarded as 5-week sham group (5S). Another group of uninjected 5-week-old mice was regarded as 5-week control group (5C). 0.5 mg/kg of intravenous cetrorelix injection was also applied to adult groups of 6-week-old female mice. The same injection protocol with pubertal mice was applied to adult mice and at the end of injections, the mice were regarded as 8Ce and 8S groups. 8C control group of adult mice was also included.

Ovaries were harvested from 5C, 5S, 5Ce, 8C, 8S and 8Ce groups of female mice and the left ovary samples were fixed by immersion in Bouin's fixative (75 mL of a saturated aqueous solution of picric acid [Sigma-Aldrich Co. LLC, Steinheim, Germany], 25 mL of formalin [Merck, NJ, USA] and 5 mL of glacial acetic acid [Sigma-Aldrich Co. LLC, Steinheim, Germany]) at room temperature for 4 h. Then tissues were dehydrated through a graded series of ethanol, cleared with xylene and finally embedded in paraffin wax for immunohistochemical investigations. Right ovary samples were preserved in − 80 °C freezer until they were processed for protein extraction for western blotting.

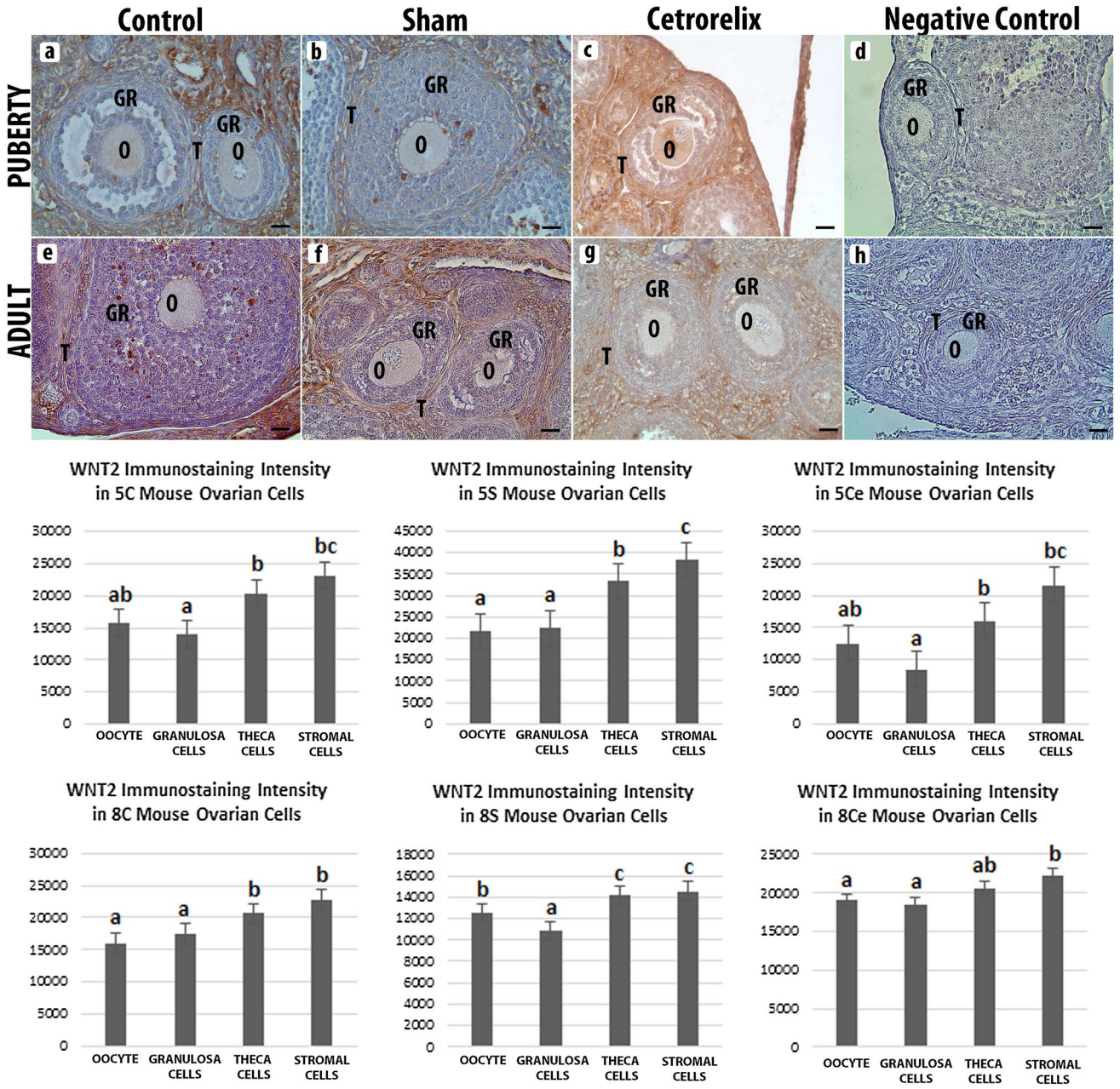


Fig. 1 WNT2 immunostainings and graphics of mathematical values of ImageJ evaluations in 5-week-old pubertal (**a**–**d**) and 8-week-old adult (**e**, **h**) mouse ovaries. **a** Ovary section from the control group (5C) of mouse with no injection. **b** Ovary section from the sham group of mouse (5S) injected with 5% methanol for 2 weeks starting from 3 weeks of age. **c** Ovary section from the cetrorelix group of mouse (5Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 3 weeks of age. **d** Representative negative control section. **e** Ovary section from the control group (8C) of mouse with no injection. **f** Ovary section from the sham group of mouse (8S) injected with 5% methanol for 2 weeks starting from 6 weeks of age. **g** Ovary section from the cetrorelix group of mouse (8Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 6 weeks of age. **h** Representative negative control section. Counterstain: hematoxylin. *GR* granulosa cell, *O* oocyte, *T* theca cell, *ST* stromal cell. Scale bars: 20 µm. Different letters above data bars mark statistical significance ($P < 0.05$) between different cell types in the same group of ovaries (one-way ANOVA, Holm–Sidak method)

Immunohistochemistry

Paraffin-embedded samples were cut into 5-µm sections and placed on Superfrost Ultra Plus Adhesion Slides (Thermo Sci., Rockford, IL, USA). After deparaffinization, the slides were boiled in citrate buffer (pH: 6.0) for 10 min for antigen retrieval and cooled for 20 min at room temperature. Then the slides were immersed in 3% hydrogen peroxide

for 20 min to block endogenous peroxidase. They were then incubated in a humidified chamber with UltraV block (Labvision, Fremont, CA, USA) for 7 min at room temperature. Excess serum was drained and the slides were incubated with rabbit polyclonal primary antibodies WNT2 (sc-50361) (1:300), WNT4 (sc-13962) (1:300), FZD4 (sc-135108) (1:300), LRP-6 (sc- 15399) (1:200) and CTNNB1 (sc-7199) (1:200) (Santa Cruz, CA, USA) and goat polyclonal primary antibody FZD1 (sc-30428) (1:300) (Santa Cruz, CA, USA) at the indicated dilutions in a humidified chamber at 4 °C overnight. Negative controls were performed by replacing the primary antibody with phosphate-buffered saline (PBS). The slides were washed three times for 5 min with PBS and then incubated with biotinylated goat anti-rabbit and horse anti-goat secondary antibodies (Vector Lab. Inc., Burlingame, CA, USA) at 1:500 dilution for 30 min at room temperature. Then the slides were washed with PBS and the peroxidase activity was visualized with 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich Co. LLC, Steinheim, Germany) for 3–5 min. The slides were counterstained with hematoxylin, dehydrated, mounted in Entellan (Merck, NJ, USA) and examined by light microscopy.

The evaluation of the immunohistochemical stainings of Wnt signaling members in mouse ovaries was accomplished by the ImageJ program (National Institutes of Health, Bethesda, MD, USA) (Tepekoy et al. 2015b). Briefly, the staining density of each cell was determined through the integrated density, area, and the mean gray value for the cell. For each cell type, the intensity of 15 cells from the same area was analyzed and the mean value for these cells was determined. For each slide, six different areas were evaluated using the images at 400× magnification. For the latter analysis, the mean values of oocytes, granulosa, theca, and stromal cell immunostaining density were used for each group of ovaries.

