Journal of Reproduction and Development, Vol. 67, No 3, 2021

—Original Article—

Sequential preovulatory expression of a gonadotropin-releasing hormone-inducible gene, *Nr4a3*, and its suppressor *Anxa5* in the pituitary gland of female rats

Ryota TERASHIMA[1)], Titaree LAOHARATCHATATHANIN[2)], Shiro KURUSU[1)] and Mitsumori KAWAMINAMI[1, 3)]

[1)]*Laboratory of Veterinary Physiology, School of Veterinary Medicine, Kitasato University, Aomori 034-8628, Japan*
[2)]*Clinic for Small Domestic Animals and Radiology, Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok 10530, Thailand*
[3)]*Laboratory of Veterinary Physiology, School of Veterinary Medicine, Okayama University of Science, Ehime 794-8555, Japan*

Abstract. Functional relationship between nuclear receptor subfamily 4 group A member 3 (*Nr4a3*) and annexin A5 (*Anxa5*), which are two gonadotropin-releasing hormone (GnRH)-inducible genes, has been established while evaluating pituitary gonadotropes in relation to follicle-stimulating hormone beta (*Fshb*) expression. However, the physiological variations that arise due to the differential expression of these genes in the pituitary gland during rat estrous cycle remain unknown. This study aimed to evaluate the *Nr4a3* and *Anxa5* mRNA expression during the estrous cycle in rats in comparison with the expression of the gonadotropin subunit genes, luteinizing hormone beta (*Lhb*) and *Fshb*. *Nr4a3* mRNA expression showed a single peak at 1400 h of proestrus during the 4-d estrous cycle. *Anxa5* mRNA level was elevated along with increased *Fshb* mRNA expression after the decline of *Nr4a3* mRNA until 2300 h. *Lhb* mRNA expression levels were not significantly changed during the estrous cycle. Notably, addition of a GnRH antagonist at 1100 h completely eradicated luteinizing hormone secretion at 1400 h and 1700 h of proestrus, and significantly reduced the *Nr4a3* mRNA expression level at both the time points. These results suggest that GnRH is, at least partly, responsible for the increase in pituitary *Nr4a3*, and that the interaction between NR4A3 and ANXA5 is required to regulate *Fshb* expression during the preovulatory gonadotropin surge.

Key words: Annexin A5, Anterior pituitary gland, Follicle-stimulating hormone beta (*Fshb*), Gonadotropin-releasing hormone (GnRH), Nuclear receptor 4 group A member 3 (NR4A3)

(**J. Reprod. Dev. 67:** 217–221, 2021)

During rat estrous cycle, the two gonadotropins, namely luteinizing hormone (LH) and follicle stimulating hormone (FSH), undergo increased secretion during the afternoon of proestrus, which is known as the gonadotropin surge [1]. This surge is closely regulated by the hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH) [2]. However, the secretion patterns of LH and FSH are not identical. On the contrary, the FSH surge consists of two distinct increases [1, 3], whereas that of LH is a single peak. It is assumed that different mechanisms regulate the GnRH-mediated FSH surge; for example, changes in the plasma concentration of inhibin (an FSH suppressor), changes in the secretion patterns of GnRH, and possible interactions with intracellular signaling pathways [4].

Annexin A5 (ANXA5) is a calcium-dependent phosphatidylserine-binding protein [5] that is linked to several functional pathways, including the pathogenesis of several cancers, membrane repair, suppression of blood coagulation, and inhibition of protein kinase C [6–9]. We previously reported that GnRH stimulates ANXA5 expression in the anterior pituitary gonadotrope, and that ANXA5 promotes the secretion of LH and FSH [10-12]. Recently, we demonstrated low expression levels of the FSH β subunit gene (*Fshb*) but not the LH β subunit gene (*Lhb*) and other hormone-encoding genes in the pituitary gland of ANXA5-deficient female mice, suggesting that ANXA5 upregulates FSH expression [13]. Furthermore, ANXA5 suppresses the expression of nuclear receptor 4 group A member 3 (NR4A3).

