Evidence for the involvement of FXR signaling in ovarian granulosa cell function

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Abstract. Farnesoid X receptor (FXR) is mainly present in enterohepatic tissues and regulates cholesterol, lipid, and glucose homeostasis in coordination with target genes such as SHP and FABP6. Although FXR has been revealed to be expressed in reproductive tissues, FXR function and expression levels in the ovary remain unknown. In this study, we investigated FXR expression in mouse ovaries and its target genes in ovarian granulosa cells. In situ hybridization and immunohistochemical staining showed that FXR was mainly distributed in secondary and tertiary follicles. The agonist-induced activation of FXR in cultured granulosa cells induced the expression of SHP and FABP6, while siRNA targeting of FXR decreased CYP19a1 and HSD17b1 expression. Upon examination of the roles of SHP and FABP6 in granulosa cells, we found that SHP overexpression significantly decreased StAR, CYP11a1, and HSD3b gene expression. In addition, siRNA targeting of FABP6 decreased CYP19a1 and HSD17b1 expression, while FABP6 overexpression increased CYP19a1 expression. In conclusion, the present study demonstrates the presence of FXR signaling in the ovary and reveals that FXR signaling may have a role in function of granulosa cells.

Key words: Farnesoid X receptor (FXR), Granulosa cell, Ovary, Steroidogenesis

Efficient production of eggs is important for the production of offspring, not only in agricultural industries, but also for the preservation of endangered animals. In the ovary, eggs are yielded as an output of oocyte growth through follicular growth and development. During follicular development, the interaction between an oocyte and the granulosa cells is crucial for normal oocyte growth. Many factors for oocyte growth are produced and regulated via the autocrine and/or paracrine pathways [1]. Various kinds of bile acids have been recently detected in human and bovine follicular fluid [2, 3], and the concentrations were 2-fold higher in follicular fluids than those in serum. Moreover, lactating cows have a 3-fold higher concentration of total bile acids in both follicular fluid and plasma, than that in heifers [4], and several enzymes in the bile acid biosynthesis pathway are present in both granulosa cells and oocytes [5].

Originally, the synthesis of bile acids was shown to be regulated in the liver by FXR. Bile acids serve as natural ligands for FXR, and activated FXR subsequently activates the transcription of several target genes, such as SHP (small heterodimer partner) and FABP6 (fatty acid-binding protein 6). SHP binds to and represses the transcriptional activities of LRH-1 (liver receptor homolog 1), which induces Cyp7a1 (cytochrome P450 7A1) expression, a liver-specific cholesterol 7α-hydroxylase [6], causing a reduction in bile acid synthesis [7, 8]. FABP6, an intracellular protein expressed in the distal ileum, plays a role in transcellular shuttling of bile acids [9–14]. Bile acid-FXR signaling and its target genes represent major regulatory factors for bile acid metabolism in enterohepatic tissues.

Recent studies have revealed the existence of FXR in non-enterohepatic tissues, such as bone marrow [15, 16], brain neurons [17], cardiomyocytes [18], blood vessels [19], and male reproductive tissues [20–22]. In the testis, the activation of FXR by bile acids affects steroidogenesis, which significantly decreases the production of sex steroids in porcine Leydig cells [23]. In addition, FXR competitively binds to the steroidogenic factor 1 response element, which regulates aromatase expression in tumor Leydig cells [21]. Although evidence for bile acid-FXR signaling in males has been established, information on this signaling in females is limited.

To investigate the role of FXR in the ovary, we examined its expression, with a focus on the follicles, and the potential activity of FXR signaling in granulosa cells. To our knowledge, this is the first study that shows the evidence for the involvement of FXR signaling in the function of ovarian granulosa cells.
Materials and Methods

Animals
All experiments were conducted in accordance with the institutional guidelines established by Shinshu University for the care and use of laboratory animals, and approved by the ethics committee for animal research of Shinshu University, Japan. ICR mice were purchased from Japan SLC (Shizuoka, Japan) and housed in a temperature-controlled room under a 12-h/12-h light-dark cycle.

