The Involvement of Granulosa Cells in the Regulation by Gonadotropins of Cyp17a1 in Theca Cells

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Abstract. Background/Aim: Theca cells produce androgen by 17α-hydroxylase-17,20-lyase encoded by Cyp17a1, and conversion of androgen to estrogen in granulosa cells is regulated by gonadotropins. Women with polycystic ovarian syndrome (PCOS) exhibit elevated levels of androgens due to high Cyp17a1 expression and alterations in gene expression in granulosa cells. The aim of this study was to examine the interaction between theca and granulosa cells in PCOS-model mice. Materials and Methods: To produce PCOS-model mice, neonatal mice were injected with 1 μg TP for 3 days from the day of birth. Gonadotropins were injected according to the superovulation protocol to 3-month-old control mice and PCOS-model mice. Histological changes and expression of genes involved in steroidogenesis, ovulation and luteinization were investigated by immunohistochemistry and real-time RT-PCR, respectively. Results: Pregnant mare serum gonadotropin (PMSG) induced the expression of genes involved in steroidogenesis in control prepubertal mice, whereas human chorionic gonadotropin (hCG) reduced Cyp17a1 expression and induced phospho-ERK1/2 in granulosa cells. Cyp17a1 was reduced in PMSG-primed PCOS-model mice regardless of hCG injection, and PMSG induced phosphorylation of ERK1/2 in granulosa cells. Conclusion: Phospho-ERK1/2 in granulosa cells can be correlated with reduced Cyp17a1 expression in theca cells, and the interaction between granulosa and theca cells may be impaired in PCOS-model mice.

In the mouse ovary, 17α-hydroxylase-17,20-lyase (CYP17A1), localized in theca cells of large antral and preovulatory follicles, converts progesterone to 17α-hydroxypregnenolone and then androstenedione by 17α-hydroxylase and C17-20 lyase activities, respectively (1, 2). The expression of Cyp17a1 mRNA in the ovary is induced by luteinizing hormone (LH) from the pituitary via LH receptor, encoded by Lhcr, and LH enhances cAMP production (3). Although LH is the main factor inducing expression of Cyp17a1, in vitro studies have also revealed that factors from granulosa cells and oocytes, such as inhibin, activin and growth differentiation factor 9 (GDF-9), regulate the expression of Cyp17a1 (4, 5). Additionally, female mice lacking extracellular signal-regulated kinases 1 and 2 (ERK1/2) in granulosa cells generated from Erk1–/– and Erk2 gc–/– mice contain preovulatory follicles with elevated levels of serum 17β-estradiol (E2), but no corpora lutea (CLs) (6). When Erk1/2 gc– mice are treated with gonadotropins, events associated with ovulation do not occur, and the levels of E2 and the expression of Cyp17a1 and Cyp19a1 remain high compared with those in the wild-type mice. These facts indicate the involvement of granulosa cells in the regulation of Cyp17a1 in theca cells.

Polycystic ovarian syndrome (PCOS) is one of the most common endocrine disorders in reproductive-aged women (7). In its typical form, there is association of hyperandrogenism with chronic anovulation (8). It is known that Cyp17a1 expression in theca cells from PCOS women is up-regulated by hypersecretion of LH (9-11). In addition to impaired steroidogenesis and gene expression in theca cells of PCOS women, gene expression in granulosa cells, especially transcription factors related to Wnt/β-catenin and mitogen-activated protein kinase (MAPK)/ERK pathways, and proteins in follicular fluid are also altered compared to healthy women (12-14). These facts suggest that not only the alterations in theca cells but also those in granulosa cells are responsible for PCOS pathology.