NR4A3 is a member of the orphan nuclear receptor family of *Nr4a* immediate early genes, namely *Nr4a1*, *Nr4a2*, and *Nr4a3*. NR4A3 is involved in various physiological processes, such as inflammatory, metabolic, cardiovascular, neurological, and immune functions [14]. The expression of NR4A3 is affected by various stimuli, including hormones, growth factors, inflammation, and cellular stimulation [15]. We recently demonstrated that GnRH stimulates NR4A3 expression in the clonal gonadotrope LβT2, and that NR4A3 specifically represses *Fshb* expression [13]. These results suggest that there is some functional interaction between ANXA5 and NR4A3 and that this interaction is involved in GnRH-dependent augmentation of FSH synthesis. Here, we investigated the transcription of pituitary *Nr4a3*, *Anxa5*, and *Fshb* during the estrous cycle of rats, and the potential

Received: February 23, 2021
Accepted: March 24, 2021
Advanced Epub: April 10, 2021

Correspondence: M Kawaminami (e-mail: m-kawaminami@vet.ous.ac.jp)

effect of GnRH on the regulation of *Nr4a3* mRNA expression.

Materials and Methods

Animals

Wistar–Imamichi female rats (200–300 g) were purchased from SLC Japan (Shizuoka, Japan). The rats were maintained under controlled temperature and light conditions (23 ± 3°C and 14-h/10-h light–dark cycle, respectively, with lights on at 0500 h), and free access to laboratory chow and tap water. Vaginal smears were examined daily for at least two weeks before the start of the experiments. Female rats with regular 4-d estrous cycles were used in our analysis. The animals were sacrificed by decapitation, and trunk blood samples were collected immediately. Pituitary tissues were immediately collected and frozen in liquid nitrogen. All animal protocols were approved by the Animal Care and Use Committee of Kitasato University (Approval no.: 19–173).

Reverse transcription–quantitative PCR (RT–qPCR)

Total RNA was extracted from the pituitary tissues using ISOGEN (Nippon Gene, Tokyo, Japan) and reverse transcribed to cDNA using a High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA). The primer and probe sequences used for all RT–qPCR assays are listed in Table 1. qPCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's protocols. Relative gene expression levels were calculated using the $^{\Delta\Delta}$CT method with ribosomal protein L19 (*Rpl19*) as the internal control for normalization.

Determination of LH levels

Plasma LH concentrations were measured using a time-resolved fluorometric immunoassay (DELFIA System; PerkinElmer, Branchburg, NJ, USA). Briefly, purified rat LH (LH-I-9, NIDDK) was labeled with europium (Eu) using a DELFIA Eu-Labeling Kit (PerkinElmer). Anti-rabbit gamma-globulin goat serum was prepared in our laboratory and purified by ammonium sulfate precipitation. A 96-well immunoplate (Nunc, Tokyo, Japan) was coated with anti-rabbit gamma globulin and then overlaid with NIDDK anti-rat LH-S-11 (1:4,000 dilution). Diluted plasma samples and LH standards (NIDDK rat LH-RP-3) were incubated at 4°C overnight, and optimally diluted Eu-labeled hormone was added for 2 h at 20°C. After washing, the plates were treated with DELFIA Enhancement Solution (Perkin Elmer) and specific fluorescence (excitation wavelength 340 nm and emission wavelength 613 nm) was measured on a Multilabel Reader 2030 ARVO X4 (Perkin Elmer). The intra- and inter-assay coefficients of variation for LH were 6.5% and 9.9%, respectively.

Treatment with GnRH antagonist

The GnRH antagonist Cetrorelix was kindly provided by Zentaris GmbH (Frankfurt, Germany). Cetrorelix (300 μg/200 μl) or saline (200 μl) was intraperitoneally injected to proestrus rats at 1100 h. Blood and pituitary samples were collected at 1400 h and 1700 h on the same day.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Differences were statistically analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by a Tukey–Kramer test. Simple linear regression analysis followed by Pearson's correlation analysis was conducted to examine the correlation between the expression levels of *Nr4a3*, *Anxa5*, and *Fshb*. All statistical analyses were performed using IBM SPSS Statistics Subscription (Build 1.0.0.1447; IBM Japan, Tokyo, Japan). Statistical significance was set at $P < 0.05$.