SDS-polyacrylamide gel electrophoresis and western blotting

To perform protein extraction and immunoblot analysis of WNT2, WNT4, FZD1, FZD4, LRP-6, and CTNNB1,k the tissues were weighed and put into homogenization buffer (10 mM Tris–HCL, 1 mM EDTA, 2.5% SDS, 1 mM phenylmethylsulfonylfluoride, 1 µg/mL leupeptin) supplemented with CompleteR protease inhibitor cocktail (Boehringer, Mannheim, Germany). After homogenization, samples were centrifuged at 10,000×*g* for 10 min. Supernatants were collected and stored at − 80 °C. The protein concentration was determined by Lowry assay (Lowry et al. 1951) and 50 µg protein was applied per lane. Prior to electrophoresis, samples were boiled for 5 min at 95 °C. Samples were subjected to SDS-polyacrylamide gel electrophoresis and then were transferred onto nitrocellulose membranes (Amersham Pharmacia, Piscat-away, NJ, USA) in a buffer containing 0.2 mol/l glycine, 25 mMTris, and 20% methanol overnight. The successful transfer was confirmed by Ponceau S (Sigma-Aldrich Co. LLC, Steinheim, Germany) staining of the blots. The membranes were blocked for 1 h with 5% non-fat dry milk (BioRad, Hercules, CA, USA) and 0.1% Tween 20 (Sigma-Aldrich Co. LLC, Steinheim, Germany) in 0.14 mol/l Tris-buffered saline (TBS), pH 7.2–7.4, at 4 °C. Blotting membranes were incubated overnight at 4 °C with primary antibodies purchased from Santa Cruz, CA, USA. The rabbit polyclonal antibodies for WNT2 (sc-50361) (1:1000) (You et al. 2004), WNT4 (sc-13962) (1:1000) (Salker et al. 2018), FZD4 (sc-135108) (1:500) (Abdul-Ghani et al. 2011), LRP-6 (sc-15399) (1:1000) (Wei et al. 2006) and CTNNB1 (sc-7199) (1:500) (Furukawa et al. 2017), and goat polyclonal antibody for FZD1 (sc-30428) (1:500) (Honda et al. 2010) were previously shown to detect the related proteins properly and were applied at the dilutions specified in the parenthesis. After washing steps, the membranes were further incubated with goat anti-rabbit IgG–horseradish peroxidase conjugate (BioRad, Her-cules, CA, USA) and horse anti-goat IgG–horseradish peroxidase conjugate (BioRad, Her-cules, CA, USA) diluted 1:3000 for 1 h at room temperature. Immunolabeling was visualized using the chemiluminescence-based SuperSignal CL HRP Substrate System (Pierce, Rockford, IL, USA) and the membranes were exposed to Hyperfilm (Amersham Pharmacia).

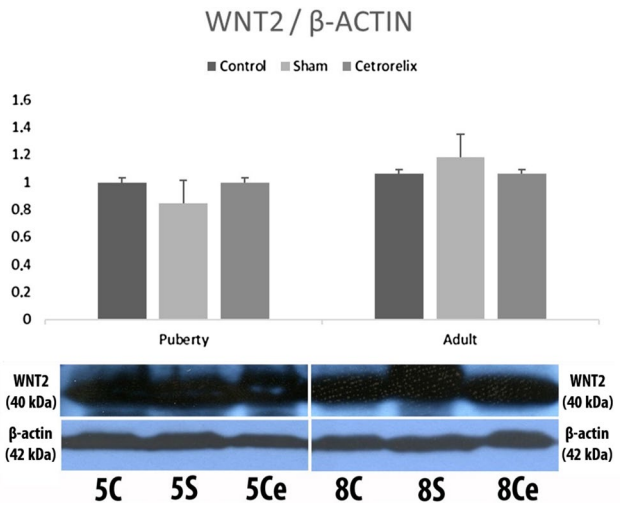


Fig. 2 Western blot bands and graphics of mathematical values of ImageJ evaluations of WNT2 protein expressions in postnatal mouse ovaries (one-way ANOVA, Holm–Sidak method)

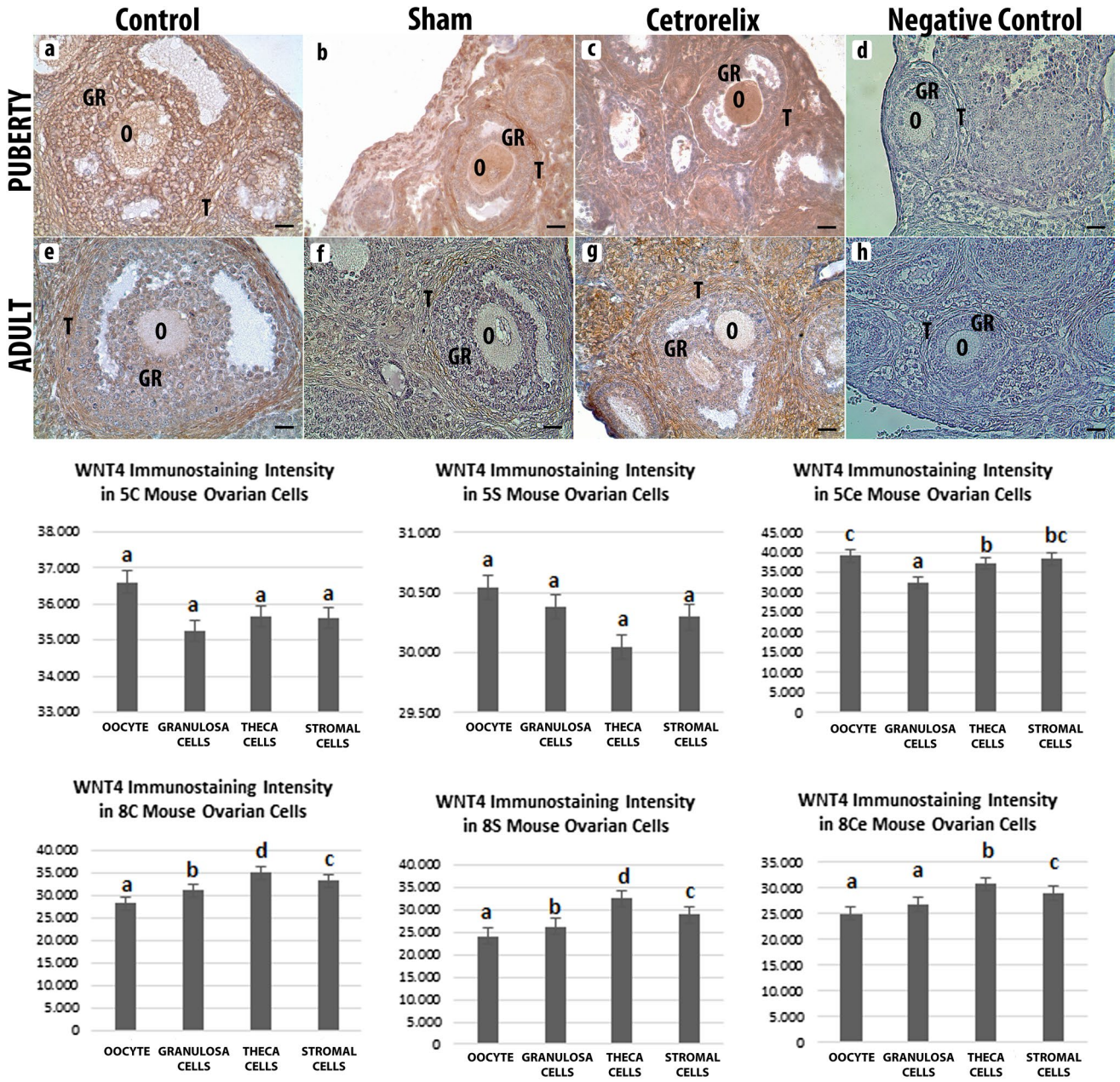


Fig. 3 WNT4 immunostainings and graphics of mathematical values of ImageJ evaluations in 5-week-old pubertal (**a**–**d**) and 8-week-old adult (**e**, **h**) mouse ovaries. **a** Ovary section from the control group (5C) of mouse with no injection. **b** Ovary section from the sham group of mouse (5S) injected with 5% methanol for 2 weeks starting from 3 weeks of age. **c** Ovary section from the cetrorelix group of mouse (5Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 3 weeks of age. **d** Representative negative control section. **e** Ovary section from the control group (8C) of mouse with no injection. **f** Ovary section from the sham group of mouse (8S) injected with 5% methanol for 2 weeks starting from 6 weeks of age. **g** Ovary section from the cetrorelix group of mouse (8Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 6 weeks of age. **h** Representative negative control section. Counterstain: hematoxylin. *GR* granulosa cell, *O* oocyte, *T* theca cell, *ST* stromal cell. Scale bars: 20 µm. Different letters above data bars mark statistical significance ($P < 0.05$) between different cell types in the same group of ovaries (one-way ANOVA, Holm–Sidak method)

