Calcineurin and CRTC2 mediate FSH and TGF β 1 upregulation of Cyp19a1 and Nr5a in ovary granulosa cells

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Abstract

Estrogens are essential for female reproduction and overall well-being, and estrogens in the circulation are largely synthesized in ovarian granulosa cells. Using primary cultures of ovarian granulosa cells from gonadotropin-primed immature rats, we have recently discovered that pituitary FSH and ovarian cytokine transforming growth factor beta 1 (TGF β 1) induce calcineurin-mediated dephosphorylation–activation of cAMP-response element-binding protein (CREB)-regulated transcription coactivator (CRTC2) to modulate the expression of *Star*, *Cyp11a1*, and *Hsd3b* leading to increased production of progesterone. This study explored the role of calcineurin and CRTC2 in FSH and TGF β 1 regulation of *Cyp19a1* expression in granulosa cells. Ovarian granulosa cells treated with FSH displayed increased aromatase protein at 24 h post-treatment, which subsided by 48 h, while TGF β 1 acting through its type 1 receptor augmented the action of FSH with a greater and longer effects. It is known that the ovary-specific *Cyp19a1* PII-promoter contains crucial response elements for CREB and nuclear receptor NR5A subfamily liver receptor homolog 1 (LRH1/NR5A2) and steroidogenic factor 1 (SF1/NR5A1), and that the *Nr5a2* promoter also has a potential CREB-binding site. Herein, we demonstrate that FSH+TGF β 1 increased LRH1 and SF1 protein levels, and their binding to the *Cyp19a1* PII-promoter evidenced, determined by chromatin immunoprecipitation analysis. Moreover, pretreatment with calcineurin auto-inhibitory peptide (CNI) abolished the FSH+TGF β 1-upregulated but not FSH-upregulated aromatase activity at 48 h, and the corresponding mRNA changes in *Cyp19a1*, and *Nr5a2* and *Nr5a1* at 24 h. In addition, FSH and TGF β 1 increased CRTC2 binding to the *Cyp19a1* PII-promoter and *Nr5a2* promoter at 24 h, with CREB bound constitutively. In summary, the results of this study indicate that calcineurin and CRTC2 have important roles in mediating FSH and TGF β 1 collateral

Key Words

- aromatase/*Cyp19a1*
- ovary
- granulosa cell
- FSH
- TGF β
- calcineurin
- CRTC2
- LRH1/*Nr5a2*
- SF1/*Nr5a1*

upregulation of *Cyp19a1* expression together with its transcription regulators *Nr5a2* and *Nr5a1* in ovarian granulosa cells.

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Introduction

The steroid hormone estrogen governs female reproductive competence and whole-body well-being (Rosenfeld *et al.* 2001). Estrogens in the circulation are largely produced in ovarian granulosa cells through aromatase conversion of theca cell-derived androgens (Rosenfeld *et al.* 2001, Britt & Findlay 2002). Imbalance of estrogen often leads to reproductive dysfunction and exacerbates certain diseases including cancers (Couse & Korach 1999, Yager & Davidson 2006). Knockout of aromatase severely impairs ovarian follicle development as has been indicated by an arrest at the antral stage and formation of hemorrhagic cysts (Fisher *et al.* 1998, Britt *et al.* 2001). In addition, deficiency of estrogen or its action is closely associated with cardiovascular, immunological, and neurological abnormalities (Couse & Korach 1999, Mendelsohn & Karas 1999, Stein 2001, Kovacs *et al.* 2002). As such, it is of crucial importance to understand the regulation of ovarian estrogen synthesis, particularly aromatase gene (*Cyp19a1*) expression.

Follicle-stimulating hormone (FSH) secreted by the pituitary is the master regulator that stimulates aromatase activity and expression and hence estrogen production in ovarian granulosa cells, and estrogen could, via feed-forward mechanisms, regulate follicle development and maturation including granulosa cell survival, proliferation, and steroidogenic differentiation (Rosenfeld *et al.* 2001, Britt & Findlay 2002). The use of different promoters confers tissue-specific transcriptional regulation of the *Cyp19a1* gene (Bulun *et al.* 2003). In granulosa cells, this is driven by the ovary-specific PII-promoter that binds cAMP-response element-binding protein (CREB) and nuclear receptor NR5A subfamily liver receptor homolog 1 (LRH1/NR5A2) and steroidogenic factor 1 (SF1/NR5A1), and these are required for FSH and its signaling second messenger cAMP to upregulate aromatase expression (Fitzpatrick & Richards 1994, Michael *et al.* 1997, Falender *et al.* 2003, Hinshelwood *et al.* 2003, Mendelson & Kamat 2007, Stocco 2008). In the ovary, LRH1 is primarily expressed in granulosa cells and luteal cells, whereas SF1 is expressed in theca/interstitial cells and granulosa cells (Falender *et al.* 2003, Hinshelwood *et al.* 2003).