In order to study the pathogenesis of PCOS, animal models including rodents are used. Rodent models of PCOS are induced by perinatal androgenization (15). A single treatment of rats with testosterone propionate (TP) (1-1,000 μg) within the first 5 days of life causes anovulation or reduced ovulation (16-19). Rats exposed to 100 μg TP on Day 1 or 5 show acyclicity and polycystic ovaries with atretic follicles which are cystic follicles with thin granulosa cell layers (19, 20).
Mice treated with TP for 3 days starting from the day of birth also exhibit anovulation and the presence of polycystic ovaries (21, 22). A nonaromatizable androgen, 5α-dihydrotestosterone (DHT), is also used to induce PCOS in animal models. Prenatal DHT exposure of rats and mice causes irregular estrous cycle and reduced ovulation (23). These treatments have shown many characteristics of PCOS including hyperandrogenism, elevated LH, disrupted cyclicity, presence of follicular cysts and altered insulin sensitivity in rodents. Therefore, the study of the interaction between granulosa and theca cells using a mouse model of PCOS is helpful to understand PCOS pathology in women.

This study aimed at studying the involvement of granulosa cells in the regulation of Cyp17a1 in theca cells in response to gonadotropins in treatments in control and PCOS-model mice. The expression of genes involved in steroidogenesis regulated by gonadotropins, and localization of CYP17A1 and phospho-ERK1/2 were investigated in immature mice.

Materials and Methods

**Animals and treatments.** C57BL/6J mice (CLEA Japan, Tokyo, Japan) were housed under a 12-h light/12-h dark cycle (lights off at 20:00 h) with controlled temperature (25°C) and given a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) and fresh tap water ad libitum. All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Experiments were approved by the Institutional Animal Care Committee of the Yokohama City University. To produce PCOS-model mice, female pups were given daily subcutaneous injections of 1 μg testosterone propionate (TP) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.02 ml sesame oil or the vehicle alone for 3 days starting on the day of birth.

Twenty-one-day-old and 3-month-old female mice at the day of diestrus and PCOS-model female mice were given 5 IU of pregnant mare serum gonadotropin (PMSG, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) intraperitoneally in the morning (10:00 h) followed by 5 IU of human chorionic gonadotropin (hCG, ASKA Pharmaceutical Co., Ltd.) 48 h later. Ovaries from mice at 48 and 56 h after PMSG and 4, 8, 12, 24 and 48 h after hCG were collected, fixed for histological analysis or frozen for quantitative real-time RT-PCR analysis (n=5-8 in each group).

Stages of the estrus cycle of 3-month-old control mice were determined by cytologic evaluation of vaginal smears. Ovaries from mice at diestrus or estrus were fixed for histological analysis or frozen for quantitative real-time RT-PCR analysis (n=5).

**Histological analysis.** Right ovaries were fixed in Bouin’s fixative overnight, dehydrated through a graded series of ethanol, embedded in paraffin and serially sectioned at 6 μm. Sections were deparaffinized and rehydrated. For antigen retrieval, sections were microwaved for 8 min in 10 mM citric acid buffer (pH 6.0) and left for 30 min at room temperature (RT) (CYP17A1) or microwaved until boiling and kept for 12 min at a sub-boiling temperature in 10 mM citric acid buffer (pH 6.0) and left for 30 min in RT (ERK1/2 and phospho-ERK1/2). Sections were then immersed in 3% H₂O₂ for 10 min to eliminate endogenous peroxidase. After washing in PBS (CYP17A1) or TBS (ERK1/2 and phospho-ERK1/2), sections were incubated with 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 45 min, and then anti-human CYP17A1 rabbit polyclonal antibody (Proteintech, Rosemont, IL, USA) at 1:1000 dilution, anti-human ERK1/2 (ERK1/2) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) at 1:100 dilution, anti-human phospho-ERK1/2 (phospho-ERK1/2, p-ERK1/2) rabbit monoclonal antibody (Cell Signaling Technology) at 1:100 dilution, or negative control rabbit immunoglobulin fraction (Dako Cytomation, Glostrup, Denmark) in PBS or TBS at 4°C overnight. After incubation with a secondary antibody, CYP17A1, ERK1/2, and p-ERK1/2 were detected by a standard Elite ABC kit (Vector Laboratories) and diaminobenzidine (Sigma-Aldrich). Hematoxylin was used for counterstaining.

