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Inhibin A inhibits follicle-stimulating hormone (FSH) action by suppressing its receptor expression in cultured rat granulosa cells

Cuiling Lu[a,b], Wei Yang[a], Min Chen[a], Tao Liu[a], Junling Yang[a], Ping Tan[a], Lei Li[a], Xiaoqian Hu[a], Cuihong Fan[a], Zhaoyuan Hu[a], Yixun Liu[a,*]

[a] State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, No. 5 Datun Road, Chaoyang District, Beijing 100101, China
[b] Graduate School of the Chinese Academy of Sciences, Beijing 100039, China

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ABSTRACT

Inhibin has long been considered as a suppresser of follicle-stimulating hormone (FSH) secretion from anterior pituitary through pituitary–gonad negative feedback to regulate follicle development. We demonstrated that addition of inhibin A could significantly suppress FSH-induced FSHR mRNA level in cultured rat granulosa cells (GCs) measured by real-time PCR. The inhibin A exerted its action mainly by inhibiting FSHR promoter activity. Furthermore, exogenous inhibin A could dramatically decrease FSH-induced P450arom and P450scc level and suppress progesterone and estradiol production in the cultured GCs, but it did not decrease forskolin-induced steroidogenesis, indicating that the inhibitory effect of inhibin A on FSH action may be upstream of cAMP signaling. Inhibin A was also capable of suppressing FSH-induced expression of steroidogenic factor 1 (SF-1) and androgen receptor, but stimulating DAX-1 expression in the culture. Our study has provided new evidence to show that inhibin A is capable of feedback antagonizing FSH action on GCs by reducing FSHR expression at ovarian level via a short feedback loop. Transcriptional factor receptors, such as SF-1, AR and DAX-1 were involved in this regulation.

1. Introduction

Inhibins are peptides predominantly produced in mammalian gonads, and their primary role is inhibition of hypophyseal follicle-stimulating hormone (FSH) secretion (Burger, 1993; Findlay, 1993; Mann et al., 1992; Woodruff and Mather, 1995). They are composed of two dissimilar, α and β disulfide-linked subunits. Heterodimerization of α-subunit with either of the two forms of the β-subunit, βA and βB, generate dimeric inhibin A and inhibin B, respectively (Baird and Smith, 1993; Campbell and Baird, 2001; Lanuza et al., 1999). Inhibin regulation of pituitary FSH secretion through its negative feedback in infantile female rat begins to operate after postnatal day (PND) 20. This negative feedback is achieved by increasing plasma level of the two dimeric forms, and the inhibin A appears to be the major negative physiological regulator of FSH secretion at the initiation of this mechanism. At PND 25 and 30, a dramatic fall in plasma FSH level coincided with rise of inhibin A, and a significant inverse correlation was observed between plasma FSH and inhibin A (Herath et al., 2001). Administration of a recombinant inhibin A during early stage of follicular development to a non-human primate resulted in decrease in serum FSH concentration (Molskness et al., 1996). Addition of increasing doses of FSH to cultured immature rat granulosa cells (GCs) dose-dependently increased both type inhibin secretion, but the rise in inhibin A was more pronounced than the rise in inhibin B, especially in presence of estrogen precursor (Lanuza et al., 1999; Ying et al., 1987). A reciprocal relationship (negative feedback) between secretion of FSH and inhibin has been established. Campbell and Baird (2001) demonstrated that the production of inhibin A by sheep granulosa cells is FSH responsive after prolonged exposure and inhibin A can augment FSH-stimulated steroid production by both granulosa and theca cells. In addition, Jimenez-Krassel et al. (2003) demonstrated that inhibin has a profoundly negative autocrine or paracrine role in regulation of estradiol production by granulosa cells from bovine healthy estrogen-active dominant follicles. However, there is no evidence available to show a short feedback role of inhibin to FSH action at rat ovarian level.

FSH is necessary for gonad development and maturation at puberty and gamete production during fertile phase (Chappel and Howles, 1991). Follicular development beyond the early stages is absolutely FSH-dependent (Schuiling et al., 1987). The hormone is produced and secreted by pituitary gland as a highly heteroge-

Abbreviations: FSHR, follicle-stimulating hormone receptor; GC, granulosa cell; SF-1, steroidogenic factor 1; AR, androgen receptor; DAX-1, dosage sensitive sex reversal (DSS), adrenal hypoplasia congenita (AHC) critical region on the X chromosome, gene 1.

* Corresponding author. Tel.: +86 10 64807038; fax: +86 10 64807583.
E-mail address: liuyx@ioz.ac.cn (Y. Liu).

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neous glycoprotein (Ulloa-Aguirre et al., 1995) and acts by binding to its specific receptors, localized exclusively in ovary and testis. FSH receptors belong to a family of G-protein-coupled receptors, complex transmembrane proteins characterized by seven hydrophobic helices inserted in plasmalemma and by intracellular and extracellular domains of variable dimensions depending on type of ligand (Gudermann et al., 1995; Simoni et al., 1997). Follicular development arrests in mice lacking FSH beta or FSH receptors in GCs (Abel et al., 2000; Dierich et al., 1998; Kumar et al., 1997).

Evidence has shown that treatment of rat granulosa cells with FSH for 48 h in vitro could increase its receptor mRNA level, addition of EGF or bFGF to the culture suppressed the FSH induced its receptor level, however, the both growth factors in lower doses did not affect the basal level of the FSH receptors. Similarly, GnRH in a dose arrange from 10 to 1000 nM attenuated the action of FSH on its receptor expression in a dose-dependent manner (Tilly et al., 1992). Evidence also showed that IGF-I enhanced FSH action in rat GCs by prolonged FSHR mRNA stability (Minegishi et al., 2000). Transforming growth factor-β (TGF-β) and activin are also known to be inducers of FSHR expression (Simoni et al., 1997).

It has been reported that activin A action on FSH receptor induction is associated with a change in FSH receptor mRNA level (Nakamura et al., 1993; Xiao et al., 1992). Activin increases FSHR mRNA level both by stimulating transcription rate and by stabilizing FSHR mRNA transcripts (Tano et al., 1997). Inhibin also belongs to TGF-β superfamily and acts as an antagonist of activin. Inhibin A is a follicle-stimulating hormone responsive marker of granulosa cell differentiation, which has an autocrine action on sheep granulose cells (Campbell and Baird, 2001). However, the exact role of inhibin on FSHR and FSH action in rat GCs is not known.

FSHR promoter in rat is known to lack the conventional TATA and CCAAT box element. A positive regulatory element therefore is needed for full activity of FSHR promoters which have been exclusively sought and located in proximal region of the 5′-untranslated region, close to their respective transcription start sites (Heckert et al., 1992; Monaco et al., 1995). The upstream stimulatory factor was shown to be a component of the complexes that interacted with the E box in the FSHR promoter (Goetz et al., 1996).

Murine FSHR promoter possesses a functional steroidogenic factor 1 (SF-1) binding site and belongs to SF-1-regulated gene (Heckert et al., 2000; Levallet et al., 2001). SF-1 stimulates rat FSHR promoter activity in a dose-dependent and promoter-specific manner (Heckert, 2001). Further evidence has also shown that SF-1 regulates a variety of cell-specific proteins involved in steroidogenesis (Andersen and Byskov, 2006; Bakke et al., 2001; Humaidan et al., 2006). Phosphorylation of SF-1 can activate expression of DAX-1, which is known to negate SF-1 activity and functions as a global negative regulator of steroid hormone production (Lalli and Sassone-Corsi, 2003).

Several reports have demonstrated that androgen is capable of stimulating follicular development at early stage, and its receptor (AR) level in GCs is positively correlated with FSHR expression and follicular growth. AR is a member of nuclear receptor superfamily, androgen-bound AR functions as a transcription factor to regulate genes involved in an array of physiological processes (Heinlein and Chang, 2002). Evidence has shown that treatment of GCs with androgen significantly increased the cell FSHR mRNA level (Wang et al., 2001; Weil et al., 1999).