By using granulosa cells isolated from ovarian antral follicles developed in rats primed with equine chorionic gonadotropin (eCG) 48 h previously, we have demonstrated that FSH acts in concert with the ovarian cytokine-transforming growth factor beta 1 (TGFβ1) to promote advanced differentiation of granulosa cells, indicated by increased production of progesterone and estrogen (Dodson & Schomberg 1987, Dorrington *et al.* 1993, Ke *et al.* 2004, Chen *et al.* 2007, 2008). FSH is known to act through the cAMP–PKA and PI3K–Akt pathways to regulate steroidogenesis in granulosa cells (Hunzicker-Dunn & Maizels 2006, Chen *et al.* 2007). In addition, FSH has been reported to activate calcium signaling that is significantly involved in FSH stimulation of progesterone production (Tsang & Carnegie 1983, Carnegie & Tsang 1984, Flores *et al.* 1990); however, the understanding of the calcium-signal-triggered mechanism remains limited. Calcineurin is a unique protein phosphatase activated by Ca²⁺–calmodulin binding, which initiates the displacement of calcineurin auto-inhibitory domain from its catalytic domain (Li *et al.* 2011). Furthermore, calcineurin could modulate CREB activity, acting indirectly through CREB-regulated transcription coactivator (CRTC), initially named transducer of regulated CREB (TORC) (Screaton *et al.* 2004, Altarejos & Montminy 2011). Upon calcineurin-mediated dephosphorylation–activation, CRTC enters the nucleus and facilitates CREB transcriptional activity through binding to the bZIP domain of CREB (Conkright *et al.* 2003, Altarejos & Montminy 2011). Interestingly, we have recently demonstrated that FSH and TGFβ1 stimulate CRTC2 activation in ovarian granulosa cells, and further revealed a pivotal role of calcineurin and CRTC2 in FSH and TGFβ1 upregulation of the expression of key steroidogenic genes, such as *Star*, *Cyp11a1*, and *Hsd3b*, responsible for progesterone production (Fang *et al.* 2012). It was not clear whether FSH-initiated calcium signaling is involved in estrogen synthesis in granulosa cells. Therefore, this study was aimed to determine whether calcineurin and CRTC2 play a central role in the induction of aromatase expression by FSH and TGFβ1 in ovarian granulosa cells. In addition, FSH has been reported to upregulate the expression of NR5A nuclear

receptors LRH1 and SF1 in granulosa cells (Falender *et al.* 2003), and both are involved in FSH stimulation of aromatase expression (Hinshelwood *et al.* 2003, Parakh *et al.* 2006). We further explored the potential involvement of calcineurin and CRTC2 in FSH and TGF β 1 regulation of LRH1 and SF1.

Materials and methods

Materials

Ovine FSH (oFSH-19-SIAFP) and eCG were purchased from the National Institute of Diabetes and Digestive and Kidney Diseases & National Hormone & Peptide Program and Dr A F Parlow (Harbor-UCLA Medical Center, Torrance, CA, USA). Recombinant human TGF β 1 and mouse MABs against LRH1 and SF1 were obtained from R&D System, Inc. (Minneapolis, MN, USA). DMEM/F12 culture medium was from Gibco Life Technologies, Inc. Engelbreth-Holm-Swarm sarcoma tumor-derived (EHS) matrigel and mouse MAB against β -actin were from Sigma Chemical Co. Rabbit polyclonal antibody against aromatase was produced and verified at the laboratory of Dr Nobuhiro Harada (Fujita Health University, Aichi, Japan). Rabbit MAB against CREB and goat polyclonal antibody against CRTC2 were respectively purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and Santa Cruz Biotechnology, Inc. Calcineurin auto-inhibitory peptide and protein G agarose were from Millipore Corp. (Billerica, MA, USA). SB431542 was obtained from Upstate Biotechnology Co. (Lake Placid, NY, USA). The Moloney murine leukemia virus reverse transcriptase set and Taq DNA polymerase were, respectively, purchased from Toyobo Biologics, Inc. (Osaka, Japan) and Protech Technology Enterprise Co. (Taipei, Taiwan).