β-Actin antibody (1:5000 dilution) (Abcam, Cambridge, UK) was used as an internal control for each blotting to confirm the equal loading of the samples. The bands were quantified using NIH image analysis software (ImageJ Version 1.36b, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The data obtained from ImageJ analysis of immunohistochemistry stainings and western blot bands were analyzed with non-parametric ANOVA on ranks (Kruskal–Wallis test) and parametric one-way ANOVA, Holm–Sidak method. The values were presented as mean ± SEM. Statistical calculations were performed using Sigma Stat for Windows, version 3.0 (Jandel Scientific Corp. San Rafael, CA, USA). Statistical significance was defined as $P < 0.05$.

Results

The effect of cetrorelix on localization and expression levels of Wnt ligands (WNT2 and WNT4) in pubertal and adult mouse ovaries

Immunohistochemistry findings revealed that WNT2 was localized in the cytoplasm of both follicular (oocyte, granulosa, and theca cells) and stromal cells of mouse ovaries in both pubertal and adult control, sham and cetrorelix groups (Fig. 1). WNT2 immunostaining intensity was found to be higher in thecal and stromal cells of all groups when compared to the oocytes and granulosa cells according to the ImageJ assessment (Fig. 1). As it was determined by western Blot (WB) findings, total WNT2 expression in both pubertal and adult mouse ovaries was not affected by cetrorelix injection significantly (Fig. 2).

WNT4 immunolocalization was detected in both follicular and stromal cells in all groups of pubertal and adult mouse ovaries (Fig. 3). Though immunostaining intensity of WNT4 was found to be similar in different cells of pubertal control (5C) and pubertal sham (5S) group mouse ovaries, in cetrorelix-injected pubertal mouse ovaries (5Ce), WNT4 immunostaining intensity in theca cells was lower compared to the oocytes, granulosa cells and stromal cells (Fig. 3). In adult control (8C) and sham (8S) mouse ovaries, WNT4 intensity showed a difference in all cell types. In both groups, theca cells had the highest level of WNT4 intensity followed by stromal cells, granulosa cells, and oocytes, respectively. In cetrorelix-injected adult mouse (8Ce) ovaries, WNT4 intensity was also higher in theca cells followed by stromal cells, granulosa cells and oocytes. In this group, WNT4 density was not significantly different in oocytes and granulosa cells (Fig. 3).

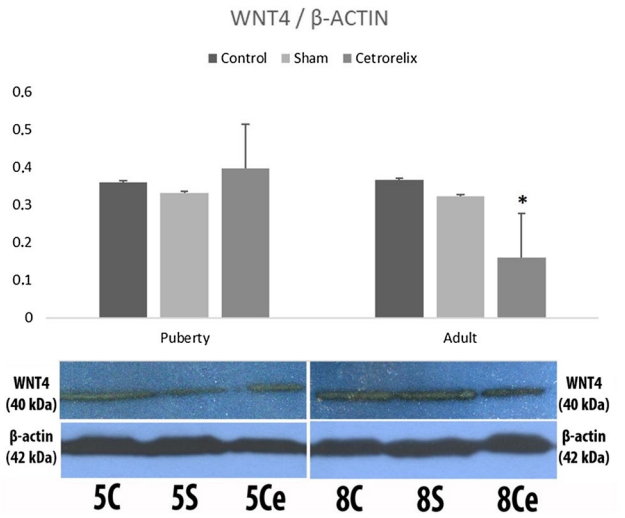


Fig. 4 Western blot bands and graphics of mathematical values of ImageJ evaluations of WNT4 protein expressions in postnatal mouse ovaries. Asterisk marks statistical significance ($P < 0.05$) between adult groups (one-way ANOVA, Holm–Sidak method)

WB findings revealed that the total WNT4 protein level in 8Ce ovaries was significantly lower when compared to 8C and 8S groups of mice. There was not a significant difference in WNT4 expression between pubertal groups (Fig. 4).

The effect of cetrorelix on localization and expression levels of Wnt receptors (FZD1, FZD4, and LRP6) in pubertal and adult mouse ovaries

FZD1 immunolocalization was detected in the cytoplasm of both follicular and stromal cells of mouse ovaries in both pubertal and adult control, sham and cetrorelix groups (Fig. 5). In pubertal mouse ovaries (5C and 5S) FZD1 was localized more intensely in oocytes compared to the other cells and granulosa cells had the lowest FZD1 intensity. However, in 5Ce mouse ovaries, FZD1 immunostaining intensity was the highest in granulosa cells compared to oocytes, theca and stroma cells (Fig. 5). In adult mouse ovaries (5C and 5S), FZD1 immunostaining level was higher in oocytes and granulosa cells compared to theca and stroma cells, whereas in 8Ce group, theca and stromal cells had