To gain insight whether inhibin A has a negative intra-follicular action on FSH function by regulating its receptor expression, the objective of the present study was designed to explore how inhibin A possibly modulates FSHR expression and FSH action, and then further to look at its molecular basis of the regulation in cultured rat GCs. Our evidence has shown that inhibin A is capable of suppressing FSHR mRNA expression and FSH-induced steroidogenesis. In addition, our evidence also showed that some nuclear transcriptional factors, such as SF-1, AR and DAX-1 may be involved in the regulation of inhibin A action.

2. Materials and methods

2.1. Reagents and hormones

Inhibin A was a gift from Dr. T.K. Woodruff. Culture materials were purchased from Corning, Inc. (Corning, NY). FSH (NIADDK-hFSH-I-3) was obtained from the National Hormone and Pituitary Distribution Program, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases. McCoy's 5a medium, diethylstilbestrol (DES), 4-androstene-3,17-dione (androstenedione), soybean trypsin inhibitor, aprotinin and leupeptin and mouse monoclonal antibody for β-actin were all purchased from Sigma. Rabbit polyclonal antibodies for SF-1, AR and DAX-1 were purchased from Santa Cruz Biotechnology, Inc.

2.2. Experimental animals

Immature female Sprage–Dawley rats (23 days old) were obtained from the Experiment Animal Center, Chinese Academy of Sciences and housed under 16 h light, 8 h dark schedule with food and water ad libitum. The animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All the protocols had the approval of the Institutional Committee on Animal Care and Use.

2.3. Primary cell culture

Granulosa cells were obtained from small antral follicles of estrogen-treated immature rats. Ovaries were punctured by 25-G needles. Ovarian debris was removed, and the remaining medium containing the granulosa cells was collected after low-speed centrifugation at $500 \times g$ for 5 min. The cells were washed twice with culture medium, and an aliquot of the cells was mixed with trypan blue stain for determining the cell number and viability. The cells were cultured overnight for adhesion in serum-free McCoy's 5a medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate at 37 °C in an atmosphere of 5% CO_2 and 95% air, and further incubated in the fresh medium with the presence or absence of the various reagents for the indicated times.

2.4. Vector preparation and transfection

Plasmid pGL3-basic is a luciferase vector lacking the eukaryotic promoter and the enhancer sequences (Promega Co.). The fragment of the 5′-flanking region of −1875 to +26 bp relative to the translational initiation site of the rat FSHR was generated from the rat genomic DNA via PCR. The specific primers were 5′-TGAAACCTAACTACACAGCTGACC-3′ and 5′-TACTCGAGGAGACCAGGAGCAAG-3′. The isolated PCR-synthesized cDNA fragments were subcloned into pMD18-T vector (Takara Co.). These fragments were then ligated to pGL3-basic vector named FSHR-Luc and characterized by nucleotide sequencing analysis. This construct included two transcriptional start sites at −80 and −98 determined previously and the relevant DNA elements (E box), which is required for full promoter function of the rat FSHR gene, at −119 bp (Goetz et al., 1996). Granulosa cells (5×10^5 viable cells/well) were cultured in the culture medium supplemented with 10% FBS for 2 h. Before transfection, the medium was changed to serum-free medium. Transient transfections using Lipofectamine 2000 (Invitrogen Co.) for 5 h followed manufacturer's recommendations. All groups were co-transfected with phRL-TK Renilla luciferase reporter vector. The cells were then lysed in the lysis buffer supplied by manufacturer before measurement of the firefly and the renilla luciferase activities on luminometer. The relative firefly luciferase activities were calculated by normalizing transfection efficiency according to the renilla luciferase activities. The experiments were performed in triplicate, and similar results were obtained from at least three independent experiments.

2.5. RNA extraction and real-time PCR

The GCs were treated with FSH (20 ng/ml) in the presence or absence of inhibin A (100 ng/ml) for 24 h. The culture medium was then removed and total cellular RNA was extracted using TRIzol (Invitrogen Co.) and quantified by measuring absorbance at 260 nm. The first-strand cDNA was synthesized (4 μg total RNA) by using random primers and SuperScript Reverse Transcriptase (Stratagene Co.). Then the cDNA was used as the template, and real-time PCR was carried out using Sybr Green (Sybr Green PCR Master Mix; Tiangen, Co.). Specific PCR settings (95 °C for 15 min and 40 cycles at 95 °C for 15 s, 58 °C for 20 s and 68 °C for 30 s) were used in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Melting curve analyses were performed after real-time PCR reactions to monitor PCR product purity. Primers for rat FSHR were forward 5′-CATCACTGTGTCCAAGGCCA-3′ and reverse 5′-TGCGGAAGTTCTTGGTGAAAA-3′; AR (forward 5′-GACACTTGAGATCCCGTCCT-3′; reverse 5′-GAGCGAGCGGAAAGTTGTAG-3′); SF-1

(forward 5′-GCAGAAGAAAGCACAGATTC-3′; reverse 5′-TGGATACTCAGACTTGATG G-3′); 18S ribosomal RNA (forward 5′-CGCGGTTCTATTTTGTTGGT-3′; reverse 5′-AGTCGGCATCGTTTATGGTC-3′) (Orisaka et al., 2006); P450scc (forward 5′-CCCCATTTACAGGGAGAAGCTGG-3′; reverse 5′-CCTCCAGGCAT-CTGAACTCTTAAAC-3′); P450arom (forward 5′-GTCCCGGAAACTGTGCCTGTC-3′; reverse 5′-AGAATCTGCCATGGGAAATGA-3′). The threshold cycle (C_T) numbers were determined for the amplified cDNA for each investigated mRNA and for the housekeeping gene, 18S rRNA in each unknown sample during real-time PCR. The relative quantification of gene expression across treatments was evaluated using the $\Delta\Delta C_T$ method. The ΔC_T is calculated as the difference between the C_T of the investigated gene and the C_T of 18S rRNA in each sample. The $\Delta\Delta C_T$ of each investigated gene is calculated as the difference between the ΔC_T in each treated sample and the ΔC_T in each control sample. The fold change in relative mRNA concentrations for treated versus control samples was calculated using the formula: $2^{-\Delta\Delta C_T}$. The effects of treatments on the two mRNAs are shown graphically as fold change compared to mRNA concentration in control (untreated) wells and expressed as percentage change from control (2-fold increase = 200% of control) in the text.

2.6. Bioactivity study

GCs (1×10^6 vial cells) were cultured overnight in a 35 mm dish with 1 ml medium. Then the cells were treated with various reagents for the indicated time. For the assessment of estradiol production, 100 nM androstenedione, a substrate for P450arom, was added to the medium. After 24 h, the conditioned media were collected and stored at −20 °C until assay. The levels of progesterone (P^4) and estradiol (E_2) in the media were measured by the commercial RIA kit at a commercial laboratory (Chemclin Co., Beijing). The intra-assay coefficient of variation (intra-assay CV) is less 10%, and the inter-assay coefficient of variation (inter-assay CV) is under 15%. The sensitivity of the E_2 assay is less than 3 pg/ml, and the sensitivity of the P assay is less than 0.05 ng/ml.

2.7. Western blotting analysis

GCs (2×10^6 viable cells) were cultured for the indicated times in a 60 mm dish with 1.5 ml McCoy's 5a alone (control) or supplemented with FSH (20 ng/ml) and/or inhibin A (100 ng/ml). The cells were then collected by pipette and centrifuged and lysed by RIPA buffer supplemented with protease inhibitors (phenylmethylsulfonyl fluoride) and aprotinin. The supernatants after centrifugation (12,000 × g, 15 min) were collected, and the total protein concentrations were determined by spectrophotometer. Protein extract (20 μg) from each sample was separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes, then probed with DAX-1-specific antibody (1:500) and reprobed with β-actin (1:5000) antibody. The bands were visualized by Supersignal West Pico chemiluminescent substrate (Pierce Co., Rockford, IL).