**RNA isolation and quantitative real-time RT-PCR.** Ovaries were homogenized in TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA isolated from ovaries was purified by DNase I (Roche, Penzberg, Germany) and with a RNeasy total RNA kit (Qiagen, Chatsworth, CA, USA) to remove genomic DNA, and reverse transcribed into cDNA by SuperScript II reverse transcriptase (Thermo Fisher Scientific) using oligo d(T) primer (Thermo Fisher Scientific). Real-time PCR was carried out using Applied Biosystems StepOnePlus™ Real Time PCR System (Thermo Fisher Scientific) with Fast SYBR Green Master Mix (Thermo Fisher Scientific). Relative mRNA expression of Cyp11a1, Cyp17a1, Cyp19a1, Hsd3b1, Hsd17b1, Lhcgr, follicle stimulating hormone receptor (Fshr), epidermal growth factor receptor (Egfr), amphotergulin (Areg), ephiregulin (Ereg), betacellulin (Btc), secreted frizzled-related protein 4 (Sfrp4), Prostaglandin-endoperoxide synthase 2 (Ptgs2) and tumor necrosis factor-alpha-induced protein 6 (Tnfaip6) was determined by the standard curve method. Peptidylprolyl isomerase A (Ppia) was chosen as an internal standard to control for variability in amplification due to differences in starting mRNA concentrations. Sequences of oligonucleotides used as primers for real-time quantitative RT-PCR are shown in Table I.

**Statistical analysis.** Data are expressed as the mean±standard error. For multiple comparisons, treatment groups were compared using analysis of variance (ANOVA) followed by Tukey’s post-hoc test. A statistically significant difference was defined at p<0.05.

Results

**Effects of PMSG and hCG injections on the ovary of pubertal mice.** Prepubertal ovaries of 21 days of age mice contained a series of developing follicles but no preovulatory follicle and CL (Figure 1A, B and C). PMSG treatment for 48 h significantly increased the total number of antral follicles (Figure 1A and D). Preovulatory follicles were found in PMSG-primed mice following treatment with PMSG or hCG.
for 48 h and 12 h, respectively (in Figure 1D and E). CLs were found in all ovaries of PMSG-primed mice following treatment with hCG for 24 and 48 h (Figure 1A and F).

The percentage of small antral follicles was significantly decreased at 48 h after PMSG treatment but it was significantly increased at 4 h after hCG treatment (Figure 1B). The percentages of large antral follicles and preovulatory follicles were significantly increased at 48 h after PMSG treatment. The percentage of preovulatory follicles was significantly decreased at 8 h after hCG compared with that at 48 h after PMSG treatment (Figure 1B).

Table I. Sequences of oligonucleotides used as primers for real-time quantitative RT-PCR.

| Gene | Forward sequence (5’ to 3’) | Reverse sequence (5’ to 3’) | PCR product (bp) |
|------|-----------------------------|-----------------------------|------------------|
| Cyp11a1 | GTGAATGACTGGTGGTTCGGT | TCGACCATGGCAGAGGCTA | 78 |
| Cyp17a1 | GATCCGGTTATAGCCTGGACG | TCCGAAGGCAAAATAACTGG | 81 |
| Cyp19 | TGTGATGAGGCCTCTTTCCTC | CCCAGCACGTCAGGACCCCT | 76 |
| Hsd3b1 | CAAGTTGCAACGGCTTCTAC | GGCCATACGGAGCACTTAT | 80 |
| Hsd17b1 | CTCGGTTGTTGATGACAGTGA | GCAGATGCAAGAAGAACGC | 77 |
| Lhcgr | GAAATGGATTGGAAGAAGCTACAAG | CCATTGTGAGCTCTTCCAG | 99 |
| Fshr | CTCCTCGAGATGCTTGTACGAC | GATGTACAGCAGATTGTTAGCCTTTTC | 103 |
| Areg | TCCCGCTATATATTAGATATTGCCTGCA | TCTCTCTTCTGTCTTTTCTTGG | 74 |
| Ereg | GCTCCCTGCTCTGTGGGTTT | TGCCCTGAGATGGGTGAGCA | 58 |
| Btc | CCAATGCCCTCTTTTGTGGAG | TGGTTTTTCTACTTTCTTTGGG | 73 |
| Egfr | AGCAGTGGCCCGCAATGTGAT | ACAGCAGCGTGTTGGGAAACAT | 120 |
| Ptg2 | GCTGTAACAGCAGGGGACAA | TTCTGCCAGCCATTTCCTTCT | 144 |
| Sfrp4 | GGCTCTATGAGCAAGCAGC | GAGCTTTTTTCTACTTTTGGCA | 110 |
| Tgfap6 | GGTTGTCCTGCTGGCCTTTAC | CGACCACGGAGACATGCAA | 71 |
| Ppia | AGGTCTCGGCATCTTGTAG | CCCATCAACATTTACATGG | 93 |