Production of ovary specimens
Adult female mice at the age of 10–12 weeks were euthanized by cervical dislocation, and the ovaries were collected and fixed in 4% paraformaldehyde in phosphate-buffered saline solution (PBS) for 24 h, followed by embedding in paraffin. Five-micrometer-thick sections were prepared from each ovary, mounted on silane-coated glass slides, and analyzed.

In situ hybridization
To determine the localization of FXR mRNA in the ovaries, in situ hybridization using an antisense oligonucleotide probe labeled at the 5′ end with digoxigenin (DIG), was performed as previously described [24]. Briefly, the glass slides containing ovary sections were placed on a warm plate and dried overnight at 39°C. The next day, specimens were dewaxed in four xylene baths for 5 min each, and rehydrated in a descending alcohol series using distilled water. The slides were then immersed in PBS. The sequence of the FXR antisense probe was 5′-CAGAGCGTACTCCTCTGAGTC-3′, and the sense probe sequence was 5′-GACTCAGGAGGAGTACGCTCTG-3′. All hybridization, washing, and visualization steps were performed using a commercial kit (IsHyb In Situ Hybridization Kit; Biochain Institute, Newark, CA, USA), according to the manufacturer's instructions. After hybridization, the sections were observed using an IX73 inverted microscope and a DP73 microscope digital camera (Olympus, Tokyo, Japan).

Immunohistochemical staining
For antigen retrieval, the slides were placed in vertical staining jars containing HistoVT One (Nacalai tesque, Tokyo, Japan) and autoclaved at 121°C for 15 min. The jars were cooled at room temperature for 30 min and rinsed with PBS. Endogenous peroxidase activity was blocked by adding 30% hydrogen peroxide at a 1:100 dilution in methanol for 20 min, after which slides were washed in PBS three times. To reduce non-specific staining, the slides were incubated in PBS containing 10% Blocking One Histo (Nacalai tesque) at room temperature for 2 h and then washed with PBS three times in PBS. The color was developed with a freshly prepared solution of 3,3’-diaminobenzidine (DAB) Peroxidase Stain DAB Kit (brown stain) (Nacalai tesque), and the sections were dehydrated and mounted. In the negative control, the sections were incubated with PBS instead of the specific primary antibody.

Collection and culture of granulosa cells
Immature mice at the age of 3 weeks were intraperitoneally injected with 5 IU of pregnant mare serum gonadotropin (PMSG, Aska Animal Health, Tokyo, Japan) followed by 5 IU of human chorionic gonadotropin (hCG, Aska Animal Health, Tokyo, Japan) 48 h later. At 0, 24, 48, and 60 h after PMSG injection, mice were euthanized by cervical dislocation, and granulosa cells were collected from ovarian follicles by puncturing them using a 26G needle and subjected to further analysis. In experiments for cell culture, granulosa cells collected at 24 h after PMSG injection were washed twice in PBS before culturing. The cells were placed onto a gelatin-coated dish and cultured in DMEM containing 1% fetal bovine serum (Sigma-Aldrich Japan, Tokyo, Japan) and 1% Insulin-Transferrin-Selenium mixture (ITS-G, Wako, Tokyo, Japan). Four to six days after culture, the confluent cells were passaged in a 12-well plate dish, and subjected to the experiments for ligand supplementation and gene knockdown. In ligand supplementation experiments, the cells were cultured for 24 h in either a medium supplemented with 50 μM chenodeoxycholic acid (CDCA, Nacalai tesque) as a natural ligand for FXR, 1 μM GW4064 (AdooQ BioScience, Irvine, CA, USA) as an agonist of FXR, or 10 μM Z-guggulsterone (Sigma) as an antagonist of FXR, and then subjected to further analysis.

siRNA knockdown of FXR, SHP, and FABP6
The cultured granulosa cells were transfected with either of 15 nM negative control siRNA (universal negative control siRNA, Nippongene, Tokyo, Japan), FXR siRNA (5′-uguucuguauacacacuccuTT-3′, Nippongene), SHP siRNA (5′-ucagcugagugcuugcTT-3′, Nippongene), or FABP6 siRNA (5′-uugguaugacacacacagaaTT-3′, Nippongene) using Lipofectamine RNAi-MAX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' instructions. After treatment with siRNA, granulosa cells were cultured in a medium with or without GW for 24 h.

