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Augmentation of *Nr4a3* and Suppression of *Fshb* Expression in the Pituitary Gland of Female Annexin A5 Null Mouse

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GnRH enhances the expression of annexin A5 (ANXA5) in pituitary gonadotropes, and ANXA5 enhances gonadotropin secretion. However, the impact of ANXA5 regulation on the expression of pituitary hormone genes remains unclear. Here, using quantitative PCR, we demonstrated that ANXA5 deficiency in female mice reduced the expression of *Fshb* and *Gh* in their pituitary glands. Transcriptome analysis confirmed a specific increase in *Nr4a3* mRNA expression in addition to lower levels of *Fshb* expression in ANXA5-deficient female pituitary glands. This gene was then found to be a GnRH-inducible immediate early gene, and its increased expression caused protein to accumulate in the nucleus after administration of a GnRH agonist in LβT2 cells, which are an in vitro pituitary gonadotrope model. The increase in ANXA5 protein levels in LβT2 cells clearly suppressed *Nr4a3* expression. siRNA-mediated inhibition of *Nr4a3* expression increased *Fshb* expression. The results revealed that GnRH stimulates *Nr4a3* and *Anxa5* sequentially. NR4A3 suppression of *Fshb* may be necessary for later massive secretion of FSH by GnRH in gonadotropes, and *Nr4a3* would be negatively regulated by ANXA5 to increase FSH secretion.

Freeform/Key Words: GnRH, annexin A5, gonadotropin, pituitary, Nr4a3, FSH

GnRH of the hypothalamus promotes the secretion of gonadotropins, FSH, and LH from the anterior pituitary gland [1-3]. FSH and LH consist of the specific beta subunits FSHβ and LHβ, respectively, and the common glycoprotein alpha subunit (CGA). GnRH stimulates the release of gonadotropin and facilitates the expression of gonadotropin subunit genes.

A transcriptome analysis of a GnRH-stimulated mouse clonal gonadotrope cell line identified hundreds of regulated genes [4], and several were proposed as required for gonadotropin secretion. For example, in vitro study but not yet in vivo showed the transcriptional complex AP-1, composed of 2 GnRH-inducible factors, c-Fos and c-Jun [5], promotes the transcriptional activity of the *Fshb* promoter [6], which is needed for the GnRH-stimulated induction of *Fshb*. In addition, GnRH stimulation of high-frequency pulse induces the

Abbreviations: ANXA5, annexin A5; CGA, glycoprotein alpha subunit; rANXA5, rat annexin A5

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expression of SKIL and TGIF1, which prevent the *Fshb* promoter activation by AP-1 [7]. Therefore, GnRH signaling may also induce the expression of negative regulators that may affect the expression of gonadotropin genes and the responsiveness of cells towards GnRH stimulation.

Annexin A5 (ANXA5), a calcium-dependent phosphatidylserine-binding protein [8], is encoded by a GnRH-inducible gene [9]. We previously demonstrated that ANXA5 is induced by GnRH signaling via protein kinase C activation of the MAPK pathway [10]. Moreover, an antisense oligonucleotide-mediated decrease in *Anxa5* suppresses the secretion of LH by GnRH signaling in primary cultures of female rat pituitary cells [9]. A recent study also revealed that the administration of the GnRH agonist stimulates the expression of ANXA5 and LHβ in the pituitary glands of female hypogonadal mice (*hpg*) that lack a functional GnRH-encoding gene [11]. These results strongly suggest that ANXA5 is involved in physiological gonadotropin secretion under the influence of GnRH, but knowledge of the role of ANXA5 is limited.

Our previous study showed that ANXA5 is expressed in some pituitary endocrine cells of female rat, not only in gonadotropes [12]. Here, we examined the regulation of pituitary hormone genes in ANXA5-deficient mice using transcriptome analysis and qPCR and identified a link between GnRH, NR4A3, ANXA5, and *Fshb* expression in pituitary gonadotrope. Sequential changes in NR4A3 and ANXA5 expression after GnRH stimulation is suggested to be beneficial for FSH secretion by GnRH.

Materials and Methods

Animals

All animal experiment protocols were approved by the President of Kitasato University through the judgment rendered by the Animal Care and Use Committee of Kitasato University (approval no. 15-032). The establishment of ANXA5-deficient mice ($Anxa5^{-/-}$, $Anxa5^{tm1Epo}/Anxa5^{tm1Epo}$) has been described previously [13]. C57BL/6J wild-type and $Anxa5^{-/-}$ mice were maintained under controlled temperature and lighting: 23 ± 3°C and 14-hour light/10-hour dark cycle (lights on at 5:00 AM). They were allowed free access to laboratory chow and tap water. Eight-week-old female mice were administered either 5 ng/50 μL of GnRH agonist (GnRHa, Des-Gly10 [Pro9]-GnRH ethylamide; Intervet K.K., Tokyo, Japan) or 50 μL of saline (control) through repeated IP injection (10 times in 30-minute intervals). Pituitary samples were collected 30 minutes after the final administration. The mice were sacrificed by cervical dislocation. Pituitary tissues were immediately collected and frozen in liquid nitrogen.

Microarray analysis

Five pituitary glands from either C57BL/6J or $Anxa5^{-/-}$ adult female mice were collected and combined. RNA was extracted using an RNeasy mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Microarray analysis was performed once each for C57BL/6J and $Anxa5^{-/-}$ mice (n = 1). Gene chip analysis and cDNA microarray data were carried out at GeneticLab Co., Ltd. (Sapporo, Japan). Briefly, the quantity and the quality of RNA samples digested with DNase was verified by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc, Rockford, IL) and Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA) (C57BL/6J: 3.12 μg, 2.11 OD, RNA integrity number score 8.3; $Anxa5^{-/-}$: 3.77 μg, 2.09 OD, RNA integrity number score 8.0). The synthesis of cDNA, cRNA, and second-cycle cDNA was performed using Ambion WT Expression Kit (Life Technologies, Tokyo, Japan). cDNA was fragmented and labeled GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA). Gene chip data were analyzed by means of GeneChip Scanner 3000 7G with GeneChip Mouse Gene 1.0 ST (Affymetrix) and GeneChip Command Console software (Affymetrix) and GeneSpring GX version 11.5.1 software (Agilent Technologies). When differences in the detected gene expression levels were greater than 2-fold, the RNA expression was further measured and statistically evaluated by quantitative real-time PCR, as described in the following section.