The localization of CYP17A1 and phospho-ERK1/2 in 21-day-old mouse ovaries. To investigate the involvement of granulosa cells in CYP17A1 expression, the localization of CYP17A1, phospho-ERK1/2 and ERK1/2 in the ovary of 21-day-old mice after the gonadotropin treatments was analyzed by immunohistochemistry. In the ovary of 21-day-old intact mice, ERK1/2 and phospho-ERK1/2 positive cells but not CYP17A1 positive cells were observed in the theca cell layer (Figure 3A, E, I, M, Q and U). PMSG injection induced strong expression of CYP17A1 in the theca cell layers of large antral and preovulatory follicles (Figure 3B and F), however, the localization of ERK1/2 positive cells was not affected and staining for phospho-ERK1/2 was observed in theca cells (Figure 3R, V, J and N). Five out of 8 mouse ovaries showed no or little CYP17A1 expression in the theca cell layer at 8 h after hCG injection (Figure 3C and G). Interestingly, preovulatory follicles which showed no staining for CYP17A1 expression contained phospho-ERK1/2 positive-granulosa cells and theca cells (Figure 3K and O). On the other hand, preovulatory follicles which did not contain phospho-ERK1/2 positive granulosa cells still expressed CYP17A1 in theca cells (Figure 3S, W, T and X).

In granulosa cells, the main factors inducing ERK1/2 phosphorylation after the LH surge or hCG treatment are EGF-like ligands such as AREG, EREG and BTC (6). In order to investigate the time course changes in the expression of Areg, Ereg and Btc by gonadotropins, real-time RT-PCR was carried out. After hCG treatment, mRNA expression of Areg and Ereg was increased at 2 h and that of Btc at 4 h, while were decreased at 4 h and 8 h after hCG, respectively.
Figure 1. Effects of PMSG and hCG injections on the ovaries of prepubertal mice at 21 days of age. A: The total numbers of antral follicles and corpora lutea (CLs) in ovaries from prepubertal mice were counted at 0 and 48 h after PMSG injection and 4, 8, 12, 24 and 48 h after hCG injection, respectively. B: Follicle stage index, preovulatory (>390 μm), large antral (250-390 μm) and small antral follicles (140-250 μm). C-F: HE staining of 21-day-old mouse ovaries at 0 h (C) and 48 h after PMSG (D), 12 h (E) and 48 h (F) after hCG in PMSG-primed mice. Bar=200 μm. Different letters (a, b and c) on A and B indicate significant changes through the experiment (p<0.05). S: Small antral follicle; L: large antral follicle; Pre: preovulatory follicle; CL: corpus luteum. n=5 in each group.
Figure 2. Time course changes in the expression of genes involved in ovarian steroidogenesis at 0, 48, and 56 h after PMSG injection and 4, 8, 12, 24, 48 h after hCG injection in 21-day-old mice. Data were expressed relative to mRNA expression in the ovaries before PMSG (=1.0). Different letters (a and b) indicate significant changes in the expression of each gene (p<0.05). *p<0.05, significant difference between PMSG-primed mice at 56 h with or without hCG. Straight line and dotted line indicate hCG treatment at 48 h after PMSG treatment and no hCG treatment, respectively. n=5 in each group.
Figure 3. The localization of CYP17A1, phospho-ERK1/2 (p-ERK1/2) and ERK1/2 by immunohistochemistry in the ovary of 21-day-old non-treated mice (A, E, I, M, Q and U), mice treated with PMSG for 48 h (B, F, J, N, R and V) and mice treated with hCG for 8 h (C, D, G, H, K, L, O, P, S, T, I W and X). Bar=100 μm. TC, theca cells; GC, granulosa cells; arrowheads, CYP17A1 positive cells; arrows, p-ERK1/2 positive cells. n=5-8 in each group.
In the absence of hCG, the expression of Areg, Ereg and Btc was not increased (Figure 4).

