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—Original Article—

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

Raj DUGGAVATHI[1], Dayananda SIDDAPPA[1], Yasmin SCHUERMANN[1], Melissa PANSERA[1], Isabelle J. MENARD[2], Dana PRASLICKOVA[2] and Luis B. AGELLON[2]

[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

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Fatty acid binding proteins are small, soluble, cytoplasmic proteins that are known to bind hydrophobic molecules [1, 2]. The fatty acid binding protein gene family consists of multiple members encoded by distinct genes and are distributed in different organs of the body [1, 2]. Fabp3 was initially detected in ovaries [3, 4], but it was later localized in macrophages of advanced atretic follicles, where this protein may be involved in the process of atresia [5]. Fabp4 is localized in granulosa cells of atretic follicles and may be involved in apoptosis of those cells potentially through its interaction with peroxisome proliferator-activated receptor γ [6]. In addition, Fabp4 variants are associated with polycystic ovary syndrome [7]. Fabp6 is generally regarded as the bile acid binding protein found in the distal portion of the small intestine, although it has been shown to be capable of also binding fatty acids [8, 9]. Though its expression has been reported in the ovary, the precise localization, regulation and the importance of *Fabp6* gene expression in the ovaries is unknown. Here, we examined the expression pattern of *Fabp6* within the murine ovary and assessed whether it plays an important role in ovarian function.

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Correspondence: L Agellon (e-mail: luis.agellon@mcgill.ca) or R Duggavathi (e-mail: raj.dugghavathi@mcgill.ca)
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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: *Fabp6* [10]; aromatase (*Cyp19a1*), 5'-TGGAGAACAATTCGCCCTTTC-3' and 5'-CCGAGGTGTCGGTGACTTC-3'; prostaglandin synthase 2 (*Ptgs2*), 5'- TGAGCAACTATTCCAAACCAGC-3' and 5'-GCACGTAGTCTTCGATCACTATC-3'; succinate dehydrogenase complex A (*Sdha*), 5'-GGAACACTCCAAAAACAGACCT-3' and 5'-CCACCACTGGGTATTGAGTAGAA-3'; β-2-microglobulin (*B2m*), 5'-TTCTGGTGCTTGGTCTCACTGA-3' and 5'-CAGTAT GTTCGGCTTCCCATTC-3'; glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-TGTAGACCATGTAGTTGAGGTCA-3'; and ribosomal protein L19 (Rpl19), 5'-ATGAGTATGCTCAGGCTACAGA-3' and 5'-GCATTGGCGATTTCATTGGTC-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-Actb (ab8227, Abcam, Cambridge, MA). The secondary antibody, goat 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 antiserum (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 *Fabp6* 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 *Fabp6* gene expression. A previous report showed gonadotropin treatment of immature rat ovaries also increased *Fabp6* expression [4], albeit this study measured *Fabp6* 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 murine luteal cells. Also, Park *et al*. [14] demonstrated that the transcription factor RUNX2 regulates *Fapb6* 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). Immnuoreactivity 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

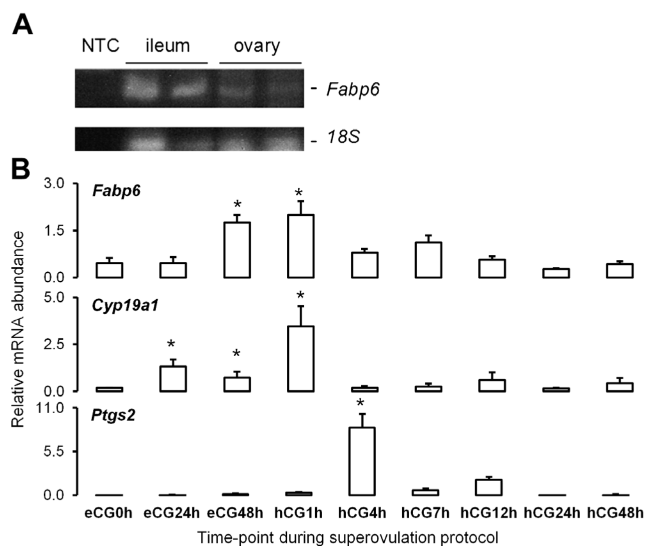

Fig. 1. Detection of *Fabp6* mRNA in the wild-type (C57BL/6) murine ovary. (A) The *Fabp6* open reading frame was amplified by conventional PCR to detect the *Fabp6* mRNA in murine ovary and ileum. Primers specific for the murine 18S ribosomal RNA sequence were used to indicate the presence of templates in the reaction. NTC, no template control. (B) Relative mRNA abundance (mean ± SEM; n = 4) of *Fabp6*, *Cyp19a1* and *Ptgs2* in pure populations of granulosa and luteal cells isolated from ovaries at specific time points during superovulation protocol as described in the Materials and Methods section. Relative mRNA abundance was determined by normalization to the average abundance of *Sdha*, *B2m*, *Gapdh* and *Rpl19* mRNAs. *denotes significant difference ($P < 0.05$) from eCG-0h.