Construction of the SHP and FABP6 overexpression vectors and lentiviral vector preparation
Mouse SHP (NM_011850.3) and FABP6 (NM_008375.2) were cloned from the mRNA of granulosa cells. A marker gene encoding Venus protein was linked by an internal ribosomal entry site (IRES), and the resultant constructs were inserted into a self-inactivating lentiviral vector carrying the CMV promoter (RIKEN, Tsukuba, Japan). Lentiviral vectors were produced as previously described [3]. Briefly, lentiviruses were generated by co-transfection of lentiviral vectors coding for CMV-SHP-IRES-Venus or CMV-FABP6-IRES-Venus, pCAG-HIVgp (RIKEN), and pCMV-VSV-G (RIKEN), into 293FT packaging cells (Invitrogen, Thermo Fisher Scientific). The medium containing viral particles was spun at 50,000 × g for 2 h at 4°C, and the viral pellet was resuspended in DMEM in 1/100 of the volume of the original lentiviral vector supernatant. The granulosa cells collected were seeded at 1 × 10^5 cells per well in a 12-well plate.
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RNA extraction and reverse transcription (RT)

RNA was isolated from granulosa cells using the NucleoSpin RNA mini kit (Takara, Tokyo, Japan) according to the manufacturer’s instructions. First-strand cDNA was synthesized from RNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Tokyo, Japan). As a negative control, RNA was allowed to react with the cDNA synthesis reaction mixture in the absence of reverse transcriptase.

PCR analysis

We used a panel of specific primers to detect the expression of each of the four FXR isoforms: α-Fwd and 1-Rev for FXRa1, α-Fwd and 2-Rev for FXRa2, β-Fwd and 1-Rev for FXRβ1, and β-Fwd and 2-Rev for FXRβ2 (Table 1). The cDNAs were subjected to PCR for 40 cycles of 98°C for 10 sec, 64°C for 30 sec, and 72°C for 30 sec using EmeraldAmp PCR Master Mix (Takara, Tokyo, Japan) according to the manufacturer’s instructions. PCR products were electrophoresed on a 1% agarose gel at 100 V for 35 min.

Real-time PCR analysis

To quantify the relative expression of total FXR, ex8-Fwd and ex9-Rev primers were used (Table 1). To detect GAPDH expression, GAPDH-Fwd and GAPDH-Rev primers were used (Table 1). The cDNAs were subjected to real-time PCR for 45 cycles of 98°C for 10 sec, 60°C for 15 sec, and 72°C for 15 sec using Ti-SYBR Green (Takara) and a LightCycler Nano (Roche, Basel, Switzerland) according to the manufacturer’s instructions. All real-time PCR results were normalized with the expression of GAPDH, and the relative transgene expression levels were shown as the mean ± standard errors (SE) of at least three repeats in each experimental group.

Statistical analysis

Statistical analysis was performed using a Dunnett’s multiple comparison test with a significance of P < 0.05.

Results

Distribution of FXR in the ovary

To examine the presence of FXR in the ovary, we performed in situ hybridization of FXR mRNA (Fig. 1A–C) and immunohistochemical staining for the FXR protein (Fig. 1D–F) in ovarian sections of adult mice. Both analyses showed that FXR was mostly distributed in secondary and tertiary follicles, particularly within granulosa cells, but not in the regressive corpus luteum and corpus albicans. From these results, we focused on granulosa cells in the following experiments.

Expression of FXR isoforms and time dependent gene expression of FXR in granulosa cells

Four isoforms of FXR mRNA have been identified (Fig. 2A), with each regulating its own target genes [25]. Therefore, we first identified the FXR isoforms that were expressed in granulosa cells. RT-PCR analysis showed that granulosa cells expressed FXRa1 and α2, but not β1 and β2, whereas liver cells expressed FXRa2 and β2, and intestine expressed FXRβ1 and β2 (Fig. 2B). These results showed that FXRa1 is specific to granulosa cells, and that these cells strongly expressed FXRa2 similar to levels seen in the liver. We next investigated the time-dependent gene expression of FXR in ovarian granulosa cells after PMSG treatment. A total FXR expression in granulosa cells increased at 24 and 48 h after PMSG treatment by 23.9- and 8.7-fold, compared with that at 0 h, respectively.