**Histology and expression of genes involved in steroidogenesis in the ovary of 3-month-old PCOS-model mice compared with those of cycling control mice.** High dose of TP (5, 10, 25, 50 and 100 μg) induced severe hypertrophy and hyperplasia in the interstitial tissue similar to that in the ovary of neonatal diethylstilbestrol-treated mice (data not shown). Mice treated with 1 μg TP for 3 days from the day of birth mimicked most of PCOS features, therefore, they were used as PCOS-model mice in the following experiments.

The expression of Cyp11a1 was reduced in the ovary of 3-month-old PCOS-model mice compared with that of age-matched cycling mice, possibly due to the lack of CL, whereas the expression of Hsd3b1 and Lhcgr was comparable among these groups. The expression of Cyp17a1 was the highest at diestrus and the lowest at estrus, and it was maintained high in PCOS-model mice. The expression of Hsd17b1 and Cyp19a1 in PCOS-model mice was significantly increased compared with that in cycling mice. The ovary of control mice at diestrus and PCOS-model mice showed similar expression of Fshr which was low in the ovary of mice at estrus (Figure 5).

Ovaries from mice at diestrus contained follicles in the variety of follicle stages and CL (Figure 6A), whereas ovaries from PCOS-model mice contained follicles in the variety of follicle stages but no CL (Figure 6E). Ovaries of PCOS-model mice contained fluid-filled cysts and hypertrophied interstitial tissues (Figure 6E).

**The histological changes in control and PCOS-model mice after gonadotropin treatments.** PMSG injection to both control and PCOS-model mice did not induce any morphological changes in the ovaries of these mice at 3 months of age (Figure 6B and F). However, hCG treatment induced the formation of the cumulus cell-oocyte complex at 8 h (Figure 6C and G) in the ovary of both control and PCOS-model mice followed by ovulation (Figure 6D and H). At 24 h after hCG treatment, all ovaries from both control and PCOS-model mice contained CLs (Figure 6D and H). There was no difference in the total number of antral follicles and the percentage of each follicle type between control and PCOS-model mice (Figure 6F and J). However, the ovary of PCOS-model mice contained cystic follicles without CL before PMSG treatment (Figure 6E and I). PMSG and hCG injections also induced hemorrhagic cysts in the ovary of PCOS-model mice (Figure 6F, G and H).

**The time-course changes in the gene expression after gonadotropin treatments in the ovary of 3-month-old cycling and PCOS-model mice.** To investigate the effects of gonadotropin treatments on the expression of genes involved in steroidogenesis, the expression of Cyp11a1, Hsd3b1, Cyp17a1, Hsd17b1, Cyp19a1, Lhcgr and Fshr was examined (Figure 7). In control mice, PMSG injection significantly induced the expression of Cyp11a1 and Lhcgr, but had no effect on the expression of Hsd3b1, Cyp17a1, Hsd17b1, Cyp19a1 and Fshr. hCG treatment decreased the expression of genes involved in androgen and estrogen synthesis, including Cyp17a1, Hsd17b1, Cyp19a1, Lhcgr and Fshr at 8 h after the treatment but had no effect on the expression of Cyp11a1 and Hsd3b1 which were expressed mainly in the CL. The expression of Lhcgr was reduced by hCG, however, its expression was significantly increased at 48 h after the treatment.