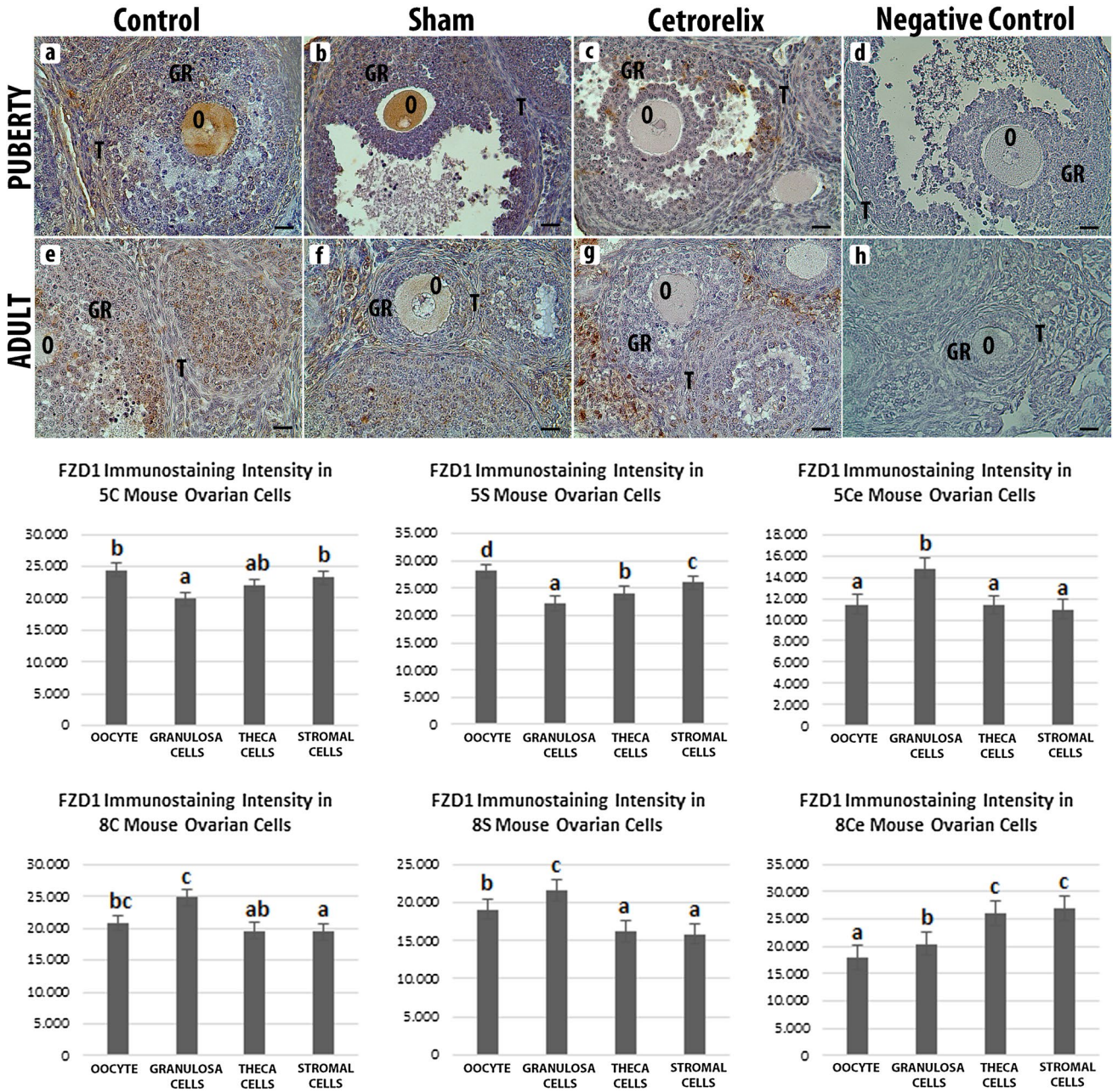


Fig. 5 FZD1 immunostainings and graphics of mathematical values of ImageJ evaluations in 5-week-old pubertal (**a**–**d**) and 8-week-old adult (**e**, **h**) mouse ovaries. **a** Ovary section from the control group (5C) of mouse with no injection. **b** Ovary section from the sham group of mouse (5S) injected with 5% methanol for 2 weeks starting from 3 weeks of age. **c** Ovary section from the cetrorelix group of mouse (5Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 3 weeks of age. **d** Representative negative control section. **e** Ovary section from the control group (8C) of mouse with no injection. **f** Ovary section from the sham group of mouse (8S) injected with 5% methanol for 2 weeks starting from 6 weeks of age. **g** Ovary section from the cetrorelix group of mouse (8Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 6 weeks of age. **h** Representative negative control section. Counterstain: hematoxylin. *GR* granulosa cell, *O* oocyte, *T* theca cell, *ST* stromal cell. Scale bars: 20 µm. Different letters above data bars mark statistical significance ($P < 0.05$) between different cell types in the same group of ovaries (one-way ANOVA, Holm–Sidak method)

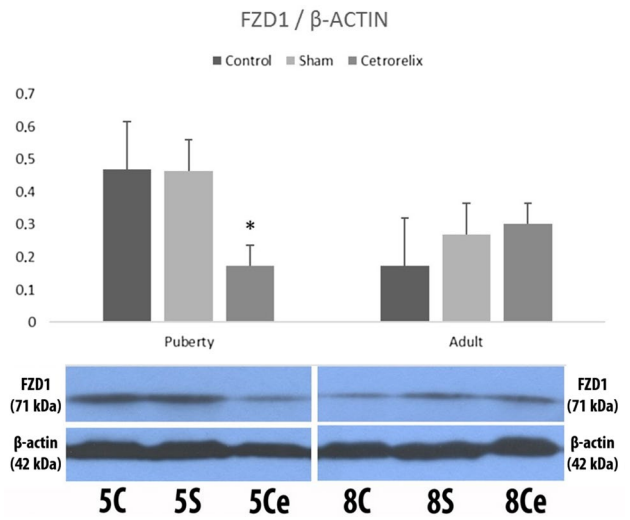


Fig. 6 Western blot bands and graphics of mathematical values of ImageJ evaluations of FZD1 protein expressions in postnatal mouse ovaries. Asterisk marks statistical significance ($P < 0.05$) between pubertal groups (one-way ANOVA, Holm–Sidak method)

the highest level of FZD1 intensity (Fig. 5). WB findings revealed that cetrorelix injection resulted in a decrease in FZD1 levels in pubertal mouse ovaries while it did not affect FZD1 expression in adult mouse ovaries (Fig. 6).

FZD4 immunolocalization was detected in the cytoplasm of both follicular and stromal cells of mouse ovaries in both pubertal and adult control, sham and cetrorelix groups (Fig. 7). FZD4 was abundantly localized in thecal and stromal cells of all groups when compared to the oocytes and granulosa cells according to the ImageJ assessment (Fig. 7). Only in the 8Ce group, there was a significant difference between theca and stroma cells (Fig. 7). According to the WB findings, FZD4 protein levels were similar in all groups of pubertal and adult mouse ovaries (Fig. 8).

LRP-6 immunolocalization was detected in the cytoplasm of both follicular and stromal cells of mouse ovaries in both pubertal and adult control, sham and cetrorelix groups (Fig. 9). LRP-6 intensity was found to be higher in oocytes and stromal cells when compared to granulosa and theca cells in 5C and 5S groups (Fig. 9). In 5Ce group mouse ovaries, LRP6 intensity levels were significantly higher in oocytes and granulosa cells when compared to theca and stromal cells (Fig. 9). In adult mouse ovaries, LRP-6 was shown to have the highest intensity level in oocytes in all groups according to the ImageJ assessment (Fig. 9). WB findings revealed that the expression of total LRP-6 was significantly decreased in ovaries of cetrorelix-injected adult (8Ce) group when there was no significant difference between pubertal mouse ovaries (Fig. 10).

The effect of cetrorelix on localization and expression levels of Wnt effector CTNNB1 in pubertal and adult mouse ovaries

CTNNB1 immunolocalization was detected in the cytoplasm of both follicular and stromal cells of mouse ovaries in both pubertal and adult controls, sham and cetrorelix groups (Fig. 11). CTNNB1 immunostaining intensity level was lower in granulosa cells compared to oocytes, theca and stromal cells in 5C and 5S mouse ovaries, whereas in 5Ce group, CTNNB1 intensity levels were similar in oocytes and granulosa cells and these levels were significantly lower when compared to theca and stroma cells (Fig. 11). In adult mouse ovaries (8C and 8S), CTNNB1 had the highest intensity level in granulosa cells, and in 8Ce group, in addition to granulosa cells, theca and stromal cells also had a higher CTNNB1 level when compared to the oocytes (Fig. 11).

WB results showed that CTNNB1 levels were similar in all pubertal groups, while cetrorelix injection in adult groups resulted in a significant decrease in CTNNB1 expression (Fig. 12).

Discussion

The gonadotropin action is dependent on downstream signaling pathways and a diverse set of intraovarian factors that are expressed in a cell-specific manner (Richards et al. 2002), one of which is Wnt signaling molecules. Though the contributions of Wnt signaling in folliculogenesis have been uncovered in recent years (Wang et al. 2009), much remains unknown about the involvement of Wnt signaling in ovarian function and the effect of gonadotropins on its activation. In the current study which is conducted on both pubertal and adult mice, the effects of GnRH antagonist cetrorelix injection on the localizations and protein levels of Wnt signaling members were investigated.

Data obtained from immunostainings in the current study revealed that the distribution of Wnt members showed a difference in mouse ovaries. Though WNT2 was formerly detected in granulosa cells throughout follicular development (Ricken et al. 2002; Wang et al. 2009, 2010), according to the results of our study, in addition to its localization in granulosa cells and oocytes, WNT2 was localized dominantly in thecal and stromal cells in all pubertal and adult groups. WNT4 ligand intensity was similar in pubertal mouse ovarian cells, whereas in adult mouse ovaries, WNT4 intensity was higher in thecal and stromal cells compared to the oocytes and granulosa cells. Previous studies also showed that WNT4 was expressed in ovarian stromal cells during embryogenesis (Vainio et al. 1999) and in