Animals

Immature Sprague-Dawley-derived rats (23–25 days of age) were obtained from the Animal Center at National Yang-Ming University (Taipei, Taiwan). The rats were maintained under controlled temperature (22–25 °C) and light conditions (12 h light:12 h darkness). Food (Lab Diet from PMI Feeds, Inc., St Louis, MO, USA) and water were available *ad libitum*. This study was conducted in accordance with the United States National Research Council's Guide for the Care and Use of Laboratory Animals and National Yang-Ming University institutional guidelines, and was approved by the Institutional Animal Care and Use Committee of National Yang-Ming University.

Cell culture and treatment

Primary cultures of ovarian granulosa cells from gonadotropin (eCG)-primed immature rats were used because FSH is well known to potently increase progesterone synthesis and cAMP level (Carnegie & Tsang 1984, Reaven *et al.* 1999, Ke *et al.* 2005, Chen *et al.* 2007, 2008, Fang *et al.* 2012). In addition, *in vivo* eCG priming for 48 h elevated FSH receptor transcript levels in ovarian follicles (Findlay & Drummond 1999). Isolation of ovarian granulosa cells from eCG-primed immature rats was carried out as described previously (Ke *et al.* 2005). In brief, immature rats received single subcutaneous injections of 15 IU of eCG to stimulate follicle development to the antral follicle stage. Forty-eight hours later, ovarian granulosa cells of mid- to large-sized antral follicles were isolated and inoculated into EHS matrigel-coated culture wares in the growth medium (DMEM/F12, 1:1, 2 μ g/ml bovine insulin, 0.1% w/v fatty acid-free BSA, 100 IU/ml penicillin, and 100 μ g/ml streptomycin) and were allowed to attach for 20 h at 37 °C, 5% CO $_2$ –95% air. The cultured cells were then incubated in an incubation medium (DMEM/F12, 1:1, 0.1% w/v lactalbumin hydrolysate, 100 IU/ml penicillin, and 100 μ g/ml streptomycin) for an additional 20 h before the beginning of treatment with FSH $\pm$ TGF β 1 and other drugs. Low-dose FSH (10 ng/ml) within physiological serum concentration (Besecke *et al.* 1997, Tébar *et al.* 1997) was used in our study.

Immunoblotting analysis

Granulosa cells ($\sim 6 \times 10^6$) were cultured in matrigel-coated 60-mm culture dishes and treated as described in the figure legends. Immunoblotting was carried out as previously described (Lai *et al.* 2013). An equal amount of protein was loaded for each sample, which was then analyzed for aromatase, LRH1, and SF1, with β -actin used as an internal control.

Aromatase activity assay

The granulosa cells ($\sim 5.5 \times 10^5$) were cultured in matrigel-coated 24-well culture plates and treated as described in the figure legends. An hour before the end of culture, androstenedione (final concentration being 10^{-7} M) was added to serve as the substrate for aromatase. At the end of culture, conditioned media were collected and 17 β -estradiol (E $_2$) content was determined by enzyme immunoassay (EIA). E $_2$ antisera and E $_2$ -HRP conjugate were produced and verified in the laboratory of Dr Leang-Shin Wu

(National Taiwan University, Taipei, Taiwan). First, a 96-well plate was coated with 150 μ l of anti-E₂ antibody (1:20 000) overnight at 4 °C. After rinsing the plate with wash buffer (150 mM NaCl and 0.05% v/v Tween-20), 100 μ l of E₂ standards (0.02–10 ng/ml) or conditioned medium samples diluted in EIA buffer (100 mM sodium phosphate, 150 mM NaCl, and 0.1% w/v BSA, pH 7) were added to the well and incubated with 50 μ l of E₂-HRP (1:20 000) for 2 h at room temperature. The reaction plate was washed and 150 μ l of peroxidase substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma Chemical Co.) at 0.1 mg/ml in reaction buffer (50 mM Na₂HPO₄ and 25 mM citric acid, pH 5) was added and the plates incubated for 2 h at room temperature. The absorbance of reaction products was measured at 410 nm using a multimode microplate reader (Infinite 200 PRO, Männedorf, Switzerland).