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 *Fapb6*$^{-/-}$ 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.

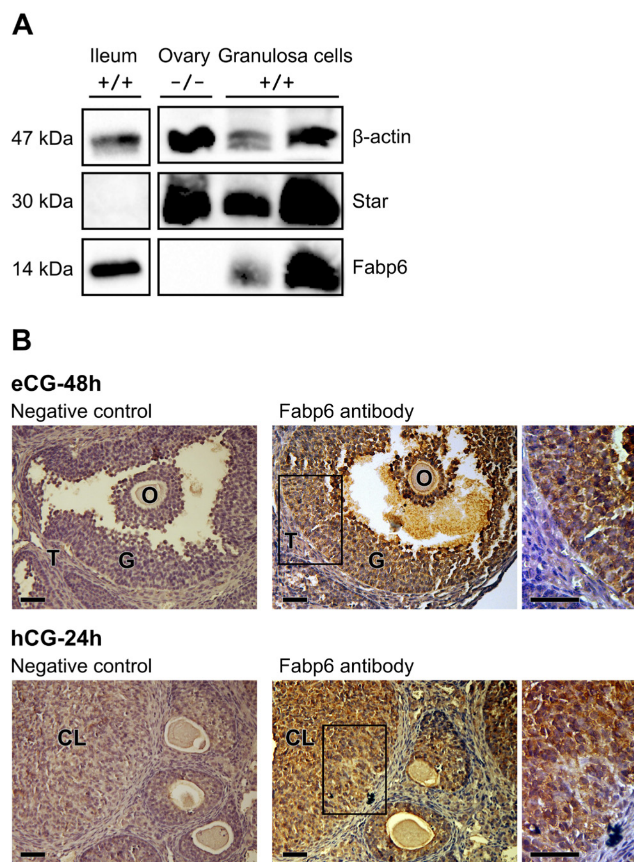

Fig. 2. Detection of Fabp6 protein in the murine ovary. (A) Homogenates of C57BL/6 mouse (+/+) ileum (8 µg), *Fabp6*$^{-/-}$ mouse ovary (100 µg; collected at 24 h after hCG) and purified C57BL/6 granulosa cells (collected at 4 h after hCG) were analyzed by immunoblotting using antiserum against recombinant murine Fabp6 [9]. Antibodies against β-actin (Actb) and steroidogenic acute regulatory protein (Star) were used as controls. (B) Immunohistochemical detection of Fabp6 in paraffin sections of the ovaries collected at specific time points of superovulation treatment from immature C57BL/6 mice. Negative control tissue sections (left) collected 48 h after eCG treatment and 24 h after hCG treatment were not incubated with anti-Fabp6. G, granulosa cells; T, theca cells; O, oocyte; CL, corpus luteum. Scale Bar, 20 µm.

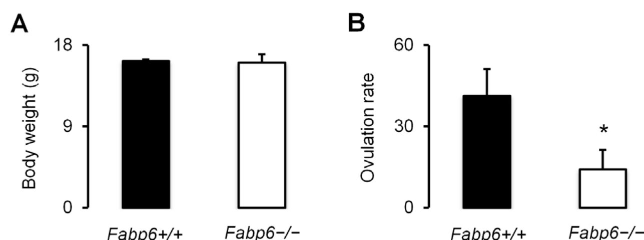

Fig. 3. Ovulatory response in mice lacking Fabp6. (A) Mean body weight of mice on the first day of superovulation treatment protocol. (B) Mean number of ovulations in response to superovulation treatment in C57BL/6 (n = 4) and *Fabp6*$^{-/-}$ (n = 7) mice as described in the Materials and Methods section. The number of oocytes in oviducts of each mouse was determined at 18–20 h after hCG stimulation. Values shown are mean ± SEM. * $P < 0.03$.

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 of lactating cows 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 Fapb6 influences the ovulatory process 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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References

