The fatty acid binding protein 6 gene (Fabp6) is expressed in murine granulosa cells and is involved in ovulatory response to superstimulation

Raj DUGGAVATHI1), Dayananda SIDDAPPA1), Yasmin SCHUERMANN1), Melissa PANSERA1), Isabelle J. MENARD2), Dana PRASLICKOVA2) and Luis B. AGELLON2)

1)Department of Animal Sciences, McGill University, Quebec H9X 3V9, Canada
2)School of Dietetics and Human Nutrition, McGill University, Quebec H9X 3V9, Canada

Abstract. The fatty acid binding protein 6 (Fabp6) is commonly regarded as a bile acid binding protein found in the distal portion of the small intestine and has been shown to be important in maintaining bile acid homeostasis. Previous studies have also reported the presence of Fabp6 in human, rat and fish ovaries, but the significance of Fabp6 in this organ is largely unknown. Therefore, we surveyed murine ovaries for Fabp6 gene expression and evaluated its role in ovarian function using mice with whole body Fabp6 deficiency. Here we show that the Fabp6 gene is expressed in granulosa and luteal cells of the mouse ovary. Treatment with gonadotropins stimulated Fabp6 gene expression in large antral follicles. The ovulation rate in response to superovulatory treatment in Fabp6-deficient mice was markedly decreased compared to wildtype (C57BL/6) mice. The results of this study suggest that expression of Fabp6 gene in granulosa cells serves an important and previously unrecognized function in fertility.

Key words: Bile acids, FABP, Fertility, Granulosa cells, Ovary, Ovulation, Reproduction, Steroid hormones (J. Reprod. Dev. 61: 237–240, 2015)

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories (Senneville, QC). The Fabp6–/– mice [10] used in this study were produced in-house and have been backcrossed to the C57BL/6 background for 8 generations. The mice were housed in a controlled environment with a 12 h light/dark cycle and were given free access to water and standard diet (2020X Teklad Global Rodent Diet). All mouse studies were approved by the Animal Care Committee of McGill University.

Superovulation and sample collection

Immature (23–25 days old) female mice were superovulated by administration of equine chorionic gonadotropin (eCG; 5 IU, i.p.) followed 48 h later by human chorionic gonadotropin (hCG; 5 IU, i.p.) [11]. These hormones function like follicle stimulating hormone and luteinizing hormone, respectively. Mice were euthanized at defined time-points during the gonadotropin-stimulated follicular and luteal development. Pure populations of granulosa cells were collected by follicular puncture as previously described [12]. Briefly, ovaries were placed in phosphate-buffered saline and large antral follicles were punctured using sterile 27.5 G needle. Ovaries were gently squeezed with a spatula to flush out remaining granulosa cells from punctured follicles. The cell suspension was passed through a cell strainer (40 µm, BD Falcon) to remove cumulus-oocyte complexes. Granulosa cells from both ovaries of each mouse were pooled for quantitative real-time PCR (qPCR) and immunoblot analyses. To determine ovulation rate, reproductive tracts were collected at 18 h post-hCG.
treatment. Ovulated oocytes were collected from both oviducts and counted as a measure of number of ovulations for each mouse.

**RNA and protein detection**

Total RNA was purified from ovaries using the TRIzol reagent (Life Technologies, Burlington, ON) and then converted to cDNA for qPCR analysis (Bio-Rad CFX384 system, Bio-Rad, Mississauga, ON). The pre-validated primers used were: Fapb6 [10]; aromatase (Cyp19a1), 5'-TGGAGAACAAATCGCCTTTC-3' and 5'-CCGAGGTGTCCGTAAGTTT-3'; prostaglandin synthase 2 (Ptgs2), 5'-TGAGCAACTTTCAACACCAGC-3' and 5'-GCACGTAAGTCTCTGATCAGATC-3'; succinate dehydrogenase complex A (Sdha), 5'-GGAACACTCTCCAAAAACAGACTT-3' and 5'-CCACACCTGTTATTGAGTAGAA-3'; β-2-microglobulin (B2m), 5'-TTCTGGTTCTTGGCTCAGCA-3' and 5'-CAGTATGTTGCTTCCATTTC-3'; glyceraldehyde-3-phosphate dehydrogenase (Gapdh), 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAAGACCTGATGTAGGTCA-3'; and ribosomal protein L19 (Rpl19), 5'-ATGAGTAGTACGCTGCTAAGCA-3' and 5'-GCATTGGCGGATATTGTATGTC-3'. The average combined abundance of Sdha, B2m, Gapdh and Rpl19 mRNAs was used to normalize the mRNA abundance of other genes. The thermal cycler profile used in the analyses is as follows: initial denaturation step of 5 min at 95°C, followed by 35 cycles of 15 sec at 95°C denaturation step and 30 sec at 58°C annealing/extension step.