Results and Discussion

Nr4a3 mRNA level in the anterior pituitary gland showed a specific increase at 1400 h of proestrus during the estrous cycle (Fig. 1A; proestrus 1400 and 1700 h versus proestrus 1100 h, $P < 0.01$), indicating a time-specific response in *Nr4a3* expression. However, *Anxa5* mRNA level in the anterior pituitary gland remained low between 1100 and 1700 h, and then increased between 2000 h and 2300 h (Fig. 1B; proestrus 2000, 2300, and estrus 0200 h versus proestrus 1100 h, $P < 0.01$). This increase was inversely correlated with a decline in *Nr4a3* expression. Previously, we showed that GnRH stimulates *Nr4a3* expression as an immediate early response

Table 1. Sequences of the primers and probes used in this study

Gene	Orientation	Sequence (5′–3′)
Nr4a3	Forward	AAAGACGGAACCTCCACAGAA
	Reverse	GTCGGGATAGGCGAAGCA
Anxa5	Forward	AAGTTCTTCGGAAGGCCATG
	Reverse	CTCAGCAATCTGCTGGCG
	Probe	FAM-CGACGAGGACAGCATCCTGAACCTGTT-TAMRA
Fshb	Forward	TGGTGTGAGGGCTACTGCTA
	Reverse	CTCGTACACCAGCTCCTTGA
Lhb	Forward	GTCTGCATCACCTTCACCAC
	Reverse	GTAGGTGCACACTGGCTGAG
Rpl19	Forward	GGAAGCCTGTGACTGTCCAT
	Reverse	ATCCTTCGCATCCAGGTCAC

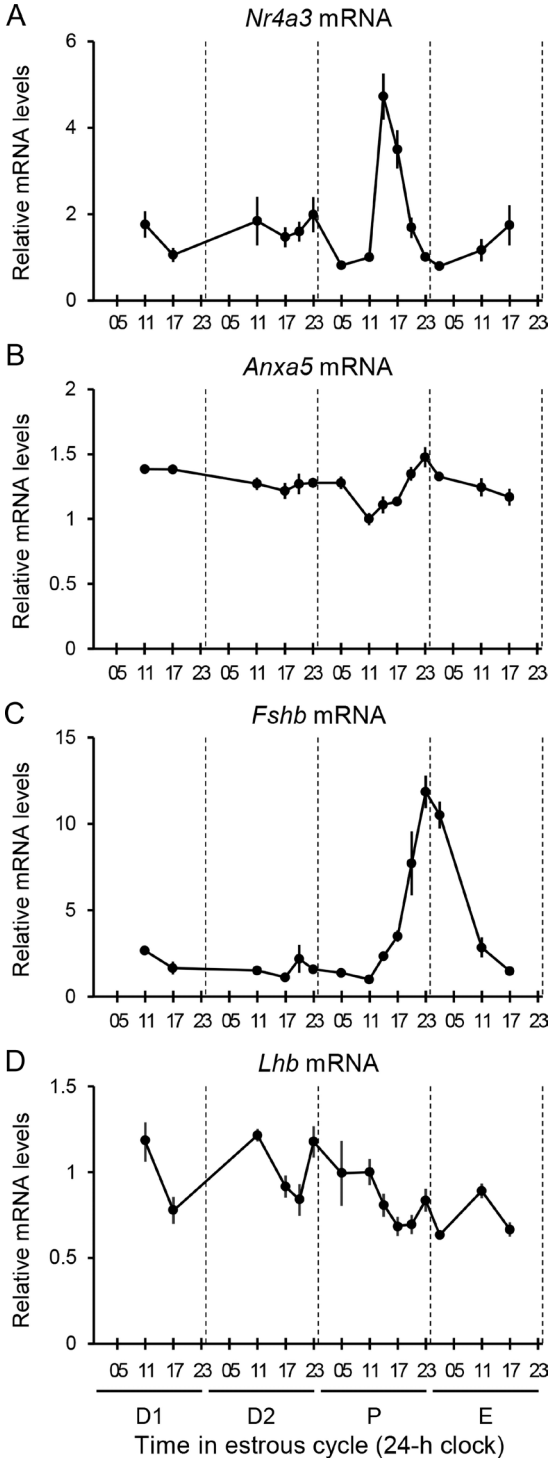

Fig. 1. Changes in the expression levels of *Nr4a3*, *Anxa5*, *Fshb*, and *Lhb* mRNA in the anterior pituitary gland during rat estrous cycle. Relative mRNA expression levels of *Nr4a3* (A), *Anxa5* (B), *Fshb* (C), and *Lhb* (D) in the anterior pituitary gland measured during diestrus 1 (D1; 1100 h, 1700 h), diestrus 2 (D2; 1100 h, 1700 h, 2000 h, and 2300 h), proestrus (P; 0500 h, 1100 h, 1400 h, 1700 h, 2000 h, and 2300 h), and estrus (E; 0200 h, 1100 h, and 1700 h) using RT–qPCR. Relative mRNA expression is quantified as a proportion of the corresponding proestrus expression at 1100 h (set to 1.0). Data are presented as mean ± SEM (n = 5).

gene and that *Anxa5* expression was augmented in the culture of the gonadotrope cell line LβT2 [13]. We also demonstrated that ANXA5 suppresses GnRH-induced *Nr4a3* expression [13], suggesting that pituitary ANXA5 expression may inhibit *Nr4a3* expression during the proestrus period.