In PCOS-model mice, PMSG treatment also significantly induced the expression of Cyp11a1 and Lhcgr, however, had no effect on the expression of Hsd3b1, Cyp17a1, Hsd17b1.
and Cyp19a1 (Figure 7). PMSG treatment also resulted in a decrease expression of Fshr in PCOS-model mice. hCG treatment reduced the expression of Hsd3b1, Cyp17a1, Hsd17b1, Cyp19a1, Lhcgr and Fshr but had no effect on Cyp11a1 expression, as that in control mice. Interestingly, Cyp17a1 expression was also reduced in the absence of hCG treatment at 56 h after PMSG treatment in PCOS-model mice, whereas in control mice the expression of Cyp17a1 remained high in the absence of hCG treatment.

The time-course changes of EGF-like ligands and genes involved in luteinization after gonadotropin treatments. The results from experiments using immature mice indicated that the expression of CYP17A1 in theca cells is possibly correlated with ERK1/2 phosphorylation in granulosa cells. EGF-like ligands are well known factors to induce phosphorylation of ERK1/2 through EGF receptor, therefore, the expression and the time-course changes of EGF-like ligands (Areg, Ereg, Btc and Egfr) and genes involved in luteinization including Ptgs2, Sfrp4 and Tnfaip6 were examined.

The expression of Areg, Ereg and Sfrp4 was significantly reduced in the ovary of PCOS-model mice (Figure 8). In both control and PCOS-model mice, hCG treatment transiently induced the expression of Areg at 2 h while the expression of Ereg, Btc and Tnfaip6 was induced at 2 h in control mice and at 4 h in PCOS-model mice (Figure 9), hCG had no effect on the expression of Sfrp4 in control mice, whereas it increased the expression of Sfrp4 in PCOS-model mice at 4 h. hCG induced the expression of Ptgs2 in control at 2 h and PCOS-model mice at 4 h (Figure 9).

The localization of CYP17A1, phospho-ERK1/2 and ERK1/2 in 3-month-old cycling and PCOS-model mice. In control mice at diestrus, CYP17A1, phospho-ERK1/2 and ERK1/2 were observed in theca cells of large antral follicles (Figure 10A, A* and A†). PMSG induced CYP17A1 expression in theca cells at 48 and 56 h (Figure 10B and C), however, phospho-ERK1/2 expression was negative and ERK1/2 expression was positive in those theca cells (Figure 10B*, C*, B‡ and C‡). At 8 h after hCG, 3 out of 5 mice showed follicles with CYP17A1 positive theca cells and no phospho-ERK1/2 positive granulosa cells (Figure 10E and E*) and 2 out of 5 mice showed phospho-ERK1/2 positive granulosa cells in follicles without CYP17A1 expression (Figure 10D and D*). ERK1/2 expression was positive in those follicles (Figure 10D§ and E§).