For immunoblot analyses, protein extracts were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After blocking with 5% milk in TBS-T (50 mM Tris-HCL, pH 7.5; 150 mM NaCl; 0.05% Tween 20), the membranes were incubated overnight at 4°C with primary antibodies followed by washing with TBS-T (3 times, 10 min each) and incubation with secondary antibody (1:10000) for 1 h at room temperature. The immunoblotted proteins were detected using Immun-Star Kit and Chemidoc Analyzer (Bio-Rad). The antibodies used in the present study are as follows. The mouse Fabp6 antiserum (used at 1:40,000 dilution) was generated in rabbits using recombinant mouse Fabp6 as antigen [9]. Other antibodies (diluted 1:1000 as recommended by the suppliers) were purchased from commercial sources: anti-Star (sc-25806, Santa Cruz Biotechnology, Dallas, TX), anti-Aceb (ab8227, Abcam, Cambridge, MA). The secondary antibody, anti-rabbit IgG (HRP-conjugated) (ab6721, Abcam), was used at 1:10,000 dilution.

**Immunohistochemistry**

Ovaries collected from superovulated mice were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 4 µm sections (Leica RM2125RT). Following initial steps of deparaffinization, dehydration, antigen retrieval and blocking (Blocking solution consisted of 3% bovine serum albumin, 0.4% Triton X-100 and 10% normal goat serum), sections were incubated with or without murine Fabp6 anti-serum (1:500) [9] for 1 h. The slides were then washed with PBS and incubated with anti-rabbit secondary antibody (1:200) dilutions for 1 h. Immunoreactivity to Fabp6 antis serum (brown staining) was visualized using the Substrate DAB chromogen system (K3467, Dako, Burlington, ON). Sections were counterstained with hematoxylin to reveal cell nuclei (blue staining). All the incubations were performed at room temperature in a humidified chamber.

**Statistical analyses**

Analyses were done using SigmaPlot 12.3 software. Differences between mRNA abundance at different time points were evaluated by one-way analysis of variance followed by Tukey’s multiple comparisons post-hoc test. Ovulation rates were compared by unpaired Student’s t-test. The values were considered significant when P < 0.05.

**Results**

Detection and semi-quantitative estimation by conventional PCR using primers specific for the entire open reading frame of the murine Fabp6 mRNA sequence [9] demonstrated its presence in ovary of wild-type (C57BL/6) mouse, but at a lower abundance compared to ileum (Fig. 1A). The expression pattern of a gene allows us to speculate its regulation and function. Therefore, we used total RNA from granulosa and luteal cells collected at specific time-points during gonadotropin-induced follicular development and ovulation. Analysis of Cyp19a1 and Ptgs2 mRNA abundance by qPCR, in eCG and hCG dependent manner, confirmed that our cell populations represented precise stages of granulosa cell differentiation through follicular and luteal development.

The pattern of Fapb6 gene expression in response to gonadotropin (eCG and hCG) treatment indicated that maximal transcript abundance (P < 0.01) was at eCG-48h and hCG-1h (Fig. 1B). Thus, the results suggest that both gonadotropins stimulate Fapb6 gene expression. A previous report showed gonadotropin treatment of immature rat ovaries also increased Fapb6 expression [4], albeit this study measured Fapb6 mRNA abundance in the whole ovary at 48 h after hCG treatment. Our data further show that gonadotropin dependent induction of Fabp6 in granulosa cells followed the expression pattern of Cyp19a1, suggesting that its expression in follicular granulosa cells is estrogen dependent. The Fabp6 mRNA was detected in rat luteal cells in previous studies [3, 13, 14]. Likewise, it was detectable in mural luteal cells. Also, Park et al. [14] demonstrated that the transcription factor RUNX2 regulates Fabp6 expression in luteinizing granulosa cells.

Immunoblot analysis confirmed that the Fabp6 protein of the expected size is present in ovarian homogenates and granulosa cell lysates of C57BL/6 mice, but at a significantly lower level than in ileal homogenates (Fig. 2A). Our findings are consistent with the results of a previous report showing lower Fabp6 abundance in human ovary and placenta compared to the ileum [15]. We recently reported the creation of a mouse strain that lacks Fabp6 [10], and the Fabp6 protein was not detectable in ovarian homogenates of these mice (Fig. 2A).