Fshb mRNA level was significantly increased along with increasing *Anxa5* level during proestrus between 1100 h and 2300 h (Fig. 1C; proestrus 2000 h, 2300 h, and estrus 0200 h versus proestrus 1100 h, $P < 0.01$). In contrast, *Lhb* expression did not change significantly over the same period (Fig. 1D). Correlation between the expression levels of *Nr4a3*, *Anxa5*, and *Fshb* from proestrus 1400 to 2300 h showed that the expression level of *Nr4a3* was negatively correlated with those of *Anxa5* (Fig. 2A; $r = –0.806$, $r^2 = 0.6489$, $P < 0.001$) and *Fshb* expression (Fig. 2B; $r = –0.774$, $r^2 = 0.5986$, $P < 0.001$), while the expression levels of *Anxa5* and *Fshb* were positively correlated (Fig. 2C; $r = 0.788$, $r^2 = 0.6207$, $P < 0.001$). Several studies have reported a dramatic increase in the *Fshb* mRNA level, which occurs at several hours after the initial gonadotropin surge [16, 17]. The *Fshb* mRNA expression level increased once the *Nr4a3* expression level decreased, which paralleled the *Anxa5* mRNA profile, suggesting that the decline in NR4A3 and increase in ANXA5 correlated with changes in *Fshb* expression during the early gonadotropin surge.

Marked increases in *Nr4a3* mRNA expression levels appeared to coincide with the onset of GnRH surge. Therefore, we further investigated the potential involvement of GnRH in the regulation of *Nr4a3* expression during proestrus using a GnRH antagonist. Plasma LH concentrations were 4.05 ng/ml at 1400 h and 20.0 ng/ml at 1700 h in saline-treated rats, indicating that these animals experienced a normal gonadotropin surge, whereas animals treated with the GnRH antagonist presented with a dramatically reduced LH level, reaching less than 0.65 ng/ml and 0.41 ng/ml, respectively (Fig. 3A). Treatment with the GnRH antagonist also significantly reduced the *Nr4a3* expression levels by 25% and 29% at 1400 h and 1700 h, respectively (Fig. 3B). Furthermore, a significant decrease was observed in *Anxa5* expression at 1700 h (Fig. 3C) and *Fshb* expression at 1400 h and 1700 h (Fig. 3D) in the GnRH antagonist-treated group. It has been suggested that the gonadotrope *Nr4a3* expression is stimulated by GnRH. Although treatment with the antagonist completely abolished LH expression, it only reduced the *Nr4a3* expression level by 25–30%, suggesting that regulation of this factor is not solely mediated by GnRH. However, the decrease in the expression of another GnRH responsive gene, *Anxa5*, was also not substantial, suggesting a limited contribution of GnRH and/or additional player of enhancement. This is plausicble since there are a variety of factors known to induce *Nr4a3* as an immediate early response gene in different cells and tissues [18]. In addition, various pituitary cells, along with gonadotrope cells, also express *Nr4a3*.

Fshb mRNA expression levels increase between bimodal plasma FSH surges [16]. Currently, two potential mechanisms have been proposed for *Fshb* augmentation. One suggests that the decline in the plasma concentration of inhibin, which is inversely correlated with increasing plasma FSH concentration, facilitates the transcriptional activation of *Fshb* [3, 19]. Although we have previously tested the effect of inhibin antiserum in proestrus rats, *Nr4a3* expression did not change despite the significantly increased expression of *Fshb* mRNA (data not shown). Therefore, it is suggested that inhibin is not involved