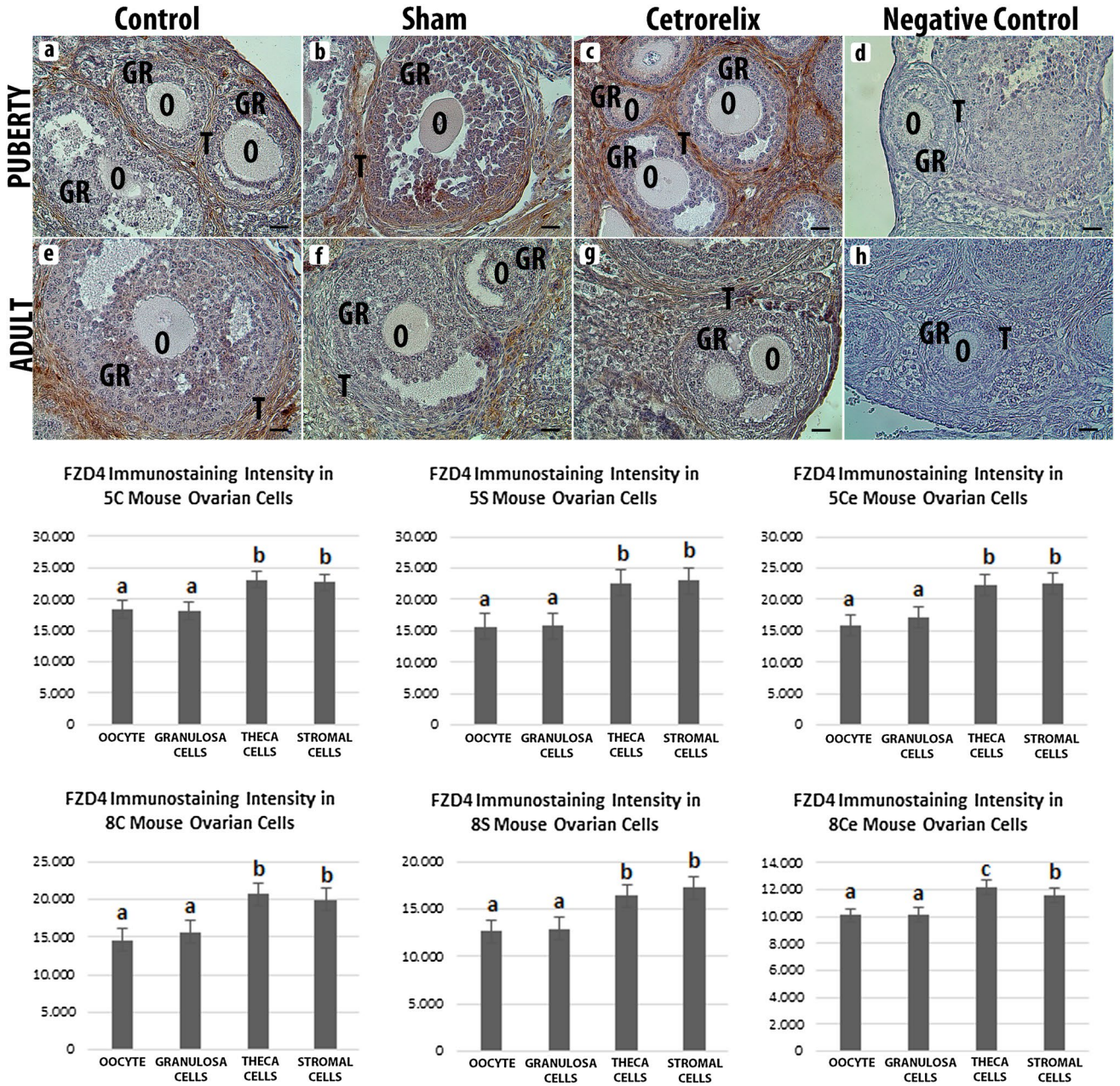


Fig. 7 FZD4 immunostainings and graphics of mathematical values of ImageJ evaluations in 5-week-old pubertal (**a**–**d**) and 8-week-old adult (**e**, **h**) mouse ovaries. **a** Ovary section from the control group (5C) of mouse with no injection. **b** Ovary section from the sham group of mouse (5S) injected with 5% methanol for 2 weeks starting from 3 weeks of age. **c** Ovary section from the cetrorelix group of mouse (5Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 3 weeks of age. **d** Representative negative control section. **e** Ovary section from the control group (8C) of mouse with no injection. **f** Ovary section from the sham group of mouse (8S) injected with 5% methanol for 2 weeks starting from 6 weeks of age. **g** Ovary section from the cetrorelix group of mouse (8Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 6 weeks of age. **h** Representative negative control section. Counterstain: hematoxylin. *GR* granulosa cell, *O* oocyte, *T* theca cell, *ST* stromal cell. Scale bars: 20 µm. Different letters above data bars mark statistical significance ($P < 0.05$) between different cell types in the same group of ovaries (one-way ANOVA, Holm–Sidak method)

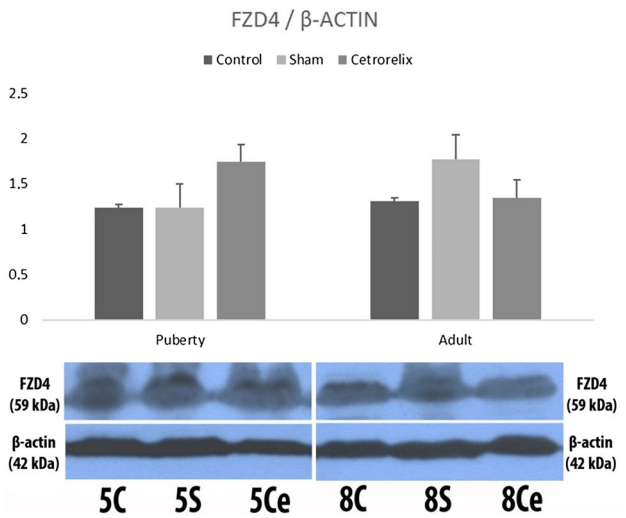


Fig. 8 Western blot bands and graphics of mathematical values of ImageJ evaluations of FZD4 protein expressions in postnatal mouse ovaries (one-way ANOVA, Holm–Sidak method)

postnatal pre-granulosa cells during the early folliculogenesis in the mouse (Hsieh et al. 2002) and also in murine cumulus–oocyte complexes (COCs) (Hernandez-Gonzalez et al. 2006). The stromal cells are known to be crucial for the development of pre-antral follicles and the interaction between stromal and granulosa cells is indispensable for follicle development (Salker et al. 2018). Thus, WNT2 and WNT4 might have roles in the functions of these cells during folliculogenesis.

In the current study, the immunolocalizations of different FZD receptors also varied in mouse ovaries. FZD1 was mainly localized in the oocytes of pubertal mouse ovaries, whereas granulosa cells in addition to oocytes showed higher FZD1 intensity levels in adult mouse ovaries. FZD1 was detected in COCs and granulosa cells of large ovulatory follicles prior to ovulation in previous studies (Hsieh et al. 2002; Hernandez-Gonzalez et al. 2006). Thus, FZD1 might be an important factor for the resumption of meiotic maturation of the oocytes.

Though FZD4 was formerly detected in follicular granulosa cells in rats (Hsieh et al. 2002; Richards et al. 2002), the findings of the current study revealed that FZD4 was dominantly localized in stromal and thecal cells of both pubertal and adult mouse ovaries. LRP-6 was also previously found to be present in granulosa cells (Harwood et al. 2008). However, in our study, it was found that the LRP-6 receptor staining intensity was higher in oocytes and stromal cells in pubertal groups, whereas in adult mouse ovaries only the oocytes had the highest LRP-6 intensity. Since oocyte maturation and meiotic competence were found to be related to Wnt signaling in recent studies (You et al. 2004), LRP-6 might also be included in this process.

The results of the current study revealed that canonical Wnt signaling effector CTNNB1 intensity was the lowest in granulosa cells of pubertal mouse ovaries, however, it was found to be more prominent in the granulosa cells of adult mouse ovaries pointing out possible roles of canonical signaling in granulosa cell function.

Previous studies revealed that the mechanism of action of GnRH agonists and antagonists on gonadotropin secretion is divergent (Horvath et al. 2002). The stimulatory effect of GnRH agonists on pituitary FSH and LH secretion results in an increase followed by a maximal decrease of about 93% in FSH and 93% in LH immunoreactive levels, respectively, and ovarian quiescence (Matikainen et al. 1992). In contrast, GnRH antagonists affect the GnRH receptor through competitive inhibition and significantly decrease gonadotropin secretion (Matikainen et al. 1992). It was found that the inhibitory effect of luteinizing hormone-releasing hormone (LHRH) antagonist on serum gonadotropins and pituitary GnRH receptors was greater than that of GnRH agonists in rodents (Horvath et al. 2002).