Real-time PCR

Total RNA was extracted from tissues of adult female mice or cells using TRIzol reagent (Life Technologies) and was reverse-transcribed to generate cDNA using a High Capacity cDNA synthesis kit (Life Technologies). Primer sequences for real-time PCR are given in Table 1. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Life Technologies) according to the manufacturer's protocol using the following amplification conditions: 95°C for 10 minutes and 50 cycles of 95°C for 15 seconds, 60°C for 1 minute. Relative gene expression levels were calculated by the delta-delta CT method using ribosomal protein L19 as an internal control for normalization. Melting curve analysis revealed no amplification of nonspecific products. Expression levels are given as the relative levels by comparing experimental levels with those of the relevant control sample.

Mouse gonadotrope LβT2 cell line

The LβT2 cell line was a kind gift of Prof. P. L. Mellon of the University of California, San Diego. The cells were maintained in DMEM with high levels of glucose (Life Technologies), 10% fetal bovine serum (Life Technologies), and antibiotic-antimycotic supplements (Life Technologies); these were maintained at 37°C with 5% CO_2. GnRHa (10^{-8} M) was added 24 hours after the cells were plated in multiwell plates, and they were further incubated for 0.5, 1, 2, 4, and 8 hours. In addition, the effect of protein synthesis inhibitors was tested by preincubating the cells with cycloheximide (50 μM; Sigma-Aldrich, St. Louis, MO) for 2 hours before GnRHa stimulation. The cells were also treated with recombinant rat ANXA5 protein [10], which was added either 30 minutes before or at the same time as GnRHa

Table 1. Primer List

Primers	Forward (5′-3′)	Reverse (5′-3′)
Lhb	GTCTGCATCACCTTCACCAC	GTAGGTGCACACTGGCTGAG
Fshb	CTGCTGCCATAGCTGTGAAT	GAGCTGGGTCCTTATACACCA
Cga	ATCACCTGCCCAGAACACAT	ACATGGACAGCATGACCAGA
Tshb	CCATCAACACCACCATCTGT	CCTGGTATTTCCACCGTTCT
Gh	GTGGACAGATCACTGCTTGG	GGAAAAGCACTAGCCTCCTG
Prl	CTCAGGCCATCTTGGAGAAG	TCGGAGAGAAGTCTGGCAGT
Pomc	GGCCACTGAACATCTTTGTC	GCGACTGTAGCAGAATCTCG
Slitrk3	CTGAGGACTCTGCCAACTGA	AATGGCATTCAGGTGTTCAA
C10orf11	TACCCCACTTGCACACCTTA	CAAGTTGACCAGCTCATTGG
Nr4a3	CCGAGCTTTAACAGATGCAA	AGCTTCTGGACACGTCAATG
Gm7120	CGGGATTTTTAGCGTCTGTT	ATGGGTATCACAGTGGAGCA
Mme	TTCTGTGGCCAGACTGATTC	ATTGGGTCATTTCGGTCTTC
Gm5148	CACGAACGCTGTGATCTTCT	CTCATGCAAAGGGAATTGTG
Mpz	TCCTTCTGGTCCAGTGAATG	AAGGTTGTCCCTTGGCATAG
Mid1	CACCATATTCACCGGACAAG	GTGGTTCTGCTTGATGTTGG
Fabp6	ACCATTGGCAAAGAATGTGA	GACCTCCGAAGTCTGGTGAT
Akr1c18	GATAGGCCAGGCCATTCTAA	AATTTTCCAAGCTGGGTCTG
Pdk4	CACCACATGCTCTTCGAACT	CTACTGGGGTCAAGGAAGGA
Cetn4	ACAACTGATCGCTGAAATCG	CCGTAGCATCGTCATCAAAT
Grp	TCAGTCTCCAGCCTACTTGG	TCCTCCCTTTTCCTTGAGAA
Anxa5	GGTACCGATGAGGACAGCAT	TCCCTGCCAAACAGAGTCTT
Rpl19	AGCCTGTGACTGTCCATTCC	GCATTGGCAGTACCCTTCCT

administration. For immunoblotting, the cells were cultured in 35-mm dishes. The cells were harvested in Laemmli sample buffer after incubation with GnRHa (10^{-8} M) or following transfection of the expression vector.

Western blotting

LβT2 cell lysates were loaded on a 12% SDS-PAGE gel and then were transferred to polyvinylidene fluoride membranes (Amersham Hybond *P* 0.45, GE Healthcare UK Ltd, Buckinghamshire, UK). After blocking with 5% nonfat milk in Tris-buffered saline and Tween 20, the membranes were probed with primary antibodies against NR4A3 (1:1000 dilution; anti-human NGFI-B gamma mouse monoclonal antibody; Perseus Proteomics Inc., Tokyo, Japan) [14], ANXA5 (1:10 000 dilution; polyclonal rabbit sera against rat ANXA5) [15], and β-actin (1:2000 dilution; mouse monoclonal sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA) [16] at 4°C overnight. Then, the membranes were incubated with an ECL peroxidase-labeled anti-mouse antibody or anti-rabbit antibody (GE Healthcare UK Ltd) [17, 18], which function as a secondary antibody. Immunoreactivity was detected by chemiluminescence with ECL Western blotting detection reagents (GE Healthcare UK Ltd), and blots were scanned using an ImageQuant LAS 4000 system (GE Healthcare UK Ltd).