Table 1. Primer sequences used in the present study

| Gene | Primer name | Primer sequences |
|------|-------------|------------------|
| FXR  | α-Fwd       | 5'- ggcctaaagctcaagagt -3' |
|      | β-Fwd       | 5'- ctaaggtgctgtcatcagtc -3' |
|      | 1-Rev       | 5'- gtttaaaacagctgctcaatc -3' |
|      | 2-Rev       | 5'- tctggtaagctgctcattgc -3' |
|      | ex8-Fwd     | 5'- gaaacttcctgccggtcatgc -3' |
|      | ex9-Rev     | 5'- ttcagttaacaaacattcagcc -3' |
| SHP  | Forward     | 5'- gccaggtgaaggcaagtctgtg -3' |
|      | Reverse     | 5'- aacgcctcatagtcacactc -3' |
| FABP6 | Forward    | 5'- gcaagaagttcaagcgttcctgt -3' |
|      | Reverse     | 5'- ctaaggatggtgatgcagtttc -3' |
| STAR | Forward     | 5'- ttcacgcgttagtgcattc -3' |
|      | Reverse     | 5'- tctggtaagctgctcattgc -3' |
| CYP19A1 | Forward   | 5'- ggtcggaaagcaagtctgag -3' |
|       | Reverse     | 5'- gcatgaccaagtctcacaacag -3' |
| HSD3B | Forward     | 5'- tccatccagcgagcaacctc -3' |
|       | Reverse     | 5'- cccctgcacaactggtcagc -3' |
| CYP11A1 | Forward   | 5'- acctgcgttggcttattcgct -3' |
|       | Reverse     | 5'- gcatgaccaagtctcacaacag -3' |
| HSD17B1 | Forward   | 5'- tggctcggttcggttcgg -3' |
|       | Reverse     | 5'- cgcctctctctctctcttc -3' |
| GAPDH | Forward     | 5'- ittggcattggtggaaggcctc -3' |
|       | Reverse     | 5'- ctaacgcctcagtcggtcttc -3' |

FXR signaling in granulosa cells

To investigate the role of FXR signaling in granulosa cells, we examined the expression of the known FXR target genes, SHP and FABP6. We treated granulosa cells with either of natural ligand (CDCA), agonist (GW), or antagonist (Gug) of FXR. As a result, FXR, SHP, and FABP6 expression were significantly increased by GW treatment, whereas the expression of each gene was decreased after Gug treatment (Fig. 3A–C). After GW treatment, SHP and FABP6 expression were significantly increased at 7.1 and 24.4-fold that of the control, respectively. After CDCA treatment, they were 2.0- and 1.7-fold higher than controls, respectively, although, this was not statistically significant. These results showed a possible involvement of FXR signaling in ovarian granulosa cells.

To further confirm that FXR mediates GW-induced upregulation of SHP and FABP6, we transfected granulosa cells with FXR siRNA prior to treatment with GW. As shown in Fig. 3E and 3F, knockdown of FXR significantly inhibited the increase of SHP and FABP6 expression by GW treatment. As the present experiment showed that FXR could activate the expression of its already-known target genes in ovarian...
granulosa cells, we were interested to know how FXR functions on granulosa cells. We therefore investigated whether FXR has any role in steroid synthesis, as it is known that steroid synthesis is one of the main functions of granulosa cells. As a first step, we analyzed the expression of progesterone synthesis-related genes (StAR, CYP11a1, and HSD3b), and estradiol synthesis-related genes (CYP19a1 and HSD17b1) by quantitative RT-PCR. As shown in Fig. 3G, FXR siRNA transfection had no effect on the expression of progesterone synthesis-related genes, whereas expression of both CYP19a1 and HSD17b1 were significantly decreased.