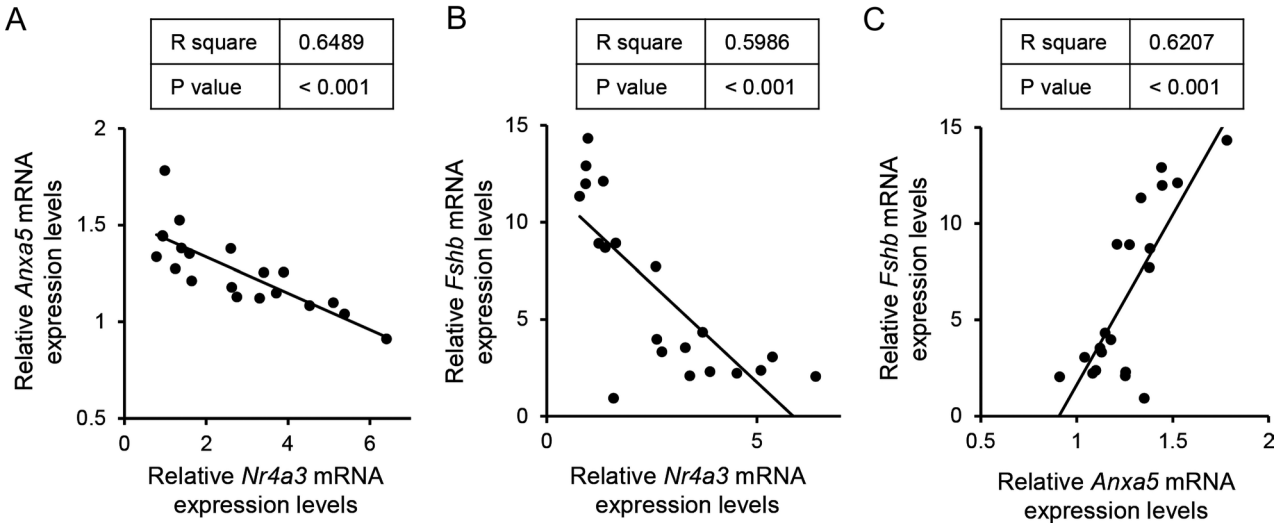

Fig. 2. Scatter plot representation of correlation between mRNA expression levels of *Nr4a3*, *Anxa5,* and *Fshb*. The plot presents simple regression line of *Nr4a3* versus *Anxa5* (A; n = 20), *Nr4a3* versus *Fshb* (B; n = 20), and *Anxa5* versus *Fshb* (C; n = 20) expression levels in rat anterior pituitary gland from proestus 1400 to 2300 h. Statistical analysis performed using Pearson's correlation method.

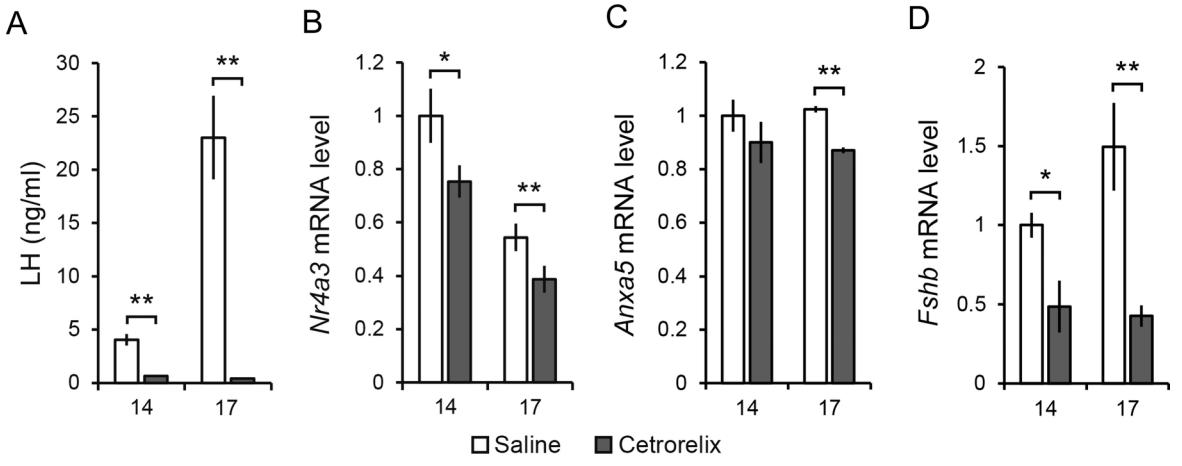

Fig. 3. Effect of GnRH antagonist on plasma LH and pituitary *Nr4a3* expression level in proestrus rats. Proestrus rats were intraperitoneally injected with the GnRH antagonist (Cetrorelix, 100 µg/200 µl) or 200 µl saline at 1100 h, and the anterior pituitary tissues and blood samples were collected at 1400 h and 1700 h. Plasma LH concentration (A), *Nr4a3* (B), *Anxa5* (C), and *Fshb* mRNA expression (D) were measured. Relative levels of mRNA are described in comparison to the saline group at 1400 h (set to 1.0). Data are presented as mean ± SEM (n = 8). Statistical analysis performed using Student's *t*-test; * $P < 0.05$, ** $P < 0.01$.