In PCOS-model mice, CYP17A1 was found not only in theca cells but also in the interstitial cells (Figure 10F). PMSG induced CYP17A1 expression in theca cells at 48 and 56 h in PCOS-model mice (Figure 10G and I) without phospho-ERK1/2 expression in granulosa cells (Figure 10G* and I*), however, the ovary of 3 out of 5 mice had follicles containing no CYP17A1 positive theca cells (Figure 10H*), and phospho-ERK1/2 positive granulosa cells at 48 h after PMSG (Figure 10H†). Especially, the granulosa cells of one PCOS-model mouse at 48 h after PMSG exhibited intense phospho-ERK1/2 staining (Figure 10H†). The ovary of 3 out of 4 PCOS-model mice at 8 h after hCG contained follicles with no CYP17A1 positive theca cells but phospho-ERK1/2 positive granulosa cells (Figure 10J and J†). The ovary of one PCOS-model mouse at 8 h after hCG contained follicles with CYP17A1 positive theca cells but no phospho-ERK1/2
Figure 6. The histology of ovaries from 3-month-old control mice at diestrus (A) and PCOS-model mice (E). Bar=200 μm. Effects of PMSG and hCG injections on the ovaries of 3-month-old control and PCOS-model mice. HE staining of 3-month-old control and PCOS model mouse ovaries at 0 (A, E), and 48 h after PMSG (B, F), 8 (C, G) and 24 h (D, H) after hCG. S: small antral follicle; L: large antral follicle; Pre: preovulatory follicle; CL: corpus luteum; HCy: hemorrhagic cyst. The total numbers of antral follicles, corpora lutea (CLs) and cysts in ovaries from control and PCOS-model mice were counted at 0 and 48 h after PMSG injection, and 4, 8, 12, 24 and 48 h after hCG injection, respectively (I and J). Follicle stage index, preovulatory (>390 μm), large antral (250-390 μm) and small antral follicles (140-250 μm), respectively. n=5 in each group.
Figure 7. Time-course changes in the expression of genes involved in ovarian steroidogenesis of 3-month-old control and PCOS-model mice at 0, 48 and 56 h after PMSG injection and 4, 8, 12, 24, 48 h after hCG injection. Data were expressed relative to mRNA expression in the ovaries at diestrus (=1.0). Different letters (a, b, c and d) indicate significant changes in the expression of each gene (p<0.05). *p<0.05 significant difference between PMSG-primed mice at 56 h with or without hCG. Straight line and dotted line indicate hCG treatment at 48 h after PMSG treatment and no hCG treatment, respectively. n=5 in each group.
positive granulosa cells (Figure 10K and K*). ERK1/2 expression was positive in all mice regardless of PMSG or hCG injections (Figure 10F#, G#, H#, I#, J# and K#).

**Discussion**

In the ovary, granulosa cells act cooperatively with theca cells in estrogen synthesis regulated by gonadotropins. Our results suggest that granulosa cells can also contribute to the LH regulated expression of \( \text{Cyp17a1} \) in theca cells.

The expression of genes involved in androgen and estrogen production was suppressed by 8 h after hCG, but this change did not occur without hCG. A decrease of preovulatory follicles occurred at 8 h after hCG due to the hCG-induced ovulation, while CLs were found at 24 h. These results indicate that hCG caused a decrease of gene expression involved in androgen and estrogen production in theca cells and granulosa cells before CL formation. In PMSG-primed rats, the fall in serum and ovarian E2 levels as well as ovarian androgen levels occurs at 6 and 3 h after LH injection, respectively (24). Thus, the expression of \( \text{Lhcgr} \) in granulosa cells is induced by PMSG pretreatment, and subsequently the gene expression involved in androgen and estrogen production can be affected in response to hCG before luteinization.

The present study showed that PMSG stimulation of prepubertal mice induced follicle growth including an increase in the number of large antral and preovulatory follicles with CYP17A1 positive cells, as well as an increase of the expression of \( \text{Cyp17a1} \) and \( \text{Cyp19a1} \). hCG injection, on the other hand, reduced the expression of \( \text{Cyp17a1} \) and \( \text{Cyp19a1} \) in both prepubertal and cycling 3-month-old mice at 8 h. Immunohistochemistry revealed that when follicles contained phospho-ERK1/2-positive granulosa cells, theca cells were negative for \( \text{CYP17A1} \) and vice versa. In \( \text{Erk1/2}^{286<} \) mice, gonadotropin treatments did not induce events associated with ovulation, and E2 levels and the expression of \( \text{Cyp17a1} \) and \( \text{Cyp19a1} \) remained high (6). Since the interaction between theca and granulosa cells is essential not only for the production of steroid hormones but also for differentiation of theca cells (24-27), it is speculated that granulosa cells can contribute to the reduction of \( \text{Cyp17a1} \) expression in theca cells after the hCG injection. EGF-like ligands, such as \( \text{AREG} \), \( \text{EREG} \) and \( \text{BTC} \), are considered as the main factors inducing phosphorylation of \( \text{ERK1/2} \) in granulosa cells upon hCG treatment. The expression of \( \text{Cyp17a1} \) in theca cells is also increased by inhibin together with LH (4). Thus, the regulation of \( \text{Cyp17a1} \) expression can be modulated by factors from granulosa cells in addition to LH.