Immunocytochemistry

LβT2 cells were seeded on poly-L-lysine-coated coverslips and were grown for 2 days. After incubation with GnRHa (10^{-8} M), the cells were fixed with 4% paraformaldehyde-PBS at room temperature for 10 minutes and then were blocked by incubating with 10% normal goat serum for 30 minutes. Immunocytochemistry was performed using an indirect immunofluorescence technique with an anti-NR4A3 primary antibody (1:2000) and an Alexa Fluor 488 goat anti-mouse IgG secondary antibody (1:200; Life Technologies) [19]. To visualize the actin cytoskeleton, F-actin was stained with Alexa Fluor 568 phalloidin (Life Technologies) for 30 minutes. The specimens were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA), and they were visualized using a confocal laser microscope (LSM710, Carl Zeiss Japan, Tokyo, Japan).

Transfection of the vector and siRNA

Rat *Anxa5* cDNA was previously cloned into BamHI site of plasmid pUC119 [20], subcloned into BamHI site of plasmid pcDNA3.1(-) (Invitrogen, Carlsbad, CA) and sequenced (LC533519). The transfection of the expression vectors for ANXA5 (pcANXA5) was performed by means of electroporation using an NEPA21 electroporator and electroporation cuvettes (Nepa Gene Co. Ltd., Chiba, Japan). A suspension of LβT2 cells (10^6 cells/100 μL) was prepared by detaching the cells with trypsin, and the cells were then mixed with 10 μg plasmid vector and electroporated by applying 2 pulses of 175 V for 5 ms at 50-ms intervals.

Nr4a3 Silencer Select siRNA (ID: s70687) and Silencer Select Negative Control #1 siRNA were obtained from Ambion (Austin, TX). LβT2 cells were transfected with each type of siRNA (66 pmol/mL final concentration) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

After transfection with the expression vectors or siRNA, the cells were incubated for 48 hours and then they were treated with GnRHa (10^{-8} M).

Statistics

The results are presented as the mean ± SEM. Significant differences were analyzed with Student *t* tests or 1- or 2-way ANOVA followed by a Tukey-Kramer test. A *P* value less than 0.05 was considered statistically significant.

Results

Expression changes in the anterior pituitary glands of Anxa5-/- *mice*

To determine the effect of ANXA5 deficiency on anterior pituitary gland hormone production, we analyzed the relative expression levels in RNA extracts from the pituitary glands of C57BL/6J mouse controls and *Anxa5*$^{-/-}$ mutants using real-time PCR. Among the 7 pituitary hormone genes analyzed, we observed that the FSH beta subunit (*Fshb*) and *Gh* expression levels were significantly reduced in the *Anxa5*$^{-/-}$ pituitary glands (Fig. 1). We also studied global expression changes and performed a transcriptome analysis of the anterior pituitary glands of C57BL/6J and *Anxa5*$^{-/-}$ mice. The results from the microarray analysis showed a 1.4-fold decrease in *Fshb* mRNA in the *Anxa5*$^{-/-}$ pituitary glands compared with the level of the control, whereas the expression of *Gh* and other pituitary hormone genes remained unchanged compared with that of the control (Table 2). Fourteen of 23 304 genes were differentially expressed, as defined by having a 2-fold or greater difference. To confirm the results, quantitative PCR analysis was performed. Here, we confirmed that *Slitrk3*, *C10orf11*, *Nr4a3*, and *Mme* were upregulated 2.9-, 7.0-, 6.8-, and 4.3-fold, respectively, and *Mid1* was downregulated 6.7-fold in the pituitary glands of *Anxa5*$^{-/-}$ mice (Fig. 2).

ANXA5-controlled genes responding to GnRH stimulus in the pituitary glands

To identify the GnRH-responsive genes within the cluster of differentially expressed genes, C57BL/6J mice were received multiple administrations of GnRHa or saline. Then, RNA from their pituitary glands was isolated, and relative gene expression was determined using quantitative PCR. Here, we observed that the administration of GnRHa significantly increased *Nr4a3* expression 2-fold and decreased *Akr1c18* expression 3-fold in the pituitary glands compared with the levels determined for the saline control (Fig. 3). AKR1C18 is a 20α-hydroxysteroid dehydrogenase that converts progesterone to the nonactive metabolite

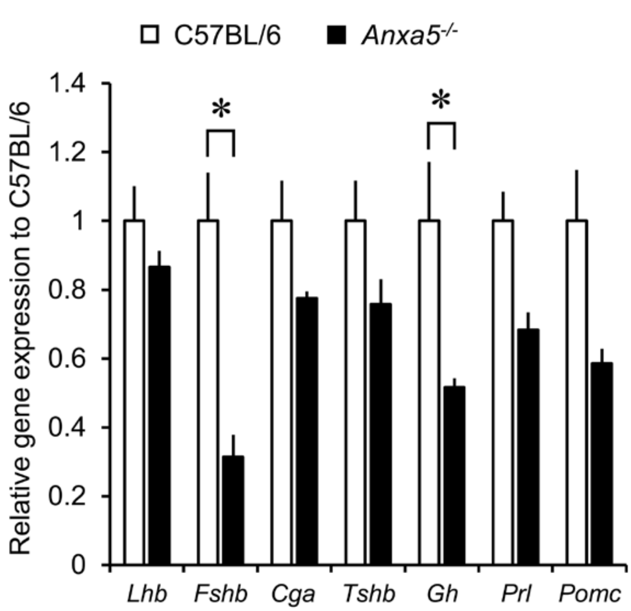

Figure 1. Pituitary hormone gene expression in *Anxa5*$^{-/-}$ mice. The levels of *Lhb*, *Fshb*, *Cga*, *Tshb*, *Gh*, *Prl*, and *Pomc* mRNA expression in wild-type (C57BL/6) and *Anxa5*$^{-/-}$ mice were analyzed by quantitative real-time PCR. Data are depicted as the mean ± SEM (n = 5). Statistical analysis was performed with Student *t* tests (**P* < 0.05). ANXA5, annexin A5; *Cga*, common alpha glycoprotein subunit; *Fshb*, follicle stimulating hormone beta subunit; *Lhb*, luteinizing hormone beta subunit; *Pomc*, pro-opiomelanocortin; *Prl*, prolactin; *Tshb*, thyroid stimulating hormone beta subunit.