in *Nr4a3* expression during proestrus. Another theory suggests that activin/follistatin is synthesized in the pituitary gonadotrope, thereby increasing *Fshb* expression [20–22]. Our data suggest an additional intracellular mechanism for the regulation of *Fshb* expression. The present animal model showed a simultaneous increase in the *Anxa5* and *Fshb* expression levels, whereas *Anxa5*-knockout mice had low *Fshb* mRNA levels [13, 23], strongly suggesting that ANXA5 positively affects *Fshb* mRNA expression. Taken together, our results suggest that the elevation of *Nr4a3* expression is inhibited by the augmentation of *Anxa5,* leading to increased *Fshb* levels, and that this mechanism regulates the bimodal FSH surge.

In summary, this study shows that *Nr4a3* mRNA expression levels are increased during the afternoon phases of proestrus and are regulated, at least partially, by GnRH. In addition, our data demonstrated a negative correlation between *Nr4a3* and *Anxa5* and *Fshb*, wherein both were upregulated at midnight. This suggests that ANXA5 suppresses *Nr4a3* mRNA expression and stimulates *Fshb* transcription.

Acknowledgments

The authors are grateful to M. Nakata for his support with the official work. This work was supported by JSPS KAKENHI [grant Number: 17K08128 to MK].

References

1. **Smith MS, Freeman ME, Neill JD.** The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* 1975; **96**: 219–226. [Medline] [CrossRef]
2. **Sarkar DK, Chiappa SA, Fink G, Sherwood NM.** Gonadotropin-releasing hormone surge in pro-oestrous rats. *Nature* 1976; **264**: 461–463. [Medline] [CrossRef]
3. **Kishi H, Taya K, Watanabe G, Sasamoto S.** Follicular dynamics and secretion of inhibin and oestradiol-17 beta during the oestrous cycle of the hamster. *J Endocrinol* 1995; **146**: 169–176. [Medline] [CrossRef]
4. **Bernard DJ, Fortin J, Wang Y, Lamba P.** Mechanisms of FSH synthesis: what we know, what we don't, and why you should care. *Fertil Steril* 2010; **93**: 2465–2485. [Medline] [CrossRef]
5. **Rosenbaum S, Kreft S, Etich J, Frie C, Stermann J, Grskovic I, Frey B, Mielenz D, Pöschl E, Gaipl U, Paulsson M, Brachvogel B.** Identification of novel binding partners (annexins) for the cell death signal phosphatidylserine and definition of their recognition motif. *J Biol Chem* 2011; **286**: 5708–5716. [Medline] [CrossRef]
6. **Peng B, Guo C, Guan H, Liu S, Sun MZ.** Annexin A5 as a potential marker in tumors. *Clin Chim Acta* 2014; **427**: 42–48. [Medline] [CrossRef]
7. **Bouter A, Gounou C, Bérat R, Tan S, Gallois B, Granier T, d'Estaintot BL, Pöschl E, Brachvogel B, Brisson AR.** Annexin-A5 assembled into two-dimensional arrays promotes cell membrane repair. *Nat Commun* 2011; **2**: 270. [Medline] [CrossRef]
8. **Ueki H, Mizushina T, Laoharatchatathanin T, Terashima R, Nishimura Y, Rieanrakwong D, Yonezawa T, Kurusu S, Hasegawa Y, Brachvogel B, Pöschl E, Kawaminami M.** Loss of maternal annexin A5 increases the likelihood of placental platelet thrombosis and foetal loss. *Sci Rep* 2012; **2**: 827. [Medline] [CrossRef]
9. **Schlaepfer DD, Jones J, Haigler HT.** Inhibition of protein kinase C by annexin V. *Biochemistry* 1992; **31**: 1886–1891. [Medline] [CrossRef]