Immunofluorescence analysis

The granulosa cells ($\sim 5 \times 10^5$) were seeded on matrigel-coated 12-mm cover slips and treated as described in the figure legends. At the end of culture, the cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.05% Triton X-100 for 5 min, and then incubated in the blocking reagent (3% BSA and 3% goat serum in PBS) for 1 h. The cells were then incubated with rabbit antibodies against LRH1/NR5A2 or SF1/NR5A1, or isotypic immunoglobulin, serving as a negative control, for 1 h, followed by Alexa 488-conjugated goat anti-rabbit IgG for 1 h. The cover slips were mounted on glass slides and viewed under a fluorescence microscope (Olympus BX50)

equipped with a mercury arc lamp and photographed using SPOT image capture system (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Only the fluorescence emission intensity, exposure time, and signal output gain value could affect the outcome signal intensity. In order not to have overexposed images, the system was set to automatically capture image of a sample that displayed the most intense fluorescence signal, that is, FSH+TGF β 1-treated cells. Then the exposure time and signal output gain value of this image were used to capture all of the subsequent samples in a single experiment. Using this technique it was possible to ensure that, the fluorescent images would reflect the actual emission signal for comparison among samples.

RT-PCR analysis

The granulosa cells ($\sim 2 \times 10^6$) were cultured in matrigel-coated 35-mm culture dishes. At the end of culture, the cells were extracted for total RNA using TRIzol reagent (Life Technologies, Inc.), and then RNA sample was analyzed using RT-PCR and agarose gel electrophoresis conducted as described previously (Lai *et al.* 2013) for *Cyp19a1*, *Nr5a1*, and *Nr5a2*, with *Gapdh* used as an internal control. Forward and reverse primer pairs used are listed in Table 1.

Chromatin immunoprecipitation analysis

The granulosa cells ($\sim 1.8 \times 10^7$) were cultured in matrigel-coated 100-mm culture dishes. At the end of culture, the

Table 1 Primer pairs used in RT-PCR and Chromatin immunoprecipitation (ChIP) analyses

Gene	Accession no.	Forward and reverse primer pair	Product length (bp)
RT-PCR analysis			
<i>Cyp19a1</i>	NM_017085.2	5'-CTGCTGATCATGGGCCTCCT-3' 5'-CTCCACAGGCTCGGGTTGTT-3'	341
<i>Nr5a1</i>	NM_001191099.1	5'-GCCCTGCTGGATTACACCTT-3' 5'-AGACATTCAGGCCACCAAG-3'	507
<i>Nr5a2</i>	NM_021742.1	5'-AAGAAAGCCCTCATTGAG-3' 5'-TGTTCCGGTTATTCTGCTC-3'	454
<i>Gapdh</i>	NM_017008.4	5'-AATGCATCCTGCACCACCAACTGT-3' 5'-GGAGGCCATGTAGGCCATGAGGTC-3'	555
ChIP analysis			
<i>Cyp19a1</i>	AC_000076.1	5'-TGAGCATGTGTGTCTAGG-3' 5'-CAGTAGTTTGGCTGTGGCTC-3'	378
<i>Nr5a2</i>	NC_005112.3	5'-CGGCCGCCGTTATCATCTAA-3' 5'-TTCAGAAAGGCCCAAGCCAT-3'	447
<i>Cyp19a1</i> exon 10	AC_000076.1	5'-ATTGGCTTTGGGCCCGCA-3' 5'-TCCACAATGGGGCTGCTCTCA-3'	180
<i>Nr5a2</i> exon 8	NC_005112.3	5'-CTCTTTTGGCGTGAGCATT-3' 5'-GCGTCCAAGAGCAGGTCAAT-3'	207

cell lysates were prepared and subjected to chromatin immunoprecipitation (ChIP) analysis conducted as described previously (Lai *et al.* 2013). In brief, 10% of each sample was saved as input, and the rest was immunoprecipitated with CRTC2, CREB, LRH1, or SF1 antibody, and isotypic normal IgG was used as a negative control. DNA was extracted from the immunoprecipitated samples and subjected to analysis by PCR and 2% w/v agarose gel electrophoresis for *Cyp19a1* PII-promoter and *Nr5a2* promoter regions containing CRE and NR5A sites. Input for each sample was used as an internal control. DNA from each sample was used as a template for amplification of the last exon of *Cyp19a1* or *Nr5a2* with the same cycle number used for the promoter regions (34 cycles) to serve as a PCR negative control. Forward and reverse primer pairs used are listed in Table 1.