Table 2. Gene Expression in the Pituitary Gland of *Anxa5*$^{-/-}$ Mice

Gene ID	Gene Description	Gene symbol	Fold change
Genes upregulated in the anterior pituitary gland of Anxa5$^{-/-}$			
NM_198864	SLIT and NTRK-like family, member 3	*Slitrk3*	2.84
NM_028275	RIKEN cDNA 1700112E06 gene (leucine-rich repeat-containing protein C10orf11 homolog)	*C10orf11*	2.64
NM_015743	Nuclear receptor subfamily 4, group A, member 3	*Nr4a3*	2.28
NM_001039244	Predicted gene 7120	*Gm7120*	2.09
NM_008604	Membrane metalloendopeptidase (CD10)	*Mme*	2.02
Genes downregulated in the anterior pituitary gland of Anxa5$^{-/-}$			
NM_198657	Predicted gene 5148	*Gm5148*	-4.71
NM_008623	Myelin protein zero	*Mpz*	-4.20
NM_010797	Midline 1 (Tripartite motif protein 18)	*Mid1*	-3.16
NM_008375	Fatty acid binding protein 6 (gastrotropin)	*Fabp6*	-2.73
NM_134066	Aldo-ketoreductase family 1, member C18	*Akr1c18*	-2.46
NM_013743	Pyruvate dehydrogenase kinase, isoenzyme 4	*Pdk4*	-2.46
NM_009673	Annexin A5	*Anxa5*	-2.19
NM_145825	Centrin 4	*Cetn4*	-2.02
NM_175012	Gastrin releasing peptide	*Grp*	-2.00
Pituitary hormone genes			
NM_008497	LH beta	*Lhb*	1.05
NM_008045	FSH beta	*Fshb*	-1.41
NM_009889	Glycoprotein hormones, alpha subunit	*Cga*	1.02
NM_00943	TSH beta	*Tshb*	1.10
NM_008117	GH	*Gh*	-1.01
NM_011164	Prolactin	*Prl*	-1.00
NM_008895	Pro-opiomelanocortin-alpha	*Pomc*	1.10

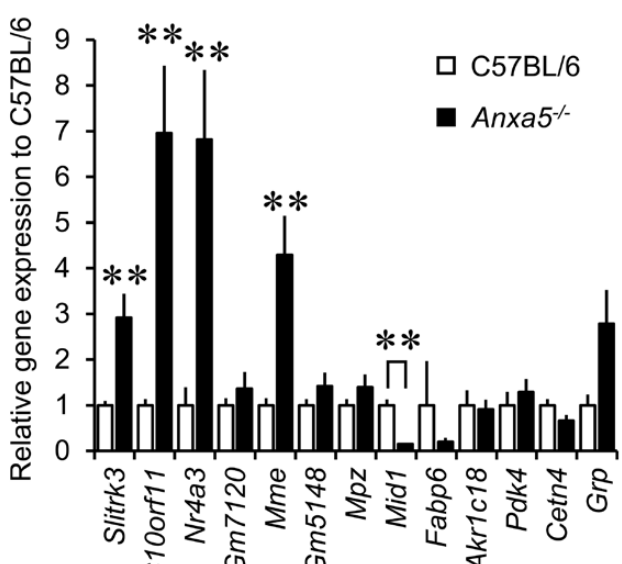

Figure 2. Expression levels of the 13 genes affected by ANXA5 deficiency in the pituitary glands listed in Table 2. Relative mRNA levels in the pituitary glands of C57BL/6 and *Anxa5*$^{-/-}$ mice were measured by real-time quantitative PCR, and the relative expression levels in the control animals were set at 1.0. Data are depicted as the mean ± SEM (n = 5). Statistical analysis was performed with Student *t* tests (**$P < 0.01$). ANXA5, annexin A5.

20α-hydroxyprogesterone in ovaries [21]. *Akr1c18* was shown to be induced by PPARalpha in the pituitary gland [22]. In addition, we confirmed a previously described increase in the expression of *Anxa5*.

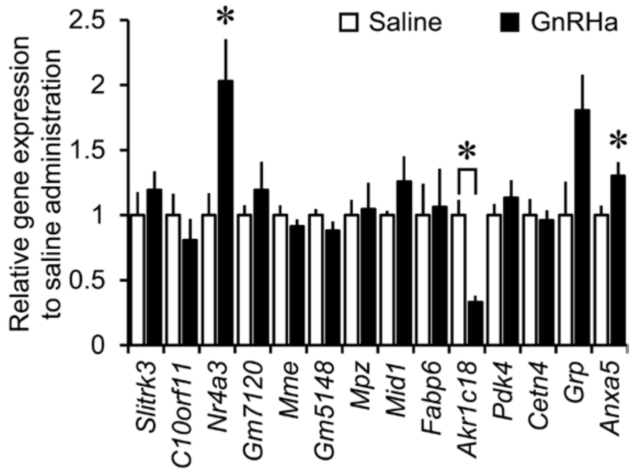

Figure 3. Effects of GnRHa stimulation on genes differentially expressed in ANXA5-deficient pituitary glands. C57BL/6 control mice were repeatedly administered a GnRH agonist (GnRHa, 5 ng/50 µL, 10 times) to obtain a large response, or they were administered saline alone; then, relative mRNA levels in the pituitary glands were measured by real-time quantitative PCR. Data are depicted as the mean ± SEM (n = 5). Statistical analysis was performed with Student *t* tests (**P* < 0.05). ANXA5, annexin A5.