Since GnRH receptors were also characterized in ovaries (Kakar, Musgrove et al. 1992), GnRH agonist or antagonist injection might also directly affect the functional dynamics in ovaries regulated through gonadotropins. GnRH agonists and antagonists are known to affect signal mechanisms related to ovarian function (Khalaf et al. 2010). Thus, Wnt signaling in the ovary might also be one of these pathways affected by cetrorelix.

In recent studies, Wnt signaling members were found to take part in granulosa cell functions and suggested to modulate the effects of gonadotropins on these cells (Abedini et al. 2016). LH was found to induce the expression of several Wnt signaling effectors in both granulosa and cumulus cells, including WNT4, the receptors FZD1 and -4, and also sFRP-2 and -4 (Edson et al. 2009). According to the results of the current study, cetrorelix injection, which is known to result in a decrease in LH levels (Matikainen et al. 1992), affected the protein levels of particular Wnt signaling members in mouse ovaries. It was previously shown that WNT2 mRNA expression was increased in response to FSH-treatment in cultured bovine granulosa cells (Castanon et al. 2012) and WNT2 levels were elevated in human cumulus cells collected after gonadotropin stimulation (Wang et al. 2009). However, in our study in which we evaluated gonadotropin antagonist actions at the protein level in vivo,

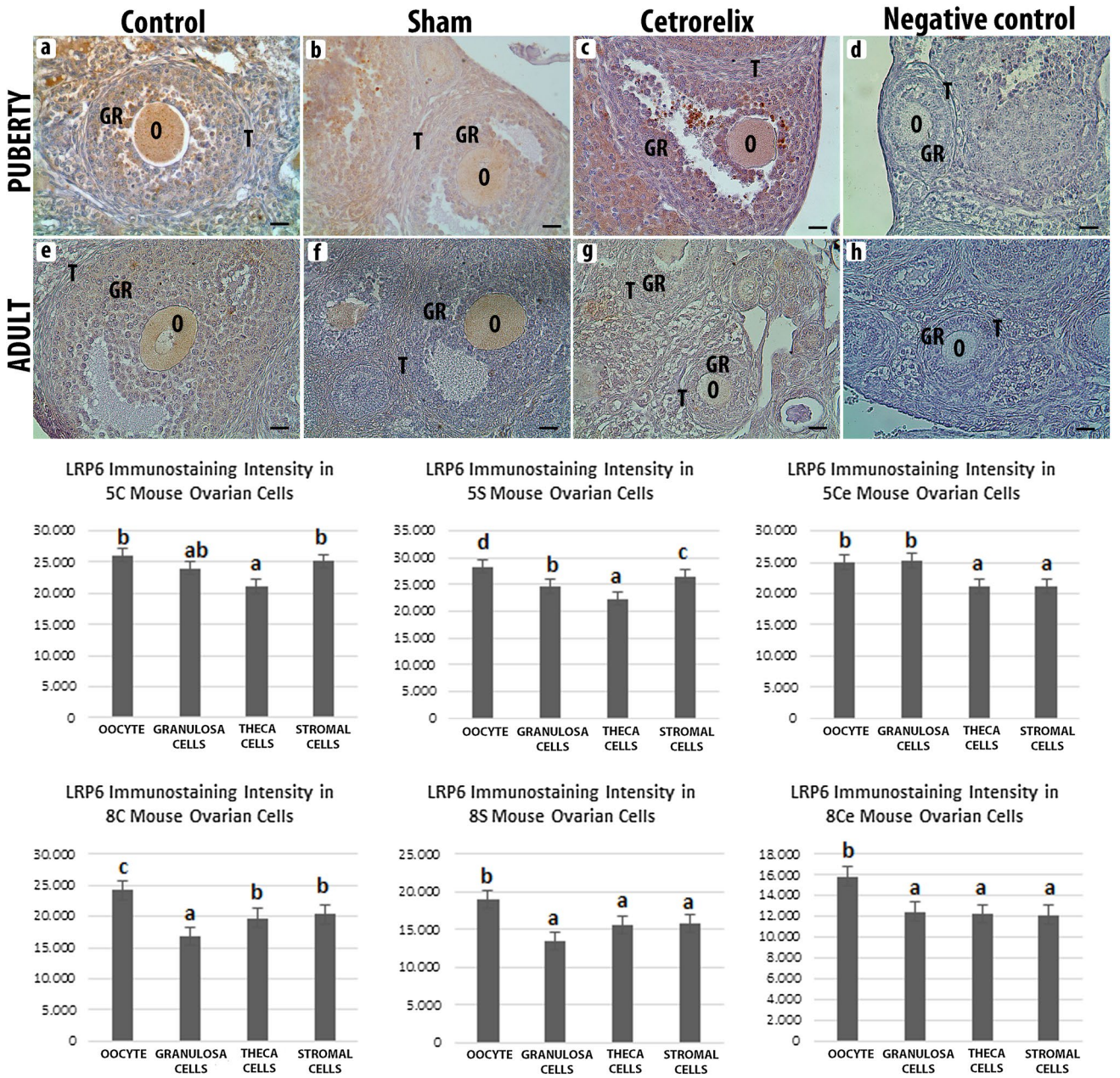


Fig. 9 LRP-6 immunostainings and graphics of mathematical values of ImageJ evaluations in 5-week-old pubertal (**a**–**d**) and 8-week-old adult (**e**, **h**) mouse ovaries. **a** Ovary section from the control group (5C) of mouse with no injection. **b** Ovary section from the sham group of mouse (5S) injected with 5% methanol for 2 weeks starting from 3 weeks of age. **c** Ovary section from the cetrorelix group of mouse (5Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 3 weeks of age. **d** Representative negative control section. **e** Ovary section from the control group (8C) of mouse with no injection. **f** Ovary section from the sham group of mouse (8S) injected with 5% methanol for 2 weeks starting from 6 weeks of age. **g** Ovary section from the cetrorelix group of mouse (8Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 6 weeks of age. **h** Representative negative control section. Counterstain: hematoxylin. *GR* granulosa cell, *O* oocyte, *T* theca cell, *ST* stromal cell. Scale bars: 20 µm. Different letters above data bars mark statistical significance ($P < 0.05$) between different cell types in the same group of ovaries (one-way anova, Holm–Sidak method)

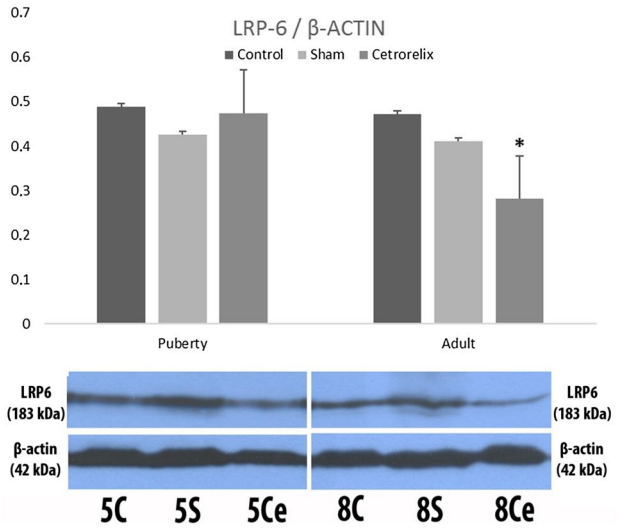


Fig. 10 Western blot bands and graphics of mathematical values of ImageJ evaluations of LRP-6 protein expressions in postnatal mouse ovaries. Asterisk marks statistical significance ($P < 0.05$) between adult groups (one-way ANOVA, Holm–Sidak method)

WB findings revealed that WNT2 and FZD4 levels were not affected by cetrorelix injection both in pubertal and adult mouse ovaries.

In the current study, cetrorelix injection significantly decreased WNT4, LRP-6 and CTNNB1 levels in adult mouse ovaries and FZD1 levels in pubertal mouse ovaries. These Wnt members might be affected directly or indirectly by cetrorelix in mouse ovaries. Since it is known that cetrorelix is related to growth factors important for the ovarian function (Chen et al. 2005), Wnt members related to these growth factors might also be affected by cetrorelix indirectly or cetrorelix might affect Wnts directly, as it was previously reported to have a direct effect on soluble factors (Grundker et al. 2000; Chen et al. 2005).