Statistical analysis

Quantitative data are presented as the mean ($\pm$ S.E.M.), and were analyzed by ANOVA and Duncan's multiple-range test at a significance level of 0.05 using the general linear model of the SAS program (SAS Institute, Inc., Cary, NC, USA). Differences between two treatment groups were analyzed using the Student's *t*-test at a significance level of 0.05.

Results

FSH and TGF β 1 regulation of aromatase and NR5A in rat ovarian granulosa cells

We first investigated FSH and TGF β 1 regulation of aromatase protein in the granulosa cells of rat ovarian antral follicles. The cells treated with FSH (10 ng/ml) for 24 h had an increase in aromatase protein level which subsided by 48 h, and FSH+TGF β 1 treatment exerted a greater and longer effect (24 and 48 h) (Fig. 1A), whereas TGF β 1 treatment alone had no effect (Supplementary Fig. 2, see section on supplementary data given at the end of this article). Consistent with results presented in an earlier report (Dorrington *et al.* 1993), we observed FSH stimulation of aromatase activity in granulosa cells at 48-h post-treatment, and TGF β 1 potentiated FSH action (Fig. 1B). Herein, we demonstrate that pretreatment with SB431542, an inhibitor of TGF β type 1 receptor (TGF β R1) kinase activity (Inman *et al.* 2002), completely suppressed TGF β 1 enhancement of FSH-induced aromatase activity at 48-h post-treatment, and this is in accordance to changes in *Cyp19a1* mRNA level at 24-h post-treatment (Fig. 1C).

Figure 1

FSH and TGF β 1 regulation of aromatase expression and activity in rat ovarian granulosa cells. (A) Cells were treated with FSH (10 ng/ml) and/or TGF β 1 (0.5 ng/ml) for 24 or 48 h. At the end of culture, cell lysates were prepared and analyzed for aromatase by immunoblotting with β -actin serving as an internal control. Relative density ratios were calculated defining the 24 h control value as one. Each bar represents mean ($\pm$ S.E.M.) relative density ratio ($n=3-4$). Different lower case letters represent significant differences among groups within the same culture period ($P<0.05$). (B and C) Cells were pretreated with vehicle or SB431542 (1 μ M) for 1 h followed by FSH and/or TGF β 1 treatment for an additional 48 h to determine aromatase activity, or 24 h to determine *Cyp19a1* gene expression. (B) For the analysis of aromatase activity, androstenedione was added 1 h before the end of culture when conditioned media were collected and 17 β -estradiol content was determined by EIA. Relative ratios of aromatase activity were calculated using FSH+TGF β 1-treated group value as one. Each bar represents mean ($\pm$ S.E.M.) relative aromatase activity ($n=6$). (C) Total RNAs were extracted and *Cyp19a1* mRNA levels were analyzed by RT-PCR using *Gapdh* as an internal control. Relative density ratios were calculated with the FSH+TGF β 1-treated group value defined as one. Each bar represents mean ($\pm$ S.E.M.) relative density ratio ($n=4$). Different lower case letters represent significant differences among groups without the inhibitor ($P<0.05$). *Significant difference compared with the respective vehicle control without the inhibitor ($P<0.05$). C, control; F, FSH; T, TGF β 1; SB, SB431542.

Besides aromatase protein level, TGF β 1 treatment alone also had no significant effect on *Cyp19a1* mRNA level (Supplementary Fig. 1). These results indicate that TGF β 1 acts through its type 1 receptor (TGF β R1) to augment FSH induction of *Cyp19a1* expression in granulosa cells.