2.8. Confocal immunohistochemistry

GCs were seeded onto 24 mm × 24 mm cover slips placed in 6-well plates at a density of 2×10^5 cells/cover slip. The cells were cultured overnight for adhesion in McCoy's 5a medium at 37 °C and then treated with FSH (20 ng/ml), and/or inhibin A (100 ng/ml) for 24 h. By the end of incubation, the media were removed and the cells were fixed with methanol and acetone (1:1) for 10 min, followed by incubation for 1 h with 10% house serum in phosphate-buffered saline (PBS). Then the cells were incubated for 2 h with an antibody for SF-1, AR or DAX-1 (1:100) at room temperature. The cells were washed with PBS buffer three times and further incubated with rabbit anti-goat antibody conjugated fluorescein (1:100) at room temperature for 1.5 h. After three washes in PBS, the cells were incubated with 10 ng/ml propidium iodide (PI) for 10 min. Slides were finally analyzed by confocal laser scanning microscopy (Carl Zeiss Inc., Thornwood, NY).

2.9. Data analysis

All the experiments were repeated at least three times with GC preparations obtained from separate groups. The values were presented as the mean ± S.E.M. Statistical significance was determined using SPSS 14.0 software for multiple group comparisons. Significance was accepted at $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Inhibin A is capable of suppressing basal and FSH-induced FSHR mRNA expression

To investigate action of inhibin A on FSH function in rat ovary, we first analyzed effect of inhibin A on FSHR mRNA level in cultured primary GCs by real-time quantitative PCR. As shown in Fig. 1, the GCs in the untreated control group expressed FSHR mRNA spontaneously. Treatment of the GCs with inhibin A for 24 h showed a weak inhibition of FSHR mRNA content as compared with the control ($p < 0.05$). The FSH-treated GCs dramatically increased FSHR mRNA level by 7–8-fold, and this increase was significantly inhibited by co-treatment with inhibin A ($p < 0.01$). Thus, both the basal and the FSH-induced FSHR mRNA expression in the GCs were negatively regulated by the addition of inhibin A in the culture.

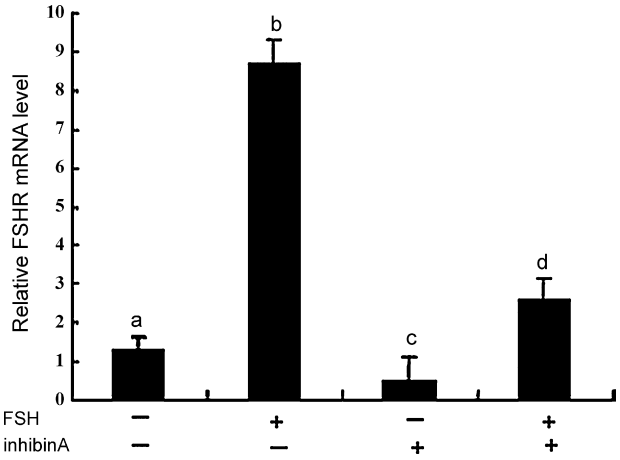

Fig. 1. Effect of inhibin A on FSH-induced FSHR mRNA expression in rat granulosa cells. Granulosa cells obtained from DES-primed rat ovaries were cultured with or without FSH (20 ng/ml) or inhibin A (100 ng/ml) for 24 h. The cell RNA was extracted and reversely transcribed. The relative mRNA level of FSHR was reflected indirectly by cDNA which was quantitated by real-time PCR. Data are presented as mean ± S.E.M. ($n = 5$). Bars with different letters among the groups indicate significantly different. $p < 0.01$(a, b; b, c; b, d); $p < 0.05$(a, c).

3.2. Inhibin A is capable of suppressing basal and FSH-induced FSHR promoter activity

To elucidate the mechanism by which inhibin A regulates FSHR mRNA expression, the promoter construct FSHR-luc and phRL-TK vector were co-transfected into the cultured primary GCs. We measured luciferase activity after addition of inhibin A and/or FSH to the culture medium for 24 h. As shown in Fig. 2, treatment with 20 ng/ml FSH could enhance activity of the 1901 bp of FSHR 5′-flanking region. Treatment with 10, 50 or 100 ng/ml inhibin A dose-dependently suppressed the FSHR promoter activity as compared to the control level. Furthermore, combined treatment of the GCs with inhibin A and FSH significantly decreased the reporter expression induced by FSH alone.

3.3. Inhibin A inhibits FSH-induced P450scc and P450arom expression

To determine whether inhibin A modulates FSH-induced genes in GCs, we examined possible effect of inhibin A on two related steroidogenic enzymes, cholesterol side-chain cleavage cytochrome P450 enzyme (P450scc) and P450 aromatase (P450arom), the key enzymes for progesterone and estrogen synthesis, respectively. As shown in Fig. 3, FSH induced a marked increase in the mRNA level for both enzymes, but inhibin A alone did not significantly affect on the basal level of their mRNAs. However, addition of inhibin A (100 ng/ml) to the cell culture in the presence of FSH totally abolished the observed FSH-induced stimulatory action.

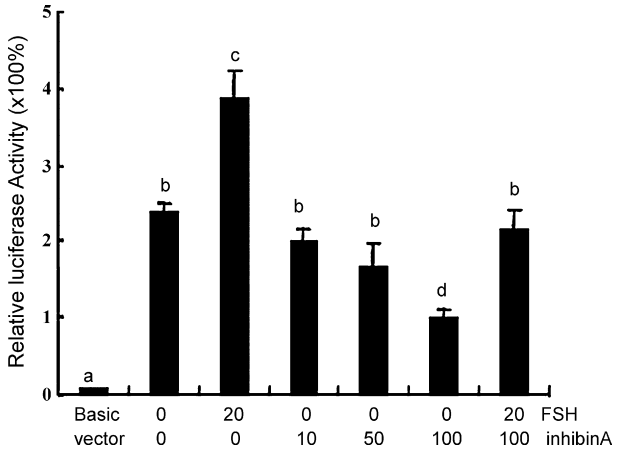

Fig. 2. Effect of inhibin A on FSH-induced FSHR-Luc expression in rat granulosa cells. Granulosa cells were co-transfected with FSHR-Luc and phRL-TK vector and were then cultured for 24 h after addition of FSH (20 ng/ml) or various concentrations of inhibin A (10, 50 and 100 ng/ml) alone or both FSH (20 ng/ml) and inhibin A (100 ng/ml). GCs were also tranfected pGL3-basic vector as a control. Cell lysate was assayed for activity of the luciferase reporter gene. Luciferase activity was presented as relative luciferase activity normalized based on renilla activity in co-transfected cells. Data are presented as mean ± S.E.M. ($n = 3$). Bars with different letters indicate significantly different. $p < 0.01$ (a, b; a, c; a, d; c, d); $p < 0.05$ (b, d).

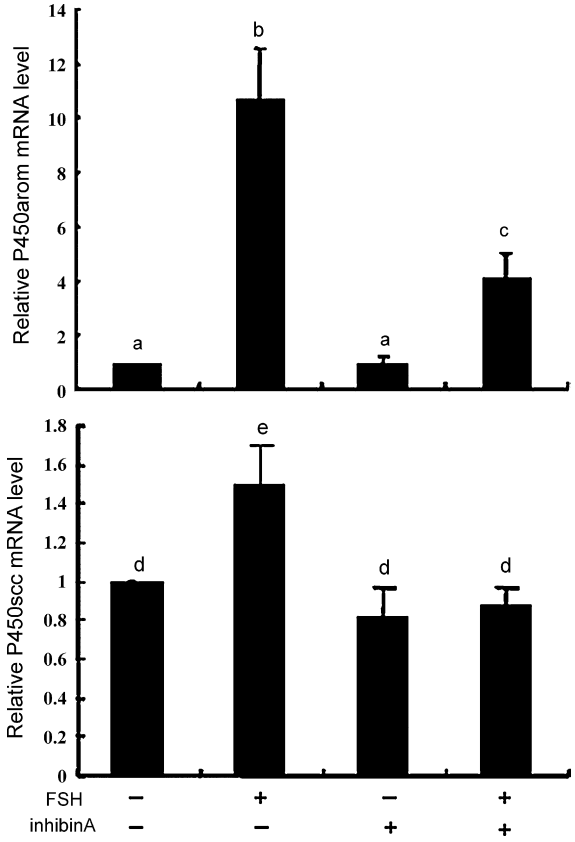

Fig. 3. Effect of inhibin A on FSH-induced P450arom and P450scc expression. Granulosa cells were treated with FSH (20 ng/ml) or inhibin A (100 ng/ml) alone or in combination. After 24 h culture, total RNA in cell lysate was extracted by TRIzol and reversely transcribed; relative levels of P450arom and P450scc mRNA were analyzed by real-time PCR. Each bar represents means ± S.E.M. ($n = 3$). Bars with different letters indicate significant different at $p < 0.01$ (a, b; b, c); $p < 0.05$ (d, e).