1. **Furuhashi M, Hotamisligil GS.** Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets. *Nat Rev Drug Discov* 2008; **7**: 489–503. [Medline] [CrossRef]
2. **Smathers RL, Petersen DR.** The human fatty acid-binding protein family: evolutionary divergences and functions. *Hum Genomics* 2011; **5**: 170–191. [Medline] [CrossRef]
3. **Iseki S, Amano O, Kanda T, Fujii H, Ono T.** Expression and localization of intestinal 15 kDa protein in the rat. *Mol Cell Biochem* 1993; **123**: 113–120. [Medline] [CrossRef]
4. **Sato E, Fujii H, Fujita M, Kanda T, Iseki S, Hatakeyama K, Tanaka T, Ono T.** Tissue-specific regulation of the expression of rat intestinal bile acid-binding protein. *FEBS Lett* 1995; **374**: 184–186. [Medline] [CrossRef]
5. **Nourani MR, Owada Y, Kitanaka N, Abdelwahab SA, Iwasa H, Sakagami H, Spener F, Kondo H.** Localization of epidermal-type fatty acid binding protein in macrophages in advanced atretic follicles of adult mice. *J Mol Histol* 2005; **36**: 391–400. [Medline] [CrossRef]
6. **Nourani MR, Owada Y, Kitanaka N, Sakagami H, Hoshi H, Iwasa H, Spener F, Kondo H.** Occurrence of immunoreactivity for adipocyte-type fatty acid binding protein in degenerating granulosa cells in atretic antral follicles of mouse ovary. *J Mol Histol* 2005; **36**: 491–497. [Medline] [CrossRef]
7. **Wang J, Tang J, Wang B, Song J, Liu J, Wei Z, Zhang F, Ma X, Cao Y.** FABP4: a novel candidate gene for polycystic ovary syndrome. *Endocrine* 2009; **36**: 392–396. [Medline] [CrossRef]
8. **Miller KR, Cistola DP.** Titration calorimetry as a binding assay for lipid-binding proteins. *Mol Cell Biochem* 1993; **123**: 29–37. [Medline] [CrossRef]
9. **Labonté ED, Li Q, Kay CM, Agellon LB.** The relative ligand binding preference of the murine ileal lipid binding protein. *Protein Expr Purif* 2003; **28**: 25–33. [Medline] [CrossRef]
10. **Praslickova D, Torchia EC, Sugiyama MG, Magrane EJ, Zwicker BL, Kolodzieyski L, Agellon LB.** The ileal lipid binding protein is required for efficient absorption and transport of bile acids in the distal portion of the murine small intestine. *PLoS ONE* 2012; **7**: e50810. [Medline] [CrossRef]
11. **Duggavathi R, Volle DH, Mataki C, Antal MC, Messaddeq N, Auwerx J, Murphy BD, Schoonjans K.** Liver receptor homolog 1 is essential for ovulation. *Genes Dev* 2008; **22**: 1871–1876. [Medline] [CrossRef]
12. **Siddappa D, Kalaiselvanraja A, Bordignon V, Dupuis L, Gasperin BG, Roux PP, Duggavathi R.** Mechanistic target of rapamycin (MTOR) signaling during ovulation in mice. *Mol Reprod Dev* 2014; **81**: 655–665. [Medline] [CrossRef]
13. **Gong YZ, Kato T, Schwartz DA, Norris JS, Wilson FA.** Ontogenic and glucocorticoid-accelerated expression of rat 14 kDa bile acid-binding protein. *Anat Rec* 1996; **245**: 532–538. [Medline] [CrossRef]
14. **Park ES, Lind AK, Dahm-Kähler P, Brännström M, Carletti MZ, Christenson LK, Curry TE Jr, Jo M.** RUNX2 transcription factor regulates gene expression in luteinizing granulosa cells of rat ovaries. *Mol Endocrinol* 2010; **24**: 846–858. [Medline] [CrossRef]
15. **Fujita M, Fujii H, Kanda T, Sato E, Hatakeyama K, Ono T.** Molecular cloning, expression, and characterization of a human intestinal 15-kDa protein. *Eur J Biochem* 1995; **233**: 406–413. [Medline] [CrossRef]
16. **Zwicker BL, Agellon LB.** Transport and biological activities of bile acids. *Int J Biochem Cell Biol* 2013; **45**: 1389–1398. [Medline] [CrossRef]
17. **Gong YZ, Everett ET, Schwartz DA, Norris JS, Wilson FA.** Molecular cloning, tissue distribution, and expression of a 14-kDa bile acid-binding protein from rat ileal cytosol. *Proc Natl Acad Sci USA* 1994; **91**: 4741–4745. [Medline] [CrossRef]
18. **Fujii H, Nomura M, Kanda T, Amano O, Iseki S, Hatakeyama K, Ono T.** Cloning of a cDNA encoding rat intestinal 15 kDa protein and its tissue distribution. *Biochem Biophys Res Commun* 1993; **190**: 175–180. [Medline] [CrossRef]
19. **Alves-Costa FA, Denovan-Wright EM, Thisse C, Thisse B, Wright JM.** Spatio-temporal distribution of fatty acid-binding protein 6 (fabp6) gene transcripts in the developing and adult zebrafish (Danio rerio). *FEBS J* 2008; **275**: 3325–3334. [Medline] [CrossRef]
20. **Smith LP, Nierstenhoefer M, Yoo SW, Penzias AS, Tobiasch E, Usheva A.** The bile acid synthesis pathway is present and functional in the human ovary. *PLoS ONE* 2009; **4**: e7333. [Medline] [CrossRef]
21. **Sanchez R, Schuermann Y, Gagnon-Duval L, Baldassarre H, Murphy BD, Gevry N, Agellon LB, Bordignon V, Duggavathi R.** Differential abundance of IGF1, bile acids, and the genes involved in their signaling in the dominant follicle microenvironment of lactating cows and nulliparous heifers. *Theriogenology* 2014; **81**: 771–779. [Medline] [CrossRef]
22. **Robker RL, Russell DL, Espey LL, Lydon JP, O'Malley BW, Richards JS.** Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. *Proc Natl Acad Sci USA* 2000; **97**: 4689–4694. [Medline] [CrossRef]