FZD1 expression was previously shown to be induced in large ovulatory follicles by an ovulatory dose of hCG in the mouse ovary (Hsieh et al. 2002). However, in the current study, FZD1 intensity in cetrorelix-injected pubertal mouse ovaries was the highest in granulosa cells, whereas cetrorelix-injected adult group had the highest level of FZD1 in stromal cells, indicating that the inhibition of gonadotropins might positively affect the distribution of FZD1 in granulosa and stromal cells. LRP-6 distribution was also affected in pubertal mouse ovaries by cetrorelix injection, in which oocytes and granulosa cells had higher LRP-6 intensity level compared to the theca and stroma cells, showing that reduction of gonadotropins might enhance the effects of LRP-6-dependent signaling on oocytes and granulosa cells.

CTNNB1 intensity levels were affected by cetrorelix injection in the adult groups. Since WB results revealed that CTNNB1 levels were decreased by cetrorelix injection, canonical Wnt signaling which is driven by CTNNB1 might be regulated through gonadotropin action. Previous studies also showed an interaction between CTNNB1 and gonadotropins, such that the increased expression of *Cyp19a1* in ovarian cell lines in response to FSH was determined to be mediated through CTNNB1 (Parakh et al. 2006). CTNNB1 was found to be required for FSH regulation of steroid production in granulosa cells of large bovine antral follicles (Castanon et al. 2012). It was also shown that FSH stimulated phosphorylation of CTNNB1 on Ser552 and Ser675 leading to its activation via protein kinase A (PKA) (Law et al. 2013). Thus, the activation of CTNNB1 facilitates FSH-mediated actions in ovarian follicular cells.

In conclusion, the distribution of Wnt signaling members in pubertal and adult mouse ovaries show a difference in different ovarian cell types, suggesting particular roles for each member in ovarian functions. Additionally, the distribution and protein levels of specific members of the Wnt signaling in the mouse ovary were found to be affected by GnRH antagonist cetrorelix injection depending on the developmental stage. Thus, possible roles of Wnt signaling in ovarian functions might be under the control of gonadotropins and this regulation might be in a cell-specific manner. To better understand the factors that might affect the Wnt signaling in the ovary, the mechanism of action of the signaling (canonical or non-canonical) including all Wnt ligands, receptors, inhibitors, and effectors must be specified for ovarian function. Also, for better consideration of short-term and long-term effects of cetrorelix injection for clinical purposes for both in adulthood and puberty, its effects on the signaling mechanisms in the ovary must be further investigated.

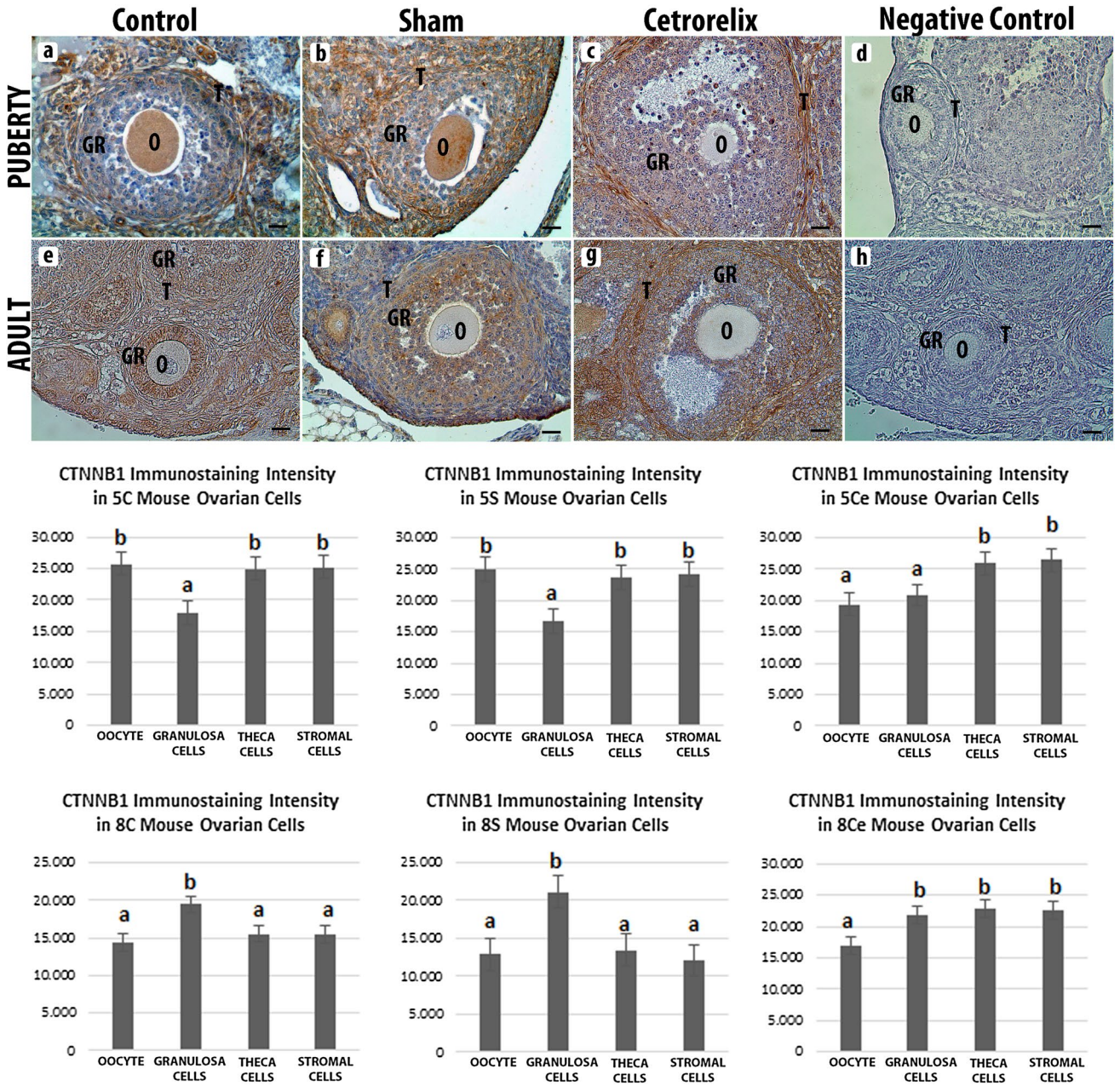


Fig. 11 CTNNB1 immunostainings and graphics of mathematical values of ImageJ evaluations in 5-week-old pubertal (**a**–**d**) and 8-week-old adult (**e**, **h**) mouse ovaries. **a** Ovary section from the control group (5C) of mouse with no injection. **b** Ovary section from the sham group of mouse (5S) injected with 5% methanol for 2 weeks starting from 3 weeks of age. **c** Ovary section from the cetrorelix group of mouse (5Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 3 weeks of age. **d** Representative negative control section. **e** Ovary section from the control group (8C) of mouse with no injection. **f** Ovary section from the sham group of mouse (8S) injected with 5% methanol for 2 weeks starting from 6 weeks of age. **g** Ovary section from the cetrorelix group of mouse (8Ce) injected with 0.5 mg/kg cetrorelix for 2 weeks starting from 6 weeks of age. **h** Representative negative control section. Counterstain: hematoxylin. *GR* granulosa cell, *O* oocyte, *T* theca cell, *ST* stromal cell. Scale bars: 20 µm. Different letters above data bars mark statistical significance ($P < 0.05$) between different cell types in the same group of ovaries (one-way ANOVA, Holm–Sidak method)

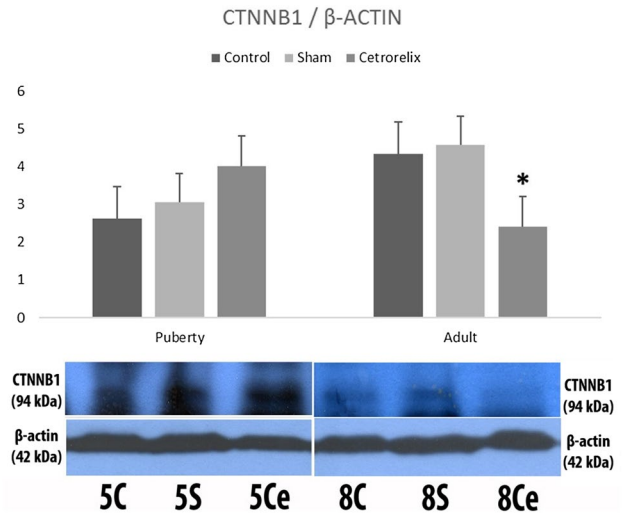


Fig. 12 Western blot bands and graphics of mathematical values of ImageJ evaluations of CTNNB1 protein expressions in postnatal mouse ovaries. Asterisk marks statistical significance ($P < 0.05$) between adult groups (one-way ANOVA, Holm–Sidak method)

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Author contributions FT and FU carried out cetrorelix injections, tissue processing, and immunohistochemistry experiments and performed evaluation and the statistical analysis for immunohistochemistry. FT and NA applied western blot experiments and performed the statistical analysis for western blot results. IU and GA conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no competing interests.