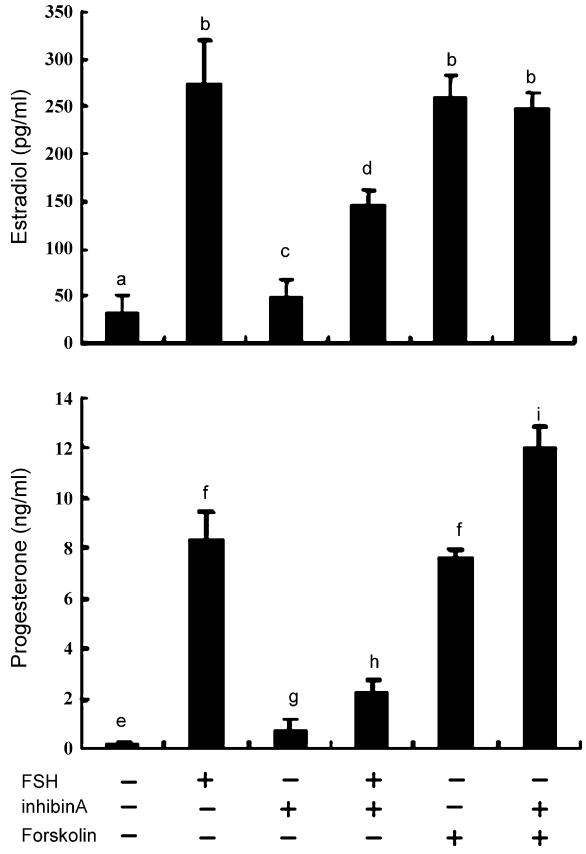

Fig. 4. Effect of inhibin A on FSH- and forskolin-induced estradiol and progesterone production. GCs (10^6 viable cells) were cultured in 35 mm dishes with McCoy's 5a medium containing 100 nM androstenedione in presence or absence of FSH (20 ng/ml), inhibin A (100 ng/ml) or forskolin (10 μM) alone or in combination. After 24 h incubation, progesterone and estradiol levels in the media were measured by radioimmunoassay. Data are presented as mean ± S.E.M. ($n = 4$). Bars with different letters indicate significant different at $p < 0.01$ (a, b; b, d; e, f; f, h; f, i); $p < 0.05$ (a, c, e, g).

3.4. Inhibin A inhibits FSH-induced, but not forskolin-induced steroidogenesis in cultured GCs

To determine the inhibitory site of inhibin A suppressing FSH action, we further examined effect of inhibin A on FSH bioactivity, specifically, on P^4 and E_2 production in the cultured GCs. As shown in Fig. 4, forskolin (10 μM) stimulated production of P^4 and E_2, similar to that observed with FSH. Addition of inhibin A to the culture significantly suppressed the FSH-induced P^4 and E_2 production. In contrast, the forskolin-induced steroidogenic increases could not be suppressed by the inhibin A. It is therefore suggested that inhibin A suppressing FSH action may be through inhibiting functional FSHR in GCs. In addition, it is interesting to note that inhibin A itself seemingly could increase the basal E_2 and P^4 production by the cultured GCs as compared with the control group.

3.5. SF-1, DAX-1 and AR are involved in inhibin A-regulated FSH action on granulosa cells

To further examine possible mechanism of inhibin A regulating FSHR expression and FSH function, we examined possible involvement of some related nuclear transcriptional regulators, such as SF-1, DAX-1 and AR in the GC culture. SF-1 mRNA level was determined by real-time PCR using SYBR green (Fig. 5A), and its protein level was estimated by immunocytochemistry (Fig. 5B). The endogenous SF-1 mRNA level was low in the unstimulated GCs, and

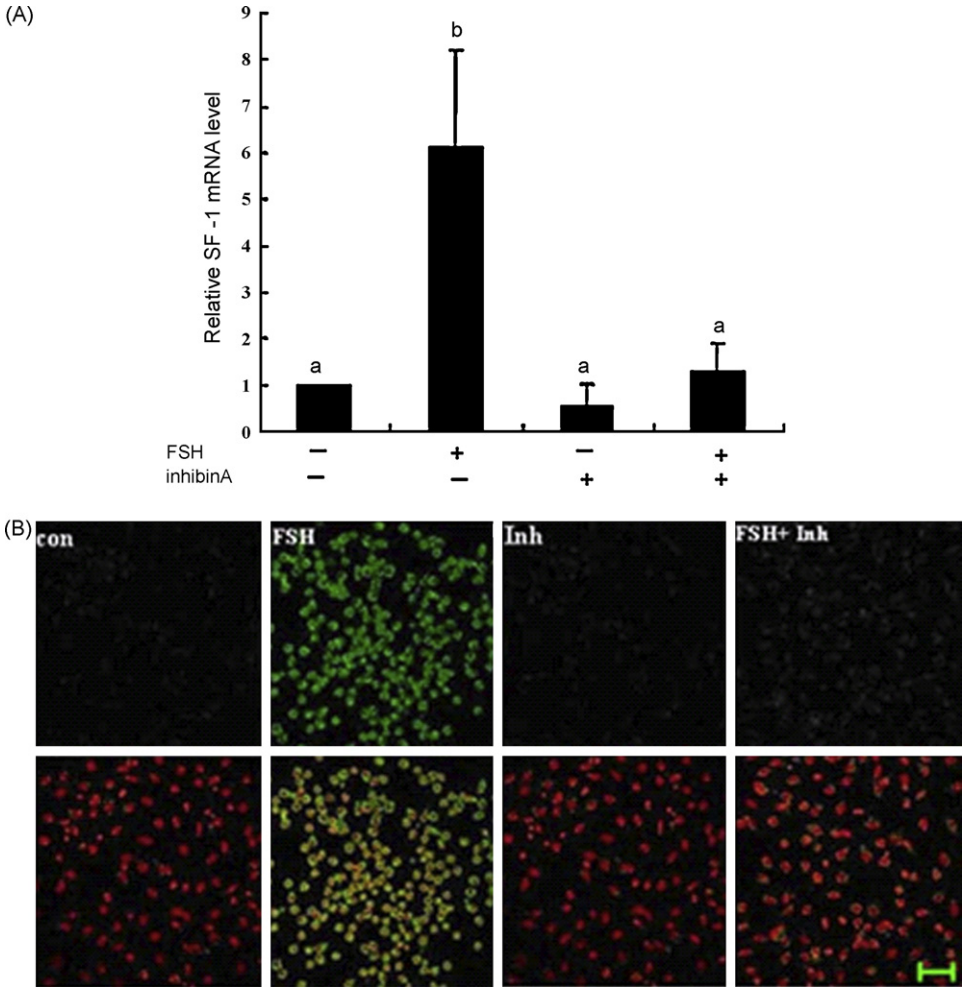

Fig. 5. Effect of inhibin A on FSH-induced SF-1 mRNA and its protein expression in rat granulosa cells. Granulosa cells were cultured and treated as described in Fig. 1. After 24 h incubation, total RNA was extracted, and SF-1 relative mRNA level was analyzed by real-time PCR. Data are presented as mean ± S.E.M. ($n = 3$). Bars with different letters indicate significant different at $p < 0.01$ (A). The expression of its protein was estimated by confocal immunocytochemistry. After treatment for 24 h, the cells were fixed with methanol and acetone (1:1), then the cells were incubated with SF-1 antibody and then goat antirabbit IgG conjugated with fluorescein. The nuclei were stained with PI (*red*). The SF-1 positive staining is green fluorescence visualized in the top row of images, while merged signals were seen in yellow in the lower row of images obtained by a confocal laser microscope. Bar = 20 μm (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

significantly increased up to 5–6-fold following the FSH (20 ng/ml) stimulation for 24 h. In contrast, both SF-1 mRNA and its protein levels induced by FSH were decreased by the co-treatment with inhibin A (100 ng/ml).