Induction and intranuclear accumulation of NR4A3 by GnRH

Interestingly, *Nr4a3* expression was increased in *Anxa5*$^{-/-}$ pituitary gland tissue, and GnRH could induce *Nr4a3* and *Anxa5* expression. Therefore, we compared the levels of *Anxa5* and *Nr4a3* expression in the mouse gonadotrope cell line LβT2. GnRHa stimulation caused a single peak of transiently increased *Nr4a3* mRNA expression 1 hour after stimulation (Fig. 4A). In contrast, *Anxa5* mRNA expression showed a constant increase 8 hours after the LβT2 cells were treated (Fig. 4B). To examine whether the synthesis of *Nr4a3* mRNA was dependent on protein synthesis, LβT2 cells were incubated with the protein synthesis inhibitor before GnRHa treatment. The kinetics of *Nr4a3* mRNA expression were not affected by the addition of the inhibitor. Hence, *Nr4a3* is a direct immediate early gene of GnRH, and stimulation of *Nr4a3* mRNA expression is independent of protein synthesis processes (Fig. 4C). The results from the immunoblot analysis showed that the increase in mRNA expression was accompanied by a peak of increased NR4A3 protein (~75 kD band) expression 2 hours after GnRHa stimulation (Fig. 4D). Immunocytochemistry clearly showed that NR4A3 protein levels were increased, and NR4A3 was detected within the nuclei of the LβT2 cells after GnRHa treatment (Fig. 4E).

Inhibitory effect of de novo synthesized and exogenous ANXA5 on Nr4a3 *expression*

Because *Nr4a3* expression is enhanced in the pituitary glands of ANXA5-deficient mice, we hypothesized that *Nr4a3* expression is inhibited in the presence of ANXA5. We therefore studied the effect of increasing ANXA5 levels on *Nr4a3* mRNA expression in LβT2 cells using transient transfection protocols (Fig. 5A). ANXA5 expression was successfully increased two days after transfection, and the *Nr4a3* mRNA levels were significantly reduced compared with the levels in the control cells after GnRH stimulation (Fig. 5B). We previously showed a stimulatory effect of externally added recombinant rat ANXA5 (rANXA5) on gonadotropin release from primary pituitary cells [9], which indicated that exogenous ANXA5 can act on gonadotropes to cause release the hormone. Therefore, the LβT2 cells were simultaneously incubated with GnRHa and rANXA5 to study the combined effect on *Nr4a3* expression. The administration of rANXA5 in addition to GnRHa significantly inhibited *Nr4a3* mRNA expression after 1 hour in a dose-dependent manner (Fig. 5C). The preincubation