Involvement of the FXR target genes, SHP and FABP6, in steroid synthesis

From the experiments shown in Fig. 3, it was revealed that FXR has a possible role in the expression of estradiol synthesis genes. As FXR was able to activate expression of both SHP and FABP6 in granulosa cells, we next sought to investigate the FXR targeted genes that are committed in that cascade. For this, we investigated the direct effects of SHP or FABP6 on progesterone- or estradiol-related gene expression. As shown in Fig. 4A, knockdown of SHP had no effect on either progesterone or estradiol synthesis-related gene expression, although SHP overexpression significantly inhibited StAR, CYP11a1, and HSD3b gene expression, but without any effect on HSD17a or CYP19a1 gene expression (Fig. 4B). In contrast, as shown in Fig. 4C, FABP6 siRNA transfection only decreased the expression of estradiol-related genes. After FABP6 overexpression, only CYP19a1 gene expression was significantly increased (Fig. 4D).

Discussion

Although recent studies have revealed evidence for the function of FXR in the testis and in male fertility [21, 22], little information on...
the function of FXR signaling in females has been reported. In the present study, we showed evidence for the existence of FXR signaling in the ovary. In situ hybridization and immunohistochemistry both revealed that FXR is distributed mainly in secondary and tertiary follicles, particularly within granulosa cells. In addition, we clarified that the FXRα1 and α2 isoforms are expressed in granulosa cells. Since Vaquero et al. reported that each isoform regulates its own target genes [25], and that FXRα2 is strongly expressed in liver, the present finding suggests the possibility that ovarian FXR may be involved in cholesterol metabolism, as in the liver. Moreover, the present study showed that FXR gene expression increases significantly 24 h after PMSG treatment, and that knockdown of FXR significantly decreases estrogen synthesis-related gene expression in ovarian granulosa cells. FXR, originally identified as an orphan nuclear hormone receptor [26], regulates bile acid synthesis, fat metabolism, and glucose homeostasis in enterohepatic tissues. Bile acids bind FXR as physiologically relevant ligands, and then the activated FXR induces the expression of many target genes. In virgin and pregnant rabbits [20], it was shown by immunostaining that FXR is present in ovarian follicles. In addition, recent studies showed the existence of various

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Fig. 2. Expression of FXR isoforms in ovarian granulosa cells. (A) FXR isoforms according to differences in the initial region (exons 1–3) of mRNA (α and β) and the presence (1) or absence (2) of a 12-bp insert in exon 4. Exon 1–3: Transactivation-independent domain. Exon 4: DNA-binding domain. Exon 5–6: Hinge region. Exon 7–10: Ligand-binding domain, transactivation-dependent domain. Black: A 12-bp insert. (B) Gene expression of FXR isoforms in ovarian granulosa cells, liver, and intestine. (C) Time-dependent gene expression of FXR in granulosa cells.
kinds of bile acids in follicular fluid, with higher concentrations than those observed in serum [2, 3]. Moreover, several bile acid biosynthesis pathway enzymes are present in both granulosa cells and oocytes [5]. Therefore, together with the present finding that FXR exists in follicles, particularly within granulosa cells, it seems that bile acid-FXR signaling acts upon the function of granulosa cells. Furthermore, in bovine, follicle size is correlated with the concentration of bile acids and the molecular subspecies are different.

Fig. 3. FXR signaling in granulosa cells. (A, B, and C) Relative gene expression levels of FXR, SHP, and FABP6 in granulosa cells treated with 50 μM CDCA, 1 μM GW4064, or 10 μM guggulsterone (Gug) for 24 h. * P < 0.05 compared with the control (Ctl). (D, E, and F) Relative gene expression levels of FXR, SHP, and FABP6 in granulosa cells with FXR siRNA (siFXR) or negative control siRNA (siNC) treatment, with or without GW4064 for 24 h. * P < 0.05 between siNC and siFXR in GW treatments. (G) Effect of siRNA targeting of FXR on the expression of steroid synthesis-related genes. In each graph, the gene expression level in the Ctl or siNC group is presented as 1. Data are shown as the mean ± SE from at least three independent experiments.
between follicles [3], suggesting a relationship between bile acid-FXR signaling and follicle growth. This speculation may be supported by the finding of a positive relationship between the concentration of bile acids and oocyte quality [2].