As reported previously that DAX-1 could antagonize SF-1 action, we then further examined changes in DAX-1 expression in the presence of inhibin A or FSH alone or in their combination in the cultured GCs. As shown in Fig. 6A, DAX-1 was low both in the control and the FSH-treated groups. However, inhibin A alone significantly increased DAX-1 expression by 10-fold, and its expression was remarkably inhibited by the co-treatment with FSH. As shown in Fig. 6B, the immunocytochemistry was consistent with the real-time PCR result. It is therefore suggested that DAX-1 may be also involved in the signaling regulation of inhibin A. However, it is not clear how FSH suppressed the inhibin A-induced DAX-1 expression, the question is remained for further study.

By bioinformatics software analysis there are at least two AR binding sites in FSHR promoter. Therefore, AR may be also involved in the regulation of FSHR expression. We then examined possible action of inhibin A on its expression in the culture. As shown in Fig. 7, AR mRNA and its protein levels were up-regulated by FSH as compared with the control group. Addition of inhibin A to the cell culture, a 5-fold inhibition of the AR mRNA production was observed (Fig. 7A). The immunocytochemistry data (Fig. 7B) were similarly to the real-time PCR results.

4. Discussion

It is well known that inhibin exerts a negative feedback suppressive action on FSH secretion by pituitary. We have hypothesized in the present study whether inhibin A could regulate FSH receptor expression at ovarian level. We have demonstrated that inhibin A markedly decreased the FSH-induced increase in FSHR mRNA expression in cultured rat granulosa cells via suppressing the FSHR promoter activity. We also provided evidence to show that inhibin A reduced FSH-induced P450scc and P450arom mRNA level. Furthermore, our evidence showed that the FSH-induced, but not forskolin-induced steroidogenesis was suppressed by the presence of inhibin A, suggesting that the inhibition of FSH-induced steroidogenesis by inhibin A may be mediated via a pre-cAMP signaling event. Therefore, we propose that inhibin A can be considered as an autocrine negative regulator of FSHR expression in the ovary, and the regulation of FSHR expression may be one of the causes of inhibin A inhibition of FSH action.

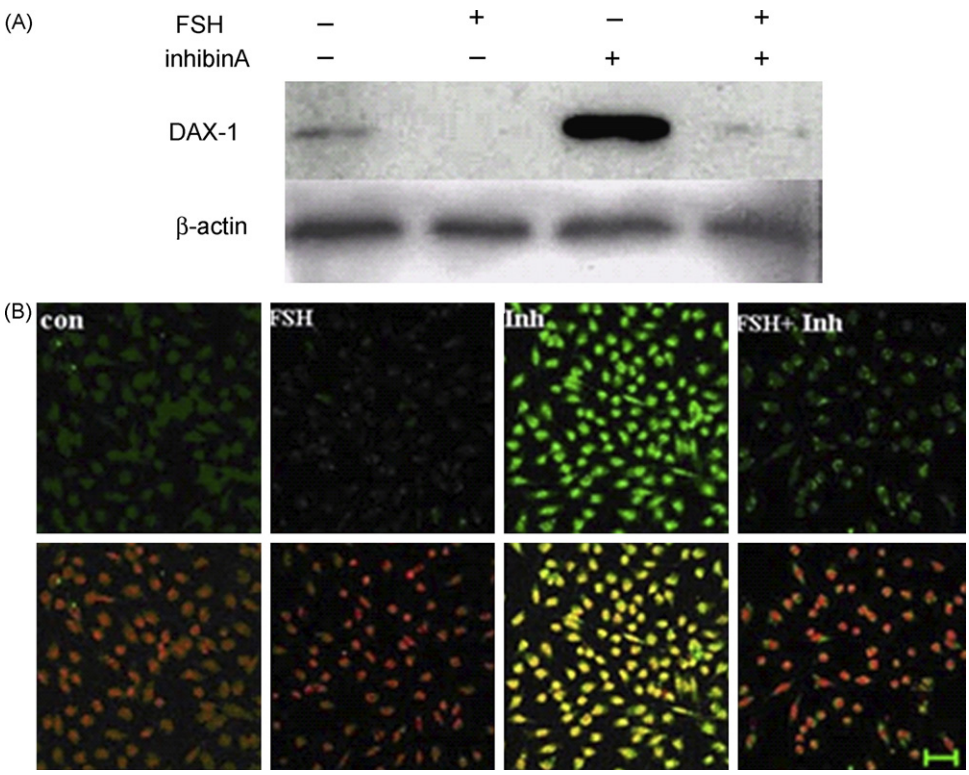

Fig. 6. Effect of inhibin A on DAX-1 protein expression in rat granulosa cells. Granulosa cells were treated as described in Fig. 1. After 24 h incubation, total protein in the cell lysate of each group were extracted by RIPA buffer, DAX-1 protein level was assayed by Western blot (A). The treated cells were fixed and incubated with DAX-1 antibody and then goat antirabbit IgG conjugated with fluorescein. The nuclei were stained with PI (*red*). The DAX-1 positive staining is *green fluorescence* visualized in the top row of images, while merged signals were seen in yellow in the lower row of images obtained by a confocal laser microscope. Bar = 20 μm (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Our observation that FSH stimulates its own receptor expression is consistent with the previous report (Minegishi et al., 2000; Tilly et al., 1992). We further demonstrated that inhibin A suppressed the FSH-induced FSHR promoter activity leading to the inhibition of FSHR transcription. However, we cannot rule out the possibility that inhibin A might also reduce FSHR mRNA stability. We observed that FSHR promoter activity measured in the basal group was higher (Fig. 2), but the FSHR mRNA was not so high similarly (Fig. 1). The reason for this observation might be due to the GCs used in the experiment before transfection had been pre-cultured in the media containing 10% FBS which might lead to partly GC differentiation and decrease its sensitivity to FSH stimulation. Inhibin itself is a physiological modulator of follicular steroidogenesis (Smyth et al., 1994). Because we did not directly examine FSHR expression at a protein level, therefore, one cannot exclude the possibility that inhibin A might also exert its effect by a mechanism other than just reducing FSHR mRNA.

Our some data in the present study are not consistent to the early reports. Shukovski and Findlay (1990) observed inhibin had no detectable effect upon progesterone production of preovulatory bovine granulosa cells in vitro. Wrathall and Knight (1995) also demonstrated that inhibin did not affect basal nor LH-stimulated secretion of progesterone in bovine theca cells. Recently, Johnson et al. (2006) in their hen experiments also observed that inhibin had no significant effect on FSHR expression at any dose in the granulosa cells or small yellow follicles. Potential explanation for the different reports may be due to species difference, the difference of hormone doses or the assay technique in detection of different inhibin isoforms. Further study is needed for clarifying these points.

It has been reported that activin is capable of up-regulating FSH receptor expression in the undifferentiated rat GCs (Nakamura et al., 1993; Xiao et al., 1992). Increasing evidence suggests that existence of an inhibin receptor or binding protein in GCs which is distinct from the activin type II receptors and inhibin is not able to antagonize activin action in all contexts (Bernard et al., 2001). Therefore, inhibin A exerting its roles may be not definitely by antagonizing activin action. Nevertheless, the finding in the present study suggested that inhibin A could be an autocrine negative regulator of FSHR expression, it not only plays an important role in regulation of pituitary FSH secretion by a long feedback via circulation (Dubey et al., 1987; Lumpkin et al., 1984; Ramaswamy et al., 1998; Ying et al., 1987), but may also be capable of inhibiting its receptor production, subsequently leading to suppress of FSH action within a follicle by a short feedback regulation.