Neonatal TP injections to female mice caused cystic follicles and anovulation, and the expression of \( \text{Cyp17a1} \) maintained high mimicking the typical physiology of women with PCOS. The ovary of PCOS-model mice showed reduced expression of \( \text{Cyp11a1} \) compared with that of control mice. This was probably caused by the lack of CL therefore; gonadotropin injections induced the expression of \( \text{Cyp11a1} \) as a result of luteinization in PCOS-model mouse ovaries. Interestingly, the expression of \( \text{Cyp17a1} \) remained high at 56 h after PMSG.
Figure 9. Time course changes in the expression of genes involved in luteinization (Sfrp4, Prgs2, Tnfaip6) and EGF-like ligands (Areg, Ereg, Btc, Egfr) in ovaries from control and PCOS-model mice at 0, 24, 48, and 56 h after PMSG treatment and 2, 4 and 8 h after hCG treatment. Data were expressed relative to mRNA expression in the ovaries at diestrus (=1.0). Different letters (a, b, c, d and e) indicate significant changes in the expression of each gene (p<0.05). Straight line and dotted line indicate with and without hCG treatment in PMSG-primed mice at 48 h, respectively. n=5 in each group.
injection in both prepubertal and cycling adult mice, whereas it was reduced in PCOS-model mice. In addition, ovaries of 3 mice had follicles containing no CYP17A1 positive theca cells and phospho-ERK1/2 positive granulosa cells at 48 h after PMSG injection. The expression of Sfrp4, normally upregulated in large antral follicles and CL after hCG injection, was also induced by PMSG in PCOS-model mice. A study using granulosa cell-specific Smad4 conditional knockout mice shows an increase of the expression of luteal markers including Sfrp4 after PMSG injection, suggesting that the lack of SMAD4 signaling in granulosa cells undergoes precocious luteinization in response to gonadotropin stimulation (28),

Figure 10. The localization of CYP17A1, phospho-ERK1/2 and ERK1/2 by immunohistochemistry in the ovary of 3-month-old control mice at diestrus (A, A* and A#) and PCOS-model mice (F, F* and F#). Control mice at 48 h after PMSG treatment (B, B* and B#), PCOS-model mice at 48 h after PMSG treatment (G, G*, G# and H#), control mice at 56 h after PMSG treatment (C, C* and C#) and PCOS-model mice at 56 h after PMSG treatment (F, F* and F#) and control mice at 8 h after hCG treatment (D, D* and D#) and PCOS-model mice at 8 h after hCG treatment (J, J*, J# and K#). Bar=100 μm. Arrowheads, CYP17A1 positive cells; arrows, phospho-ERK1/2 positive cells. n=4-6 in each group.
understand steroidogenesis in the ovary of PCOS women.

Histological analysis revealed that phosphorylation of ERK1/2 in granulosa cells was induced regardless of hCG in PCOS-model mouse ovaries. EGF-like ligands are well-known factors causing phosphorylation of ERK1/2 in granulosa cells after hCG (30). However, PMSG did not induce the expression of \( Cyp17a1 \) expression in theca cells. Furthermore, \( Cyp17a1 \) expression is elevated in PCOS-model mouse ovaries but it may be caused by other factors, such as VEGF.

In conclusion, hCG injection reduced the expression of \( Cyp17a1 \) in ovaries from both prepubertal and cycling adult mice and the reduction of \( Cyp17a1 \) may be correlated with the phosphorylation of ERK1/2 in granulosa cells after hCG. These facts suggest that granulosa cells may be involved in the regulation of \( Cyp17a1 \) in granulosa cells. Furthermore, PCOS-model mouse ovaries were established by neonatal TP injections. PMSG reduced the expression of \( Cyp17a1 \) at 48 h only in PCOS-model mouse ovaries, suggesting that phosphorylation of granulosa cells can be induced by PMSG. Thus, the interaction between granulosa and theca cells may be impaired in PCOS-model mice and the study of granulosa cells is important to understand steroidogenesis in the ovary of PCOS women.

Conflicts of Interest

The Authors declare that there is no conflict of interest.

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