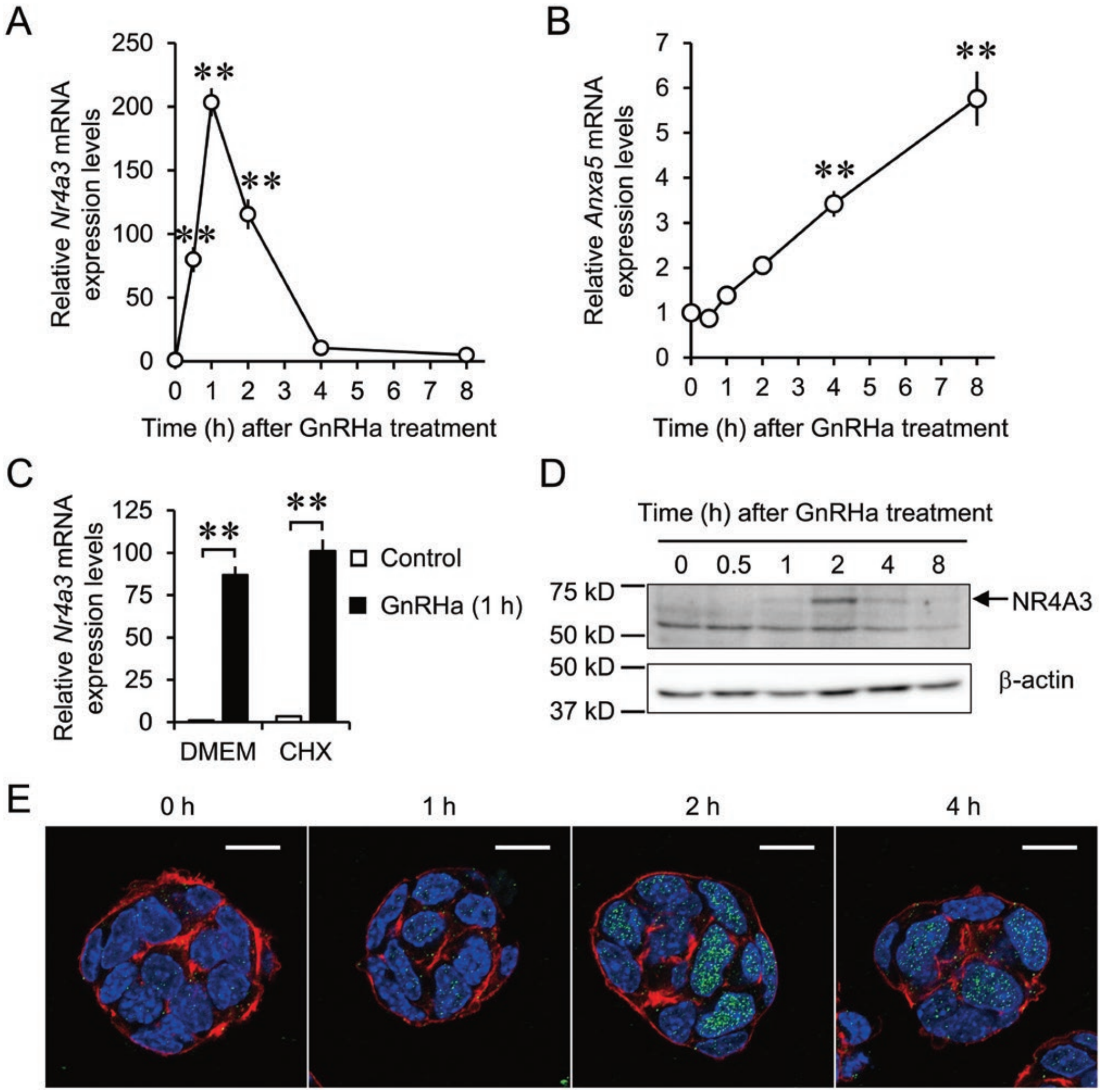

Figure 4. Induction of *Nr4a3* expression and accumulation of NR4A3 protein in the nuclei after GnRH stimulation in gonadotrope cell line LβT2. (A) *Nr4a3* and (B) *Anxa5* mRNA expression was observed after LβT2 cells were incubated with GnRHa (10^{-8} M) for 0 to 8 hours (mean ± SEM; n = 4). Statistical analysis was performed with 1-way ANOVA and a Tukey-Kramer test (***P* < 0.01 vs 0 hours). (C) LβT2 cells were preincubated with or without cycloheximide (CHX, 50 µM) for 2 hours. *Nr4a3* mRNA expression was measured after GnRHa (10^{-8} M) treatment for 1 hour (mean ± SEM; n = 4). Statistical analysis was performed with 2-way ANOVA and a Tukey-Kramer test (***P* < 0.01). NR4A3 protein expression was observed by (D) Western blotting and (E) immunocytochemistry of LβT2 cells in the presence of GnRHa (10^{-8} M) for 0 to 8 hours and 0 to 4 hours, respectively. (E) Distribution of the NR4A3 protein (green), actin cytoskeleton (red) and nuclei (blue) in cell aggregates was observed by confocal laser scanning microscopy. Scale bars indicate 10 µm (E). ANXA5, annexin A5.

with rANXA5 for 30 minutes (Fig. 5D) or the preincubation and simultaneous incubation with GnRHa (Fig. 5E) showed a similar inhibitory effect on *Nr4a3* mRNA expression.

Effect of Nr4a3 on gonadotropin gene expression

Next, we used siRNA to knock down *Nr4a3* in LβT2 cells so that we could determine whether NR4A3 could regulate gonadotropin gene expression. The *Nr4a3* mRNA levels were significantly

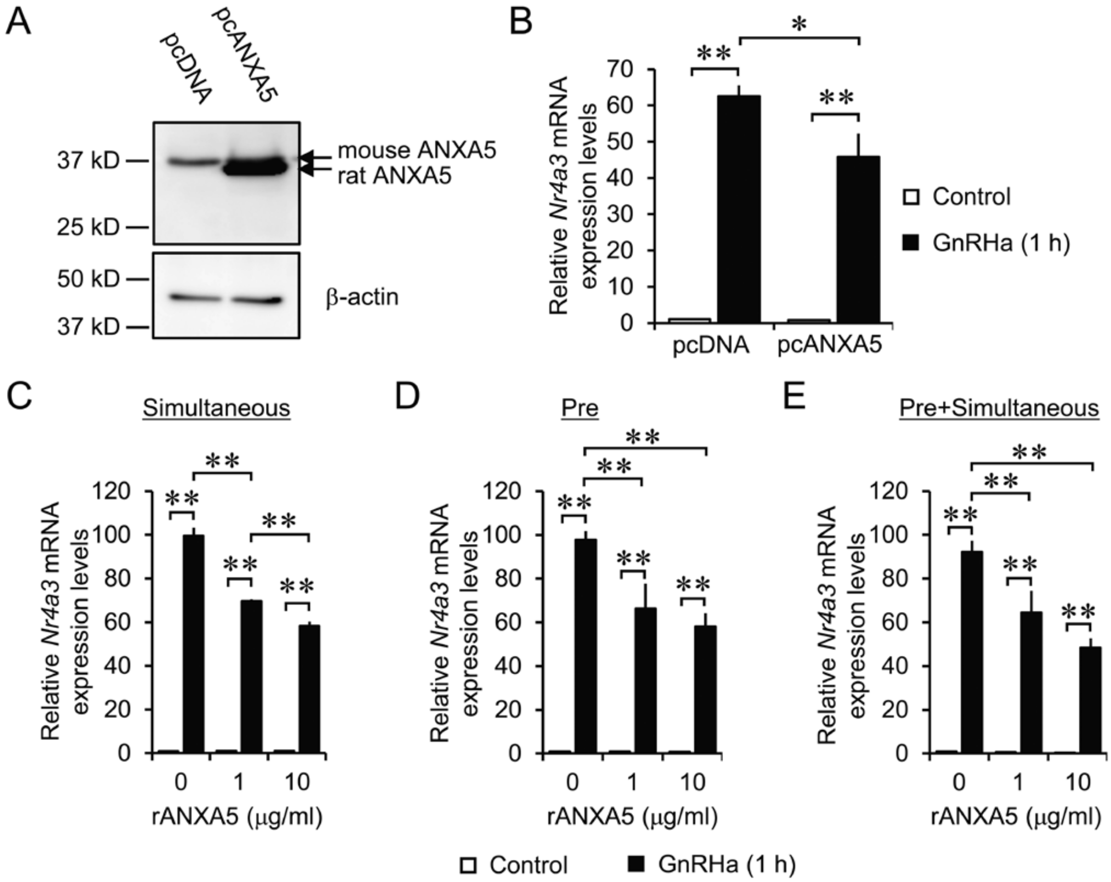

Figure 5. Suppression of *Nr4a3* expression by ANXA5. (A) LβT2 cells were transfected with an ANXA5 expression vector (pcANXA5) or an empty vector (pcDNA), and the expression of the ANXA5 protein was detected by Western blotting for mouse and rat ANXA5 protein. (B) *Nr4a3* mRNA expression in the cells transfected with pcANXA5 or pcDNA was measured after induction with GnRHa (10^{-8} M) for 1 hour (mean ± SEM; n = 4; **P* < 0.05, ***P* < 0.01). (C-E) LβT2 cells were incubated for 1 hour in the absence or presence of GnRHa (10^{-8} M), and the *Nr4a3* mRNA levels were measured (mean ± SEM; n = 4; ***P* < 0.01). Recombinant ANXA5 (rANXA5, 0-10 μg/mL) was used as a treatment in the 3 conditions: simultaneous administration with GnRHa (C, simultaneous), preadministration 30 minutes before incubation with GnRHa alone (D, pre), or preadministration and simultaneous administration (E, pre+simultaneous). Statistical analysis was performed with 2-way ANOVA and a Tukey-Kramer test. ANXA5, annexin A5.