It has been reported that nuclear transcriptional factors, such as AR, SF-1 and DAX-1, are involved in regulation of FSH-induced function in GCs (Bakke et al., 2001; Su et al., 2002; Tai et al., 2001; von Bubnoff and Cho, 2001). In our previous studies, we observed that FSH could significantly induce GC LRH-1 expression. LRH-1 has 60% amino acid similarity to SF-1 which contains a virtually identical DNA binding domain (Yu et al., 2005). FSH could induce SF-1 expression in the cultured GCs observed in the present study is consistent with the finding of Shapiro et al. (1996). We have further demonstrated that inhibin A is capable of suppressing the FSH-induced SF-1 expression and steroidogenesis.

DAX-1 has been extensively described as a potent repressor of SF-1 action (Crawford et al., 1998; Ito et al., 1997; Zazopoulos et al., 1997). Evidence by Yazawa et al. (2003) showed that FSH suppressed DAX-1 expression in rat GCs. Our observation showed that inhibin A itself was capable of enhancing DAX-1 expression in the cells. Interestingly, addition of FSH to the culture remarkably suppressed the inhibin A-induced DAX-1 expression, the molecu-

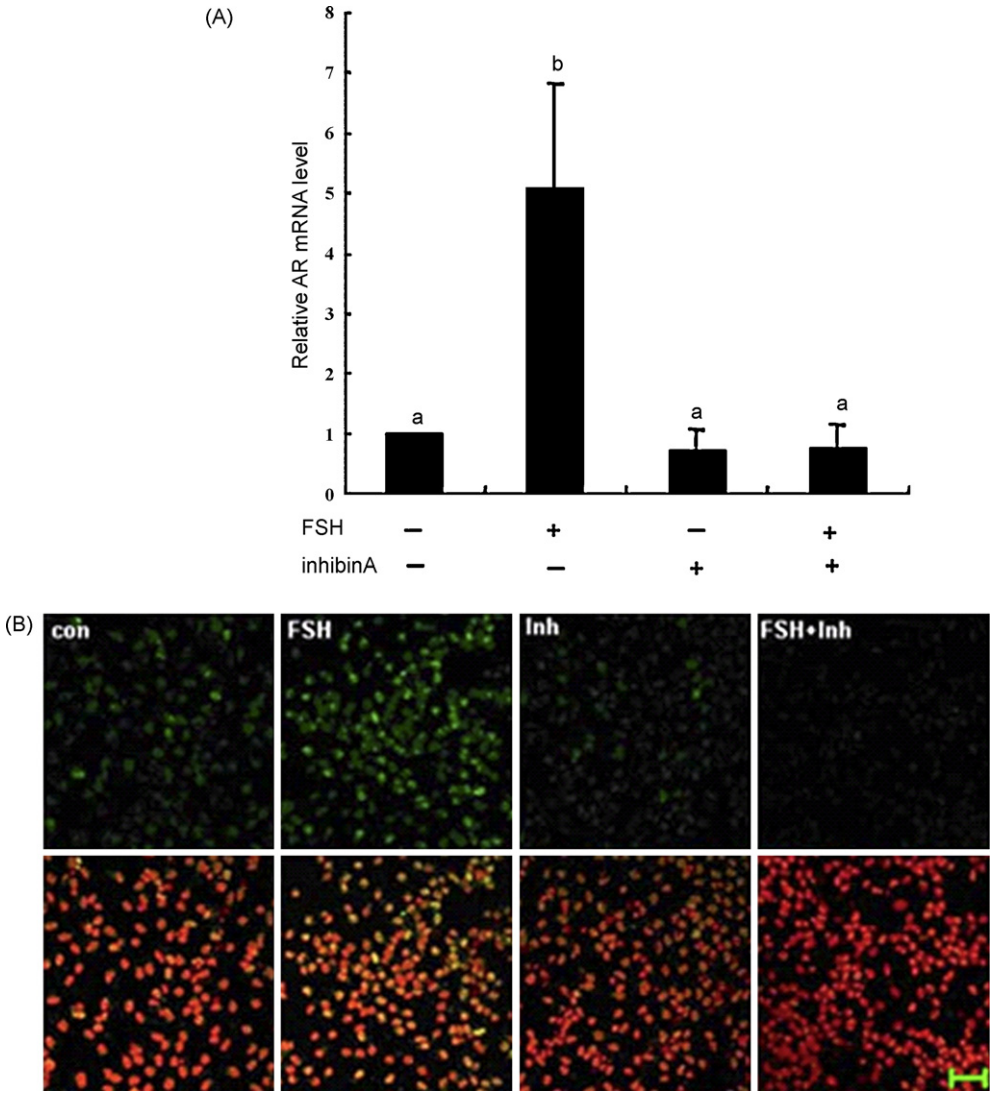

Fig. 7. Effect of inhibin A on FSH-induced AR mRNA and its protein expression in rat granulosa cells. Granulosa cells were cultured and treated as described in Fig. 1. After 24 h incubation, total RNA was extracted, and AR relative mRNA level was analyzed by real-time PCR. Data are presented as mean ± S.E.M. ($n = 3$). Bars with different letters indicate significant different at $p < 0.01$ (A). The expression of AR protein was estimated by confocal immunocytochemistry. After fixed for 10 min, the cells were incubated with AR antibody and then goat antirabbit IgG conjugated with fluorescein. The nuclei were stained with PI (*red*). The AR positive staining is *green fluorescence* visualized in the top row of images, while merged signals were seen in yellow in the lower row of images obtained by a confocal laser microscope. Bar = 20 μm (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

lar mechanism is not known. Previous evidence has shown that in addition to a direct interaction of DAX-1 with SF-1, DAX-1 could also affect steroidogenic cascade at multiple signaling levels by inhibition of enzymes involved in steroidogenesis (Lalli et al., 1998). An increase in DAX-1 expression and a down-regulation of SF-1 production observed may explain the decreased steroidogenesis in response to prolonged stimulation of ovarian GCs by the gonadotropin (Iyer and McCabe, 2004; Tajima et al., 2003). Therefore, interaction between transcriptional factor SF-1 and DAX-1 may play important roles not only in regulating FSHR mRNA expression, but also in steroidogenesis observed in the present study.

FSH treatment of GCs up-regulated AR expression, and addition of inhibin A to the culture could suppress the FSH-induced AR expression. Our data suggest that AR may play a role in regulation of FSHR expression. However, Tetsuka et al. (1995) reported AR expression in rat GCs was developmentally regulated, and FSH was not directly responsible for the down-regulation of AR mRNA expression. There are also evidence that androgen down-regulated AR mRNA expression in immature GCs, and this effect was reversed by FSH (Tetsuka and Hillier, 1996; Tetsuka et al., 1995). The reason for the different results obtained from our study might be due to a low dose of estrogen precursor, androstenedione (10^{-7} M) used in the culture, which might influence the basal level of AR in the control. DAX-1 is one of the inhibitors of AR-mediated transcription (Agoulnik et al., 2003; Jouravel et al., 2007) and has also been reported to be an AR negative co-regulator (Holter et al., 2002). DAX-1 may play a role in limiting AR activity (Agoulnik et al., 2003). An important relationship between DAX-1 and AR expression has been reported in human breast cancer. AR acting as a suppressor of DAX-1 in adrenal cortex was demonstrated through interaction with SF-1 (Mukai et al., 2002). Jorgensen and Nilson (2001) reported AR suppressing bovine LH-β promoter activity was through protein–protein interactions with SF-1. Therefore, interactions may exist among the three transcriptional factors in inhibin A regulating FSH-induced FSHR expression and steroidogenesis.