Aromatase is also regulated by the NR5A family members LRH1/NR5A2 and SF1/NR5A1, as both are expressed in granulosa cells and could bind to the two NR5A-response element half-sites within the *Cyp19a1* PII-promoter (Falender *et al.* 2003, Hinshelwood *et al.* 2003). We therefore investigated whether FSH and TGF β 1 regulate the expression of *Nr5a*, and whether the resulting LRH1 and SF1 modulate *Cyp19a1* expression. The granulosa cells treated with FSH for 24 and 48 h had increased levels of SF1 protein but not of LRH1, and treatment with FSH+TGF β 1 markedly increased both LRH1 and SF1 protein and mRNA levels (Figs 2A, and Supplementary Figs 1 and 2). Also, treatment with TGF β 1 alone had no effect on LRH1 and SF1 protein and mRNA levels

Figure 2

FSH and TGF β 1 regulation of LRH1/NR5A2 and SF1/NR5A1 protein and their binding to *Cyp19a1* PII-promoter in rat ovarian granulosa cells. (A) Cells were treated with FSH (10 ng/ml) and/or TGF β 1 (0.5 ng/ml) for 24 or 48 h. At the end of culture, cell lysates were prepared and analyzed for LRH1 and SF1 by immunoblotting, with β -actin used as an internal control. Relative density ratios were calculated using the 48-h FSH+TGF β 1-treated group value as one. Each bar represents mean ($\pm$ S.E.M.) relative density ratio ($n=3-4$). Different lower case letters represent significant differences among groups within the same culture period ($P<0.05$). (B) Cells were treated with FSH and/or TGF β 1 for 24 h. Cell lysates were analyzed by ChIP assay using LRH1 or SF1 antibody with isotopic normal IgG serving as a negative control, and input DNA as an internal control. DNA from each sample was amplified by PCR with a primer pair flanking the promoter region, and another primer pair flanking the exon region used as a PCR negative control (Table 1). PCR product intensity was determined and normalized to input DNA. Relative density ratios were calculated with the FSH+TGF β 1-treated group value defined as one. Each bar represents mean ($\pm$ S.E.M.) relative density ratio ($n=4$). *Significant difference compared with the control ($P<0.05$). C, control; F, FSH; T, TGF β 1.

Figure 3

Localization of LRH1 and SF1 in rat ovarian granulosa cells. Cells were treated with FSH (10 ng/ml) and/or TGF β 1 (0.5 ng/ml) for 24 h. At the end of culture, cells were subjected to immunofluorescence analysis for LRH1 and SF1 with normal mouse IgG used as a negative control. Cells were also stained with DAPI for visualization of cell nuclei.

(Supplementary Figs 1 and 2). In addition, we noticed that both LRH1 and SF1 were predominantly localized in the cell nucleus (Fig. 3), with relative immunostaining intensities among treatment groups similar to those observed in the immunoblotting analysis (Fig. 2). Furthermore, ChIP analysis reveals that FSH+TGF β 1 treatment for 24 h increased LRH1 and SF1 binding to *Cyp19a1* PII-promoter (Fig. 2B). These results indicate the important roles of the two NR5As in FSH and TGF β 1 upregulation of *Cyp19a1* expression.

Involvement of calcineurin in FSH and TGFβ1 regulation of aromatase and NR5A in rat ovarian granulosa cells

Results from our recent work has demonstrated that Ca^{2+} -dependent calcineurin signaling mediates FSH and TGFβ1-stimulated progesterone production and expression of *Star*, *Cyp11a1*, and *Hsd3b* (Fang *et al.* 2012). Herein, we sought to understand whether calcineurin mediates FSH and TGFβ1 upregulation of aromatase by employing calcineurin auto-inhibitory peptide (CNI). The cell-permeable CNI peptide contains the calcineurin auto-inhibitory domain, and thus is a highly selective inhibitor of calcineurin phosphatase activity. Pretreatment with CNI completely suppressed the additional increase in aromatase activity induced by FSH+TGFβ1 but not the increase induced by FSH alone at 48-h post-treatment, and this is in accordance with changes in *Cyp19a1* mRNA levels at 24 h (Fig. 4). Next, we examined FSH and TGFβ1 regulation of *Nr5a* expression, and the involvement of calcineurin in the process. The granulosa cells treated with FSH+TGFβ1 for 24 h, but not FSH or TGFβ1 alone, had increased mRNA levels of both *Nr5a2*/LRH1 and *Nr5a1*/SF1, and pretreatment with CNI completely abrogated such effect (Fig. 5 and Supplementary Fig. 1). These results together indicate that calcineurin crucially mediates FSH and TGFβ1 induction of the expression of *Cyp19a1*, *Nr5a2*, and *Nr5a1* in ovarian granulosa cells.