reduced between 1 and 2 hours after GnRHa administration (expression ratio of NR4A3 siRNA transfected cells to control siRNA transfected cells: 0 hours, 55%; 1 hours, 66%; 2 hours, 47%; 4 hours, 56%; 8 hours, 62%) (Fig. 6A); further, *Fshb* mRNA expression, which is known to be increased after stimulation, was increased in the siRNA for NR4A3 transfected cells between 1 and 8 hours after GnRHa administration compared with the expression levels observed in the control cells (Fig. 6B). *Lhb* mRNA expression levels showed a 40% decrease within the first 2 hours of GnRHa administration, and thereafter, normal levels were reestablished (Fig. 6C); however, the expression of the common alpha subunit (*Cga*) mRNA was not significantly altered in the NR4A3 siRNA transfected LβT2 cells (Fig. 6D). This finding suggests that the induction of NR4A3 by GnRH can suppress *Fshb* gene expression.

Discussion

Here, we demonstrated a unique system for regulating *Fshb* expression among gonadotropin subunits through GnRH and ANXA5 in pituitary gonadotropes. Although GnRH

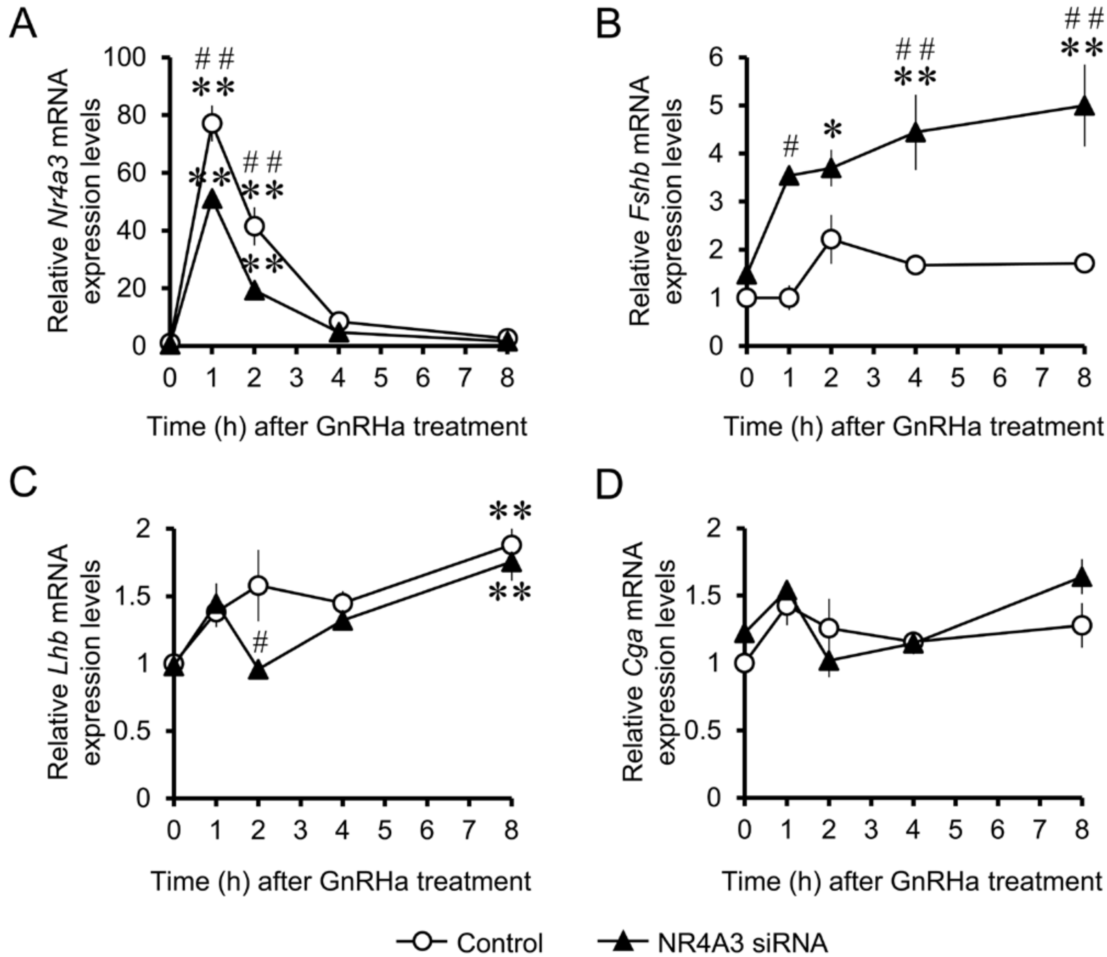

Figure 6. Effect of RNA interference targeting *Nr4a3* on gonadotropin genes. LβT2 cells were transfected with an *Nr4a3*-specific siRNA (Nr4a3 siRNA; filled triangle) or a negative control siRNA (control; open circle), and then the cells were incubated for 48 hours. (A) *Nr4a3*, (B) *Fshb*, (C) *Lhb*, and (D) *Cga* mRNA expression in cells treated with GnRHa (10^{-8} M) was measured by quantitative PCR at 0 to 8 hours (mean ± SEM; n = 4). Relative levels of mRNA are indicated compared with levels of the control group at the start (0 hours) of treatment. Statistical analysis was performed with 2-way ANOVA and a Tukey-Kramer test (**$P < 0.01$). *$P < 0.05$ and **$P < 0.01$ indicate comparison to the levels at 0 hours under the same conditions as those of the siRNA transfected cells; #$P < 0.05$ and ##$P < 0.01$ indicate comparison to the control group at each time point.

stimulates both LH and FSH secretion, the secretion patterns of these hormones are not always identical. Therefore, specific regulation for each subunit has been presumed.