In conclusion, our study has demonstrated for the first time that inhibin A is capable of antagonizing FSH action on GCs by reducing FSHR mRNA expression and some orphan receptors, such as SF-1,

AR and DAX-1 may be involved in the regulation. It is therefore suggested that inhibin may be considered as an autocrine negative regulator of FSHR expression within a follicle by a short feedback regulation.

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References

Abel, M.H., Wootton, A.N., Wilkins, V., Huhtaniemi, I., Knight, P.G., Charlton, H.M., 2000. The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. Endocrinology 141, 1795–1803.

Agoulnik, I.U., Krause, W.C., Bingman III, W.E., Rahman, H.T., Amrikachi, M., Ayala, G.E., Weigel, N.L., 2003. Repressors of androgen and progesterone receptor action. J. Biol. Chem. 278, 31136–31148.

Andersen, C.Y., Byskov, A., 2006. Estradiol and regulation of anti-Mullerian hormone inhibin-A and inhibin-B secretion: analysis of small antral and preovulatory human follicles' fluid. J. Clin. Endocrinol. Metab. 91, 4064–4069.

Baird, D.T., Smith, K.B., 1993. Inhibin and related peptides in the regulation of reproduction. Oxf. Rev. Reprod. Biol. 15, 191–232.

Bakke, M., Zhao, L., Hanley, N.A., Parker, K.L., 2001. SF-1 a critical mediator of steroidogenesis. Mol. Cell. Endocrinol. 171, 5–7.

Bernard, D.J., Chapman, S.C., Woodruff, T.K., 2001. Mechanisms of inhibin signal transduction. Recent Prog. Horm. Res. 56, 417–450.

Burger, H.G., 1993. Evidence for a negative feedback role of inhibin in follicle stimulating hormone regulation in women. Hum. Reprod. 8 (Suppl. 2), 129–132.

Campbell, B.K., Baird, D.T., 2001. Inhibin A is a follicle stimulating hormone-responsive marker of granulosa cell differentiation, which has both autocrine and paracrine actions in sheep. J. Endocrinol. 169, 333–345.

Chappel, S.C., Howles, C., 1991. Reevaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process. Hum. Reprod. (Oxford, England) 6, 1206–1212.

Crawford, P.A., Dorn, C., Sadovsky, Y., Milbrandt, J., 1998. Nuclear receptor DAX-1 recruits nuclear receptor corepressor N-CoR to steroidogenic factor 1. Mol. Cell. Biol. 18, 2949–2956.

Dierich, A., Sairam, M.R., Monaco, L., Fimia, G.M., Gansmuller, A., LeMeur, M., Sassone-Corsi, P., 1998. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc. Natl. Acad. Sci. U.S.A. 95, 13612–13617.

Dubey, A.K., Zeleznik, A.J., Plant, T.M., 1987. In the rhesus monkey (*Macaca mulatta*), the negative feedback regulation of follicle-stimulating hormone secretion by an action of testicular hormone directly at the level of the anterior pituitary gland cannot be accounted for by either testosterone or estradiol. Endocrinology 121, 2229–2237.

Findlay, J.K., 1993. An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. Biol. Reprod. 48, 15–23.

Goetz, T.L., Lloyd, T.L., Griswold, M.D., 1996. Role of E box and initiator region in the expression of the rat follicle-stimulating hormone receptor. J. Biol. Chem. 271, 33317–33324.

Gudermann, T., Nurnberg, B., Schultz, G., 1995. Receptors and G proteins as primary components of transmembrane signal transduction. Part 1. G-protein-coupled receptors: structure and function. J. Mol. Med. (Berlin, Germany) 73, 51–63.

Heckert, L.L., 2001. Activation of the rat follicle-stimulating hormone receptor promoter by steroidogenic factor 1 is blocked by protein kinase a and requires upstream stimulatory factor binding to a proximal E box element. Mol. Endocrinol. 15, 704–715.

Heckert, L.L., Daley, I.J., Griswold, M.D., 1992. Structural organization of the follicle-stimulating hormone receptor gene. Mol. Endocrinol. 6, 70–80.

Heckert, L.L., Sawadogo, M., Daggett, M.A., Chen, J.K., 2000. The USF proteins regulate transcription of the follicle-stimulating hormone receptor but are insufficient for cell-specific expression. Mol. Endocrinol. 14, 1836–1848.

Heinlein, C.A., Chang, C., 2002. Androgen receptor (AR) coregulators: an overview. Endocr. Rev. 23, 175–200.

Herath, C.B., Yamashita, M., Watanabe, G., Jin, W., Tangtrongsup, S., Kojima, A., Groome, N.P., Suzuki, A.K., Taya, K., 2001. Regulation of follicle-stimulating hormone secretion by estradiol and dimeric inhibins in the infantile female rat. Biol. Reprod. 65, 1623–1633.

Holter, E., Kotaja, N., Makela, S., Strauss, L., Kietz, S., Janne, O.A., Gustafsson, J.A., Palvimo, J.J., Treuter, E., 2002. Inhibition of androgen receptor (AR) function by the reproductive orphan nuclear receptor DAX-1. Mol. Endocrinol. 16, 515–528.

Humaidan, P., Bungum, L., Bungum, M., Yding Andersen, C., 2006. Rescue of corpus luteum function with peri-ovulatory HCG supplementation in IVF/ICSI GnRH antagonist cycles in which ovulation was triggered with a GnRH agonist: a pilot study. Reprod. Biomed. Online 13, 173–178.

Ito, M., Yu, R., Jameson, J.L., 1997. DAX-1 inhibits SF-1-mediated transactivation via a carboxy-terminal domain that is deleted in adrenal hypoplasia congenita. Mol. Cell. Biol. 17, 1476–1483.

Iyer, A.K., McCabe, E.R., 2004. Molecular mechanisms of DAX1 action. Mol. Genet. Metab. 83, 60–73.

Jimenez-Krassel, F., Winn, M.E., Burns, D., Ireland, J.L., Ireland, J.J., 2003. Evidence for a negative intrafollicular role for inhibin in regulation of estradiol production by granulosa cells. Endocrinology 144, 1876–1886.

Johnson, P.A., Woodcock, J.R., Kent, T.R., 2006. Effect of activin A and inhibin A on expression of the inhibin/activin beta-B-subunit and gonadotropin receptors in granulosa cells of the hen. Gen. Comp. Endocrinol. 147, 102–107.

Jorgensen, J.S., Nilson, J.H., 2001. AR suppresses transcription of the LHbeta subunit by interacting with steroidogenic factor-1. Mol. Endocrinol. 15, 1505–1516.

Jouravel, N., Sablin, E., Arnold, L.A., Guy, R.K., Fletterick, R.J., 2007. Interaction between the androgen receptor and a segment of its corepressor SHP. Acta Crystallogr. D Biol. Crystallogr. 63, 1198–1200.

Kumar, T.R., Wang, Y., Lu, N., Matzuk, M.M., 1997. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat. Genet. 15, 201–204.

Lalli, E., Sassone-Corsi, P., 2003. DAX-1, an unusual orphan receptor at the crossroads of steroidogenic function and sexual differentiation. Mol. Endocrinol. 17, 1445–1453.

Lalli, E., Melner, M.H., Stocco, D.M., Sassone-Corsi, P., 1998. DAX-1 blocks steroid production at multiple levels. Endocrinology 139, 4237–4243.

Lanuza, G.M., Groome, N.P., Baranao, J.L., Campo, S., 1999. Dimeric inhibin A and B production are differentially regulated by hormones and local factors in rat granulosa cells. Endocrinology 140, 2549–2554.

Levallet, J., Koskimies, P., Rahman, N., Huhtaniemi, I., 2001. The promoter of murine follicle-stimulating hormone receptor: functional characterization and regulation by transcription factor steroidogenic factor 1. Mol. Endocrinol. 15, 80–92.

Lumpkin, M.D., DePaolo, L.V., Negro-Vilar, A., 1984. Pulsatile release of follicle-stimulating hormone in ovariectomized rats is inhibited by porcine follicular fluid (inhibin). Endocrinology 114, 201–206.