In this study, *in vitro* experiments with the natural ligand and agonist showed that in granulosa cells, FXR upregulates *SHP* and
**FABP6** gene expression as it does in enterohepatic tissues, but that the stimulatory levels were different between two drugs. This difference may be related to their optimal concentrations under *in vitro* conditions. In the present study, the concentration of CDCA used was 50 μM. One of the reasons for this may be that FXR in enterohepatic tissues binds to higher concentration of bile acid, and that more than 100 μM of bile acid is generally used in such tissue culture experiments, although bile acid concentration of human follicular fluid is about 10 μM [2], on which the present study was based. Besides bile acid acting as a natural ligand, there are likely to be other, unknown ligands in ovarian granulosa cells, such as sulfated progesterone metabolites [27] and androsterone [28].

It is known that FXR functions mainly via its target genes. When we examined the functions of FXR-targeted genes, knockdown of FXR with siRNA suppressed the expression of both *SHP* and *FABP6* by the agonist. In addition, such treatment had less of an effect on the expression of *CYP19a1* and *HSD17b1*, but not of *StAR*, *CYP11a1*, and *HSD3b*. In a further experiment involving direct suppression by siRNA, we found that there was no effect on steroidogenesis-related gene expression, except upon *CYP19a1* and *HSD17b1*. The present findings first showed that on ovarian granulosa cells, FXR functions via its targeted gene. In liver, FXR induces the expression of *SHP*, and *SHP* subsequently binds to and represses the transcriptional activities of *LRH-1*, inducing *Cyp7a1* gene expression [6], and resulting in the reduction of bile acid synthesis [7, 8]. In granulosa cells, cholesterol are converted to sex hormones by steroidogenic enzymes, whose expression are regulated by LRH-1 [29, 30]. LRH-1 is an essential factor for both ovulation and pregnancy [31, 32], and its transcriptional activity is repressed by SHP through direct binding [7, 8]. This suggests that the FXR-SHP cascade in granulosa cells downregulates progesterone synthesis-related genes via a repression of LRH-1-dependent transcriptional activity.

Besides the finding that treatment with *FABP6* siRNA suppressed the expression of both *CYP19a1* and *HSD17b1* in cultures of granulosa cells, the overexpression of *FABP6* upregulated the *CYP19a1* gene, which is an estrogen synthesis-related gene. Together with our previous reports that FABP6 is distributed in granulosa and luteal cells of rat ovaries [33–36], it seems quite probable that FXR-FABP6 is related to steroidogenesis in granulosa cells, especially in estrogen biosynthesis. FABP6 is an intracellular protein expressed in the distal ileum and plays a role in transcellular shuttling of bile acids [9–14]. Although the function of FABP6 is not fully elucidated, even in enterohepatic tissues, it may be possible that FABP6 has a role in transcellular shuttling of sex hormones in the ovary, as previous studies have demonstrated that FABP6 is involved in intracellular transport of bile acids in enterocytes [14, 37, 38]. In FABP6-deficient mice [39], the ovulation rate after supersululatory treatment was markedly decreased compared to that in wild-type mice, which suggests that FABP6 is involved in fertility via a function in granulosa cells.

In conclusion, the present study showed evidence for the existence of FXR signaling in ovarian granulosa cells. In addition, reproduction and metabolism are deeply related to each other; metabolic syndrome induces dysfunction of the ovary, such as menstrual abnormalities and ovulation disorder via an association with insulin resistance. FXR is a major regulatory factor for bile acid metabolism in enterohepatic tissues, and its deficiency results in abnormalities in glucose homeostasis as well as in insulin resistance. In addition, intestine-specific FXR activation corrects numerous obesity-related defects, enhances glucose tolerance, and lowers hepatic glucose production, indicating that these physiologic changes are dependent on FXR expression [40]. Therefore, besides the action of FXR signaling in granulosa cells revealed in the present study, FXR signaling may have multiple functions in ovarian events. These findings suggest that potential new therapeutic targets in ovarian-related diseases may be developed by investigating ovarian FXR signaling.

**Conflict of interests:** The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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