Involvement of CRTC2 in FSH and TGFβ1 regulation of aromatase and NR5A in rat ovarian granulosa cells

CRTC2 is a calcineurin-activated CREB coactivator (Screaton *et al.* 2004, Altarejos & Montminy 2011). Results from our recent study indicated that calcineurin auto-inhibitory peptide CNI could effectively block the FSH and TGFβ1-induced CRTC2 activation in granulosa cells of rat ovarian antral follicles (Fang *et al.* 2012). Herein, we further investigated whether CRTC2 mediates the action of calcineurin in induction of *Cyp19a1* expression by FSH and TGFβ1. In a previous study it was reported that CREB binds to a CRE-like site within the *Cyp19a1* PII-promoter (Fitzpatrick & Richards 1994); accordingly, a primer pair flanking such region was designed (Table 1) for ChIP analysis. The granulosa cells treated with FSH for 24 h had increased CRTC2 binding to this region containing CRE-like-site in *Cyp19a1* PII-promoter, and TGFβ1 further enhanced the effect of FSH (Fig. 6). In addition, results shown in Fig. 5 indicated that FSH and TGFβ1 increased the mRNA levels of both *Nr5a2* and *Nr5a1*, and that this requires calcineurin activity. We therefore performed

Figure 4

Effect of calcineurin inhibitor (CNI) on FSH and TGFβ1 regulation of aromatase in rat ovarian granulosa cells. Cells were pretreated with vehicle or CNI (50 μM) for 1 h followed by treatment with FSH (10 ng/ml) and/or TGFβ1 (0.5 ng/ml) for an additional 48 h to determine aromatase activity, or 24 h to determine *Cyp19a1* gene expression. (A) Aromatase activity was analyzed as described in Fig. 1B. Relative ratios of aromatase activity were calculated using the FSH + TGFβ1-treated group value as one. Each bar represents mean (± s.e.m.) relative aromatase activity (n=6). (B) *Cyp19a1* mRNA levels were analyzed by RT-PCR as described in Fig. 1C. Relative density ratios were calculated using the FSH + TGFβ1-treated group value as one. Each bar represents mean (± s.e.m.) relative density ratio (n=4). Different lower case letters indicate significant differences among groups without the inhibitor ($P < 0.05$). *Significant difference compared to the respective vehicle control without the inhibitor ($P < 0.05$). C, control; F, FSH; T, TGFβ1.

promoter sequence analysis of *Nr5a2* and *Nr5a1* genes, searching for potential CRTC2 regulatory regions containing CRE sites, and identified a putative CREB-binding motif 438 bp upstream of the initiation site in *Nr5a2* that is highly conserved among species (Fig. 7A), whereas no potential CRE site was found in the *Nr5a1* promoter. ChIP assay was further conducted and revealed that granulosa cells treated with FSH for 24 h had increased CRTC2 binding to the *Nr5a2* promoter region containing the potential CRE site, and TGFβ1 further enhanced the effect of FSH (Fig. 7B). Interestingly, CREB exhibited constitutive binding to these promoter regions of *Cyp19a1*

Figure 5

Effect of calcineurin inhibitor (CNI) on FSH and TGFβ1 regulation of NR5A expression in rat ovarian granulosa cells. Cells were pretreated with vehicle or CNI (50 μM) for 1 h followed by treatment with FSH (10 ng/ml) and/or TGFβ1 (0.5 ng/ml) for an additional 24 h. Total RNAs were extracted and analyzed for *Nr5a2*/LRH1 and *Nr5a1*/SF1 mRNA levels using RT-PCR as described in Fig. 1C. Relative density ratios were calculated using the FSH + TGFβ1-treated group value as one. Each bar represents mean (±s.e.m.) relative density ratio ($n=4$). Different lower case letters indicate significant differences among groups without the inhibitor ($P<0.05$). *Significant difference compared with the respective vehicle control without the inhibitor ($P<0.05$). C, control; F, FSH; T, TGFβ1.

and *Nr5a2* independent of FSH and/or TGFβ1 treatment (Figs 6 and 7). This indicates that with CREB constitutively occupying at the CRE-like site in the *Cyp19a1* PII-promoter and *Nr5a2* promoter, FSH and TGFβ1-activated CRTC2 may play a key role in enhancement of CREB transcriptional activity to turn on the expression of both genes.