Mann, G.E., Campbell, B.K., McNeilly, A.S., Baird, D.T., 1992. The role of inhibin and oestradiol in the control of FSH secretion in the sheep. J. Endocrinol. 133, 381–391.

Minegishi, T., Hirakawa, T., Kishi, H., Abe, K., Abe, Y., Mizutani, T., Miyamoto, K., 2000. A role of insulin-like growth factor I for follicle-stimulating hormone receptor expression in rat granulosa cells. Biol. Reprod. 62, 325–333.

Molskness, T.A., Woodruff, T.K., Hess, D.L., Dahl, K.D., Stouffer, R.L., 1996. Recombinant human inhibin-A administered early in the menstrual cycle alters concurrent pituitary and follicular, plus subsequent luteal, function in rhesus monkeys. J. Clin. Endocrinol. Metab. 81, 4002–4006.

Monaco, L., Foulkes, N.S., Sassone-Corsi, P., 1995. Pituitary follicle-stimulating hormone (FSH) induces CREM gene expression in Sertoli cells: involvement in long-term desensitization of the FSH receptor. Proc. Natl. Acad. Sci. U.S.A. 92, 10673–10677.

Mukai, T., Kusaka, M., Kawabe, K., Goto, K., Nawata, H., Fujieda, K., Morohashi, K., 2002. Sexually dimorphic expression of Dax-1 in the adrenal cortex. Genes Cells 7, 717–729.

Nakamura, M., Minegishi, T., Hasegawa, Y., Nakamura, K., Igarashi, S., Ito, I., Shinozaki, H., Miyamoto, K., Eto, Y., Ibuki, Y., 1993. Effect of an activin A on follicle-stimulating hormone (FSH) receptor messenger ribonucleic acid levels and FSH receptor expressions in cultured rat granulosa cells. Endocrinology 133, 538–544.

Orisaka, M., Orisaka, S., Jiang, J.Y., Craig, J., Wang, Y., Kotsuji, F., Tsang, B.K., 2006. Growth differentiation factor-9 is anti-apoptotic during follicular development from preantral to early antral stage. Mol. Endocrinol..

Ramaswamy, S., Pohl, C.R., McNeilly, A.S., Winters, S.J., Plant, T.M., 1998. The time course of follicle-stimulating hormone suppression by recombinant human inhibin A in the adult male rhesus monkey (*Macaca mulatta*). Endocrinology 139, 3409–3415.

Schuiling, G.A., Pols-Valkhof, N., van der Schaaf-Verdonk, G.C., de Jong, F.H., Koiter, T.R., 1987. Decidua and the control of corpus luteum function, follicular development and pituitary LHRH-responsiveness in pseudopregnant and pregnant rats. Experientia 43, 895–898.

Shapiro, D.B., Pappalardo, A., White, B.A., Peluso, J.J., 1996. Steroidogenic factor-1 as a positive regulator of rat granulosa cell differentiation and a negative regulator of mitosis. Endocrinology 137, 1187–1195.

Shukovski, L., Findlay, J.K., 1990. Activin-A inhibits oxytocin and progesterone production by preovulatory bovine granulosa cells in vitro. Endocrinology 126, 2222–2224.

Simoni, M., Gromoll, J., Nieschlag, E., 1997. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr. Rev. 18, 739–773.

Smyth, C.D., Gosden, R.G., McNeilly, A.S., Hillier, S.G., 1994. Effect of inhibin immunoneutralization on steroidogenesis in rat ovarian follicles in vitro. J. Endocrinol. 140, 437–443.

Su, Y.Q., Wigglesworth, K., Pendola, F.L., O'Brien, M.J., Eppig, J.J., 2002. Mitogen-activated protein kinase activity in cumulus cells is essential for gonadotropin-induced oocyte meiotic resumption and cumulus expansion in the mouse. Endocrinology 143, 2221–2232.

Tai, C.J., Kang, S.K., Tzeng, C.R., Leung, P.C., 2001. Adenosine triphosphate activates mitogen-activated protein kinase in human granulose–luteal cells. Endocrinology 142, 1554–1560.

Tajima, K., Dantes, A., Yao, Z., Sorokina, K., Kotsuji, F., Seger, R., Amsterdam, A., 2003. Down-regulation of steroidogenic response to gonadotropins in human and rat preovulatory granulosa cells involves mitogen-activated protein kinase activation and modulation of DAX-1 and steroidogenic factor-1. J. Clin. Endocrinol. Metab. 88, 2288–2299.

Tano, M., Minegishi, T., Nakamura, K., Karino, S., Ibuki, Y., Miyamoto, K., 1997. Transcriptional and post-transcriptional regulation of FSH receptor in rat granulosa cells by cyclic AMP and activin. J. Endocrinol. 153, 465–473.

Tetsuka, M., Hillier, S.G., 1996. Androgen receptor gene expression in rat granulosa cells: the role of follicle-stimulating hormone and steroid hormones. Endocrinology 137, 4392–4397.

Tetsuka, M., Whitelaw, P.F., Bremner, W.J., Millar, M.R., Smyth, C.D., Hillier, S.G., 1995. Developmental regulation of androgen receptor in rat ovary. J. Endocrinol. 145, 535–543.

Tilly, J.L., LaPolt, P.S., Hsueh, A.J., 1992. Hormonal regulation of follicle-stimulating hormone receptor messenger ribonucleic acid levels in cultured rat granulosa cells. Endocrinology 130, 1296–1302.

Ulloa-Aguirre, A., Midgley Jr., A.R., Beitins, I.Z., Padmanabhan, V., 1995. Follicle-stimulating isohormones: characterization and physiological relevance. Endocr. Rev. 16, 765–787.

von Bubnoff, A., Cho, K.W., 2001. Intracellular BMP signaling regulation in vertebrates: pathway or network? Dev. Biol. 239, 1–14.

Wang, H., Andoh, K., Hagiwara, H., Xiaowei, L., Kikuchi, N., Abe, Y., Yamada, K., Fatima, R., Mizunuma, H., 2001. Effect of adrenal and ovarian androgens on type 4 follicles unresponsive to FSH in immature mice. Endocrinology 142, 4930–4936.

Weil, S., Vendola, K., Zhou, J., Bondy, C.A., 1999. Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. J. Clin. Endocrinol. Metab. 84, 2951–2956.

Woodruff, T.K., Mather, J.P., 1995. Inhibin, activin and the female reproductive axis. Annu. Rev. Physiol. 57, 219–244.

Wrathall, J.H., Knight, P.G., 1995. Effects of inhibin-related peptides and oestradiol on androstenedione and progesterone secretion by bovine theca cells in vitro. J. Endocrinol. 145, 491–500.

Xiao, S., Robertson, D.M., Findlay, J.K., 1992. Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology 131, 1009–1016.

Yazawa, T., Mizutani, T., Yamada, K., Kawata, H., Sekiguchi, T., Yoshino, M., Kajitani, T., Shou, Z., Miyamoto, K., 2003. Involvement of cyclic adenosine 5′-monophosphate response element-binding protein, steroidogenic factor 1, and Dax-1 in the regulation of gonadotropin-inducible ovarian transcription factor 1 gene expression by follicle-stimulating hormone in ovarian granulosa cells. Endocrinology 144, 1920–1930.

Ying, S.Y., Czvik, J., Becker, A., Ling, N., Ueno, N., Guillemin, R., 1987. Secretion of follicle-stimulating hormone and production of inhibin are reciprocally related. Proc. Natl. Acad. Sci. U.S.A. 84, 4631–4635.

Yu, F.Q., Han, C.S., Yang, W., Jin, X., Hu, Z.Y., Liu, Y.X., 2005. Activation of the p38 MAPK pathway by follicle-stimulating hormone regulates steroidogenesis in granulosa cells differentially. J. Endocrinol. 186, 85–96.

Zazopoulos, E., Lalli, E., Stocco, D.M., Sassone-Corsi, P., 1997. DNA binding and transcriptional repression by DAX-1 blocks steroidogenesis. Nature 390, 311–315.