To examine the localization of Fabp6 within the murine ovary, we analyzed thin sections of ovaries collected at specific time-points of follicle and luteal development by immunohistochemistry using our validated antiserum to murine Fabp6 [9] (Fig. 2B). Immunoreactivity was observed only in tissue sections incubated with anti-Fabp6, but not in negative control tissue sections. Granulosa cells of follicles at all stages and luteal cells of corpora lutea showed immunoreactivity to Fabp6 antibody. Fabp6 was abundant in the cytoplasm of the...
granulosa, cumulus and luteal cells compared to the cytoplasm of theca cells, interstitial cells and oocytes which showed very little or no immunoreactivity. Granulosa cells close to the antrum showed more immunoreactivity compared to those near the basement membrane. Our data partially agree with the previous report [13], which showed Fabp6 in corpora lutea of unstimulated rats of unknown estrous cycle stage. It is also worth mentioning that we used a validated Fabp6 antibody and pure cell populations to determine the presence of Fabp6 protein in specific cell-types. Overall, these results provide in vivo evidence for the expression of Fabp6 specifically in granulosa and luteal cells.

To determine if Fabp6 plays a role in ovarian function, we compared the ovulatory response of C57BL/6 mice and Fabp6–/– mice to superovulatory treatment with eCG and hCG. Mean body weight of mice of both genotypes was similar (P > 0.05; Fig. 3A) on the day of eCG treatment suggesting that overall growth and development of Fabp6–/– mice was normal. The number of oocytes ovulated in Fabp6–/– mice was significantly lower (P < 0.03) than C57BL/6 mice (Fig. 3B). Nonetheless, the ovulated cumulus-oocyte-complexes of both genotypes appeared normal with expanded cumulus cell layers and mature oocytes. These data suggest that Fabp6 plays a significant role in granulosa cell differentiation required for follicular rupture, but is dispensable for meiotic maturation of the oocyte.
Discussion

It is generally accepted that Fabp6 (also known as ileal lipid binding protein; ileal bile acid binding protein) is involved in intracellular transport of bile acids in enterocytes [10, 16]. However, the Fabp6 mRNA and protein have also been detected in the ovaries of several species [3, 14, 15, 17–19], albeit the precise tissue localization and expression pattern in dynamic structures of the ovary were not established. Further, the importance of this protein in ovarian function remains unknown since the ovary is not a major site for bile acid metabolism. In the present study, we surveyed murine ovaries for Fabp6 distribution and explored its potential importance in ovarian function.

Fabp6 is known to preferentially bind bile acids but the ovaries are not regarded as an active site for bile acid metabolism, although a recent study reported that some genes involved in bile acid synthesis are expressed in human granulosa cells [20]. We reported recently that bile acids are found in the follicular fluid of the dominant follicle and that granulosa cells of the dominant follicles express SLC10A2 and GPBAR1 (bile acid transporter and receptor, respectively) [21], suggesting the novel idea that bile acid signaling might be occurring in ovarian cells.

On the other hand, Fabp6 has been shown to be capable of binding other ligands, including fatty acids [8, 9] and progesterone [17], and therefore may be involved in metabolic pathways beyond bile acid metabolism. It is well established that the progesterone receptor is induced in granulosa cells of ovulating follicles by hCG and mice lacking this receptor do not ovulate [22]. Along with these observations, our data showing high mRNA abundance of Fabp6 at eCG-48h and hCG-1h are indicative of its role in progesterone signaling, which is needed for efficient ovulation. Interestingly, despite the attenuated ovulation response displayed by Fabp6−/− mice to superovulatory stimulation, they do not exhibit overt defects in fertility or fecundity [10]. The loss of Fabp6 function may not seriously impact the overall fecundity of polytocous animals (such as mice), but may be important for species that release only one egg per cycle (such as humans). Detailed understanding of mechanisms by which Fabp6 influences the oocyte development requires further research.

In summary, we demonstrated here that Fabp6 is present in distinct cell populations within the murine ovaries and loss of Fabp6 results in marked reduction in ovulatory response to superstimulation. These findings suggest that Fabp6 may have important roles in ovarian physiology that are distinct from its function as an intracellular bile acid transporter in the ileum.

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