References

Abdul-Ghani M et al (2011) Wnt11 promotes cardiomyocyte development by caspase-mediated suppression of canonical Wnt signals. Mol Cell Biol 31(1):163–178

Abedini A et al (2016) WNT5a is required for normal ovarian follicle development and antagonizes gonadotropin responsiveness in granulosa cells by suppressing canonical WNT signaling. FASEB J 30(4):1534–1547

Bienz M, Clevers H (2003) Armadillo/beta-catenin signals in the nucleus–proof beyond a reasonable doubt? Nat Cell Biol 5(3):179–182

Boyer A et al (2010) WNT4 is required for normal ovarian follicle development and female fertility. FASEB J 24(8):3010–3025

Castanon BI et al (2012) Follicle-stimulating hormone regulation of estradiol production: possible involvement of WNT2 and beta-catenin in bovine granulosa cells. J Anim Sci 90(11):3789–3797

Chen W et al (2005) Gonadotropin-releasing hormone antagonist cetrorelix down-regulates proliferating cell nuclear antigen and epidermal growth factor expression and up-regulates apoptosis in association with enhanced poly(adenosine 5′-diphosphate-ribose) polymerase expression in cultured human leiomyoma cells. J Clin Endocrinol Metab 90(2):884–892

Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell 127(3):469–480

Edson MA et al (2009) The mammalian ovary from genesis to revelation. Endocr Rev 30(6):624–712

Fan HY et al (2010) Beta-catenin (CTNNB1) promotes preovulatory follicular development but represses LH-mediated ovulation and luteinization. Mol Endocrinol 24(8):1529–1542

Furukawa KT, Yamashita K et al (2017) The epithelial circumferential actin belt regulates YAP/TAZ through nucleocytoplasmic shuttling of Merlin. Cell Rep 20(6):1435–1447

Grundker C et al (2000) Luteinizing hormone-releasing hormone agonist triptorelin and antagonist cetrorelix inhibit EGF-induced c-fos expression in human gynecological cancers. Gynecol Oncol 78(2):194–202

Gupta PS et al (2014) Regulation and regulatory role of WNT signaling in potentiating FSH action during bovine dominant follicle selection. PLoS One 9(6):e100201

Harwood BN et al (2008) Members of the WNT signaling pathways are widely expressed in mouse ovaries, oocytes, and cleavage stage embryos. Dev Dyn 237(4):1099–1111

Hernandez Gifford JA et al (2009) Conditional deletion of beta-catenin mediated by Amhr2cre in mice causes female infertility. Biol Reprod 80(6):1282–1292

Hernandez-Gonzalez I et al (2006) Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? Mol Endocrinol 20(6):1300–1321

Honda T et al (2010) PDZRN3 negatively regulates BMP-2-induced osteoblast differentiation through inhibition of Wnt signaling. Mol Biol Cell 21(18):3269–3277

Horvath JE et al (2002) Effects of long-term treatment with the luteinizing hormone-releasing hormone (LHRH) agonist Decapeptyl and the LHRH antagonist Cetrorelix on the levels of pituitary LHRH receptors and their mRNA expression in rats. Proc Natl Acad Sci USA 99(23):15048–15053

Hsieh M et al (2002) Regulated expression of Wnts and Frizzleds at specific stages of follicular development in the rodent ovary. Endocrinology 143(3):898–908

Khalaf M et al (2010) GnRH agonist and GnRH antagonist protocols in ovarian stimulation: differential regulation pathway of aromatase expression in human granulosa cells. Reprod Biomed Online 21(1):56–65

Lapointe E et al (2012) FZD1 regulates cumulus expansion genes and is required for normal female fertility in mice. Biol Reprod 87(5):104

Law NC et al (2013) Lhcgr expression in granulosa cells: roles for PKA-phosphorylated beta-catenin, TCF3, and FOXO1. Mol Endocrinol 27(8):1295–1310

Lee HY et al (2004) Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. Science 303(5660):1020–1023

Lowry OH et al (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1):265–275

Maman E et al (2011) Expression and regulation of sFRP family members in human granulosa cells. Mol Hum Reprod 17(7):399–404

Matikainen T et al (1992) Differing responses of plasma bioactive and immunoreactive follicle-stimulating hormone and luteinizing hormone to gonadotropin-releasing hormone antagonist and agonist

treatments in postmenopausal women. J Clin Endocrinol Metab 75(3):820–825
Nusse R, Varmus HE (1992) Wnt genes. Cell 69(7):1073–1087
Parakh TN et al (2006) Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires beta-catenin. Proc Natl Acad Sci USA 103(33):12435–12440
Pinson KI et al (2000) An LDL-receptor-related protein mediates Wnt signalling in mice. Nature 407(6803):535–538
Prunskaite-Hyyrylainen R et al (2014) Wnt4, a pleiotropic signal for controlling cell polarity, basement membrane integrity, and antimullerian hormone expression during oocyte maturation in the female follicle. FASEB J 28(4):1568–1581
Richards JS et al (2002) Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. Recent Prog Horm Res 57:195–220
Ricken A et al (2002) Wnt signaling in the ovary: identification and compartmentalized expression of wnt-2, wnt-2b, and frizzled-4 mRNAs. Endocrinology 143(7):2741–2749
Roose J et al (1998) The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature 395(6702):608–612
Saatli B et al (2009) Long-term effects of GnRH agonist, GnRH antagonist, and estrogen plus progesterone treatment on apoptosis related genes in rat ovary. Fertil Steril 91(5 Suppl):2006–2011
Salker MS et al (2018) LEFTY2 inhibits endometrial receptivity by downregulating Orai1 expression and store-operated Ca(2+) entry. J Mol Med (Berl) 96(2):173–182
Spate LD et al (2014) Dickkopf-related protein 1 inhibits the WNT signaling pathway and improves pig oocyte maturation. PLoS One 9(4):e95114
Stapp AD et al (2014) Canonical WNT signaling inhibits follicle stimulating hormone mediated steroidogenesis in primary cultures of rat granulosa cells. PLoS One 9(1):e86432
Tepekoy F et al (2015a) The role of Wnt signaling members in the uterus and embryo during pre-implantation and implantation. J Assist Reprod Genet 32(3):337–346
Tepekoy F et al (2015b) CD90 and CD105 expression in the mouse ovary and testis at different stages of postnatal development. Reprod Biol 15(4):195–204
Usongo M et al (2012) beta-Catenin/Tcf signaling in murine oocytes identifies nonovulatory follicles. Reproduction 144(6):669–676
Vainio S et al (1999) Female development in mammals is regulated by Wnt-4 signalling. Nature 397(6718):405–409
Wang HX et al (2009) Identification of WNT/beta-CATENIN signaling pathway components in human cumulus cells. Mol Hum Reprod 15(1):11–17
Wang HX et al (2010) WNT2 regulates DNA synthesis in mouse granulosa cells through beta-catenin. Biol Reprod 82(5):865–875
Wei W et al (2006) The LDL receptor-related protein LRP6 mediates internalization and lethality of anthrax toxin. Cell 124(6):1141–1154
Wu MH et al (2005) Clinical suppression of precocious puberty with cetrorelix after failed treatment with GnRH agonist in a girl with gonadotrophin-independent precocious puberty. Reprod Biomed Online 11(1):18–21
You L et al (2004) Inhibition of Wnt-2-mediated signaling induces programmed cell death in non-small-cell lung cancer cells. Oncogene 23(36):6170–6174

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