Mittag et al. first reported that *Fshb* mRNA expression levels were specifically lowered among anterior pituitary hormone genes in immature 3-week-old male *Anxa5*$^{-/-}$ mice [23]. Because ANXA5 was suggested to enhance the expression of *Fshb* mRNA, we have now identified the genes specifically regulated in ANXA5-deficient pituitary glands of adult female mice. The present study shows a regulatory network involving ANXA5 and the transcription factor NR4A3, and both were enhanced by GnRH in pituitary gonadotrope cells. NR4A3 is proposed to suppress the expression of *Fshb* mRNA.

The expression of *Nr4a3* is enhanced in the ANXA5-deficient pituitary glands, and, in contrast, its expression is suppressed by the overexpressed ANXA5 protein such that ANXA5 represents a negative regulator of *Nr4a3* expression. The effect of ANXA5 on NR4A3 was highly effective as preadministration of ANXA5 elicited the same effect as when ANXA5 was given with GnRHa. In turn, NR4A3 was suggested to suppress *Fshb* mRNA because overexpression of *Nr4a3* in ANXA5-deficient mice accompanied decreased *Fshb* expression and the suppression of *Nr4a3* by siRNA augmented *Fshb* mRNA expression.

Here, we demonstrated the influence of ANXA5 and NR4A3 on *Fshb* mRNA expression. *Nr4a3* was shown to be an immediate early gene induced by the GnRH receptor. NR4A3 (also known as neuron-derived orphan receptor 1) is known as a nuclear orphan receptor in the NR4A protein family, which includes NR4A1 and NR4A2. It has been reported that the NR4A protein is induced by various stimuli, including the activation of GPCRs [24-31]. In the present study, the NR4A3 protein immediately accumulated in the nucleus upon GnRHa administration. The NR4A protein was also reported to translocate into nuclei in a ligand-independent manner [32]. Our data further support the idea that NR4A3 has a primary role in regulating gene expression upon GnRH stimulation. Because ANXA5 expression is also stimulated by GnRH after NR4A3, we assume that the sequential expression of NR4A3 and ANXA5 may define a regulatory network linked with GnRH effects on FSH secretion.

In *Nr4a3* knockdown experiments, the increase in *Fshb* mRNA following GnRHa administration continued for at least 8 hours after administration. This result suggests that the transient increase in NR4A3 after GnRHa stimulation elicits long-lasting effects, such as the following: (1) direct inhibition of *Fshb* transcription activity, (2) repression of the effector of *Fshb* expression, and (3) suppression of *Fshb* mRNA stability. Verification of multiple aspects of this mechanism, such as measuring transcription activity by reporter gene assay and analyzing direct DNA binding by chromatin immunoprecipitation assay, will be helpful for elucidating the effect of NR4A3 on *Fshb* mRNA expression.

NR4A3 is known as a nuclear receptor that is predicted to interact with a specific sequence. To date, we do not know how NR4A3 affects *Fshb* mRNA expression, and we need to examine the relationship between NR4A3 and the reported gene products that affected *Fshb* mRNA. Because NR4A3 was shown to interact with the SIX3 homeodomain transcription factor and SIX3 is expressed in gonadotropes [33, 34], we assume the involvement of the interaction between NR4A3 and SIX3 in the functional network of GnRH-ANXA5-NR4A3-*Fshb*.

Although various molecular functions have been proposed for ANXA5, including the inhibition of protein kinase C [35], the formation of calcium channels in phospholipid membranes [36], the binding of calcium ions, and the interaction with actin and collagen [37, 38], the physiological significance of these functions is still unknown. Therefore, the functional relationship between ANXA5 and NR4A3 expression is a part of the next subject of investigation into the molecular mechanism for ANXA5 function. Although the present study showed that ANXA5 enhances FSH synthesis, it is not known how it works physiologically. We have already revealed that ANXA5 augments gonadotropin secretion [9], but ANXA5-deficient mice exhibit regular estrous cycles and ovulation numbers [39]. Brachvogel et al. also did not observe any apparent phenotype in reproductive function in ANXA5$^{-/-}$ [13]. This is probably because of the presence of a redundant mechanism of the other annexin family members, ANXA1 through 13, 12 does not exist. Recently, we demonstrated that the expression of ANXA1 is also facilitated by GnRH [40]. Further analysis of ANXA5 function as it relates to gonadotropin secretion is needed.

It is well known that FSH and LH have different secretion patterns. This difference is sometimes explained by the difference in the pulsatile pattern of GnRH release. The increase in GnRH pulse frequency leads to downregulation of FSH synthesis and release, which is recognized as one of the switching processes between follicular development and maturation [41-43]. This inhibitory mechanism is suggested to be involved in the suppression of *Fshb* under high-frequency GnRH stimulation, in which negative effectors on *Fshb* gene transcription, such as ICER, SKIL, and TGIF1, are induced [7, 44, 45]. Because *Nr4a3* is transiently induced by GnRH as an immediate early gene and is suggested to specifically suppress the *Fshb* gene expression level, NR4A3 would also be involved in the frequency-dependent mechanism by which GnRH regulates FSH secretion.

The present results clearly demonstrate a specific functional relationship among GnRH, ANXA5, and NR4A3. GnRH stimulation of NR4A3 expression that is suggested to suppress

FSH expression is followed by augmentation of ANXA5 that downregulates NR4A3 expression. This proposed relationship would consist of a regulatory network that controls *Fshb* expression through association with the mechanisms that establish the harmonized secretion of LH and FSH.

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