10. **Kawaminami M, Etoh S, Miyaoka H, Sakai M, Nishida M, Kurusu S, Hashimoto I.** Annexin 5 messenger ribonucleic acid expression in pituitary gonadotropes is induced by gonadotropin-releasing hormone (GnRH) and modulates GnRH stimulation of gonadotropin release. *Neuroendocrinology* 2002; **75**: 2–11. [Medline] [CrossRef]
11. **Kawaminami M, Tsuchiyama Y, Saito S, Katayama M, Kurusu S, Hashimoto I.** Gonadotropin-releasing hormone stimulates annexin 5 messenger ribonucleic acid expression in the anterior pituitary cells. *Biochem Biophys Res Commun* 2002; **291**: 915–920. [Medline] [CrossRef]
12. **Kawaminami M, Uematsu N, Funahashi K, Kokubun R, Kurusu S.** Gonadotropin releasing hormone (GnRH) enhances annexin A5 mRNA expression through mitogen activated protein kinase (MAPK) in LbetaT2 pituitary gonadotrope cells. *Endocr J* 2008; **55**: 1005–1014. [Medline] [CrossRef]
13. **Terashima R, Saigo T, Laoharatchatathanin T, Kurusu S, Brachvogel B, Pöschl E, Kawaminami M.** Augmentation of Nr4a3 and suppression of Fshb expression in the pituitary gland of female annexin A5 null mouse. *J Endocr Soc* 2020; **4**: bvaa096. [Medline] [CrossRef]
14. **Safe S, Jin UH, Morpurgo B, Abudayyeh A, Singh M, Tjalkens RB.** Nuclear receptor 4A (NR4A) family - orphans no more. *J Steroid Biochem Mol Biol* 2016; **157**: 48–60. [Medline] [CrossRef]
15. **Maxwell MA, Muscat GE.** The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. *Nucl Recept Signal* 2006; **4**: e002. [Medline] [CrossRef]
16. **Haisenleder DJ, Ortolano GA, Jolly D, Dalkin AC, Landefeld TD, Vale WW, Marshall JC.** Inhibin secretion during the rat estrous cycle: relationships to FSH secretion and FSH beta subunit mRNA concentrations. *Life Sci* 1990; **47**: 1769–1773. [Medline] [CrossRef]
17. **Marshall JC, Dalkin AC, Haisenleder DJ, Paul SJ, Ortolano GA, Kelch RP.** Gonadotropin-releasing hormone pulses: regulators of gonadotropin synthesis and ovulatory cycles. *Recent Prog Horm Res* 1991; **47**: 155–187, discussion :188–189. [Medline]
18. **Pearen MA, Muscat GE.** Minireview: Nuclear hormone receptor 4A signaling: implications for metabolic disease. *Mol Endocrinol* 2010; **24**: 1891–1903. [Medline] [CrossRef]
19. **Woodruff TK, D'Agostino J, Schwartz NB, Mayo KE.** Decreased inhibin gene expression in preovulatory follicles requires primary gonadotropin surges. *Endocrinology* 1989; **124**: 2193–2199. [Medline] [CrossRef]
20. **Bauer-Dantoin AC, Weiss J, Jameson JL.** Gonadotropin-releasing hormone regulation of pituitary follistatin gene expression during the primary follicle-stimulating hormone surge. *Endocrinology* 1996; **137**: 1634–1639. [Medline] [CrossRef]
21. **Besecke LM, Guendner MJ, Schneyer AL, Bauer-Dantoin AC, Jameson JL, Weiss J.** Gonadotropin-releasing hormone regulates follicle-stimulating hormone-beta gene expression through an activin/follistatin autocrine or paracrine loop. *Endocrinology* 1996; **137**: 3667–3673. [Medline] [CrossRef]
22. **Besecke LM, Guendner MJ, Sluss PA, Polak AG, Woodruff TK, Jameson JL, Bauer-Dantoin AC, Weiss J.** Pituitary follistatin regulates activin-mediated production of follicle-stimulating hormone during the rat estrous cycle. *Endocrinology* 1997; **138**: 2841–2848. [Medline] [CrossRef]
23. **Mittag J, Oehr W, Heuer H, Hämäläinen T, Brachvogel B, Pöschl E, Bauer K.** Expression and thyroid hormone regulation of annexins in the anterior pituitary. *J Endocrinol* 2007; **195**: 385–392. [Medline] [CrossRef]