Discussion

The master regulator FSH is well known to act via cAMP-PKA to stimulate aromatase activity and *Cyp19a1* expression in ovarian granulosa cells involving key transcription factors, CREB and nuclear receptors LRH1/NR5A2 and SF1/NR5A1 (Fitzpatrick & Richards 1994, Michael *et al.* 1997, Falender *et al.* 2003, Hinshelwood *et al.* 2003, Mendelson & Kamat 2007, Stocco 2008). Using primary cultures of granulosa cells from ovarian antral follicles of gonadotropin-primed immature rats, this study

revealed that calcium-dependent calcineurin critically mediates FSH and TGFβ1-marked induction of *Cyp19a1* expression through direct and indirect mechanisms discussed as described below. In addition, TGFβ1 acts through its type 1 receptor (TGFβR1) to augment the effect of FSH. The proposed working model is illustrated in Fig. 8.

Calcineurin could act directly through activation of CRTC2 that may escalate CREB activity to upregulate *Cyp19a1* expression in granulosa cells in response to FSH and TGFβ1 stimulation. This is supported by the following lines of evidence. Our recent study using granulosa cells has revealed that FSH induces an early-phase (0.5–1 h) and FSH + TGFβ1 induces a late-phase (24 and 48 h) calcineurin-dependent dephosphorylation-activation of CRTC2 that complexes with CREB to upregulate the expression of the steroidogenic genes *Star*, *Cyp11a1*, and *Hsd3b* (Fang *et al.* 2012). The results indicated that FSH ± TGFβ1 upregulate *Cyp19a1* mRNA at 24-h post-treatment with corresponding changes in aromatase protein level and enzymatic activity at 48 h (control < FSH < FSH + TGFβ1;

Figure 6

FSH and TGFβ1 regulation of CRTC2 and CREB binding to *Cyp19a1* PII-promoter in rat ovarian granulosa cells. Cells were treated with FSH (10 ng/ml) and/or TGFβ1 (0.5 ng/ml) for 24 h. Cell lysates were analyzed by ChIP assay using CRTC2 or CREB antibody with isotypic normal IgG serving as a negative control, and input DNA as an internal control. DNA from each sample was amplified by PCR with a primer pair flanking the promoter region, and another primer pair flanking the exon region used as a PCR negative control (Table 1). PCR product intensity was determined and normalized to input DNA. Relative density ratios were calculated using the FSH + TGFβ1-treated group value as one. Each bar represents mean (±s.e.m.) relative density ratio ($n=3$). Different lower case letters indicate significant differences among groups ($P<0.05$). C, control; F, FSH; T, TGFβ1.

Figure 7

FSH and TGFβ1 regulation of CRTC2 and CREB binding to the *Nr5a2* promoter in rat ovarian granulosa cells. (A) The *Nr5a2* promoter region contains a potential CREB-binding site (shaded area) and is conserved among rats, mice, and humans. *100% identity among species. (B) Cells were treated with FSH (10 ng/ml) and/or TGFβ1 (0.5 ng/ml) for 24 h. Cell lysates were analyzed by ChIP assay as described in Fig. 5. Relative density ratios were calculated using the FSH+TGFβ1-treated group value as one. Each bar represents mean ($\pm$ s.e.m.) relative density ratio ($n=3$). Different lower case letters indicate significant differences among groups ($P<0.05$). C, control; F, FSH; T, TGFβ1.

Figs 1 and 4). Also, at 24-h post-treatment TGFβ1 potentiated the FSH-induced increase in CRTC2 binding to the *Cyp19a1* PII-promoter region containing a CREB-binding site (control < FSH < FSH+TGFβ1; Fig. 6). Another report using primary breast adipose stromal cells also described how treatment with the adenylyl cyclase activator forskolin increased CRTC2 binding to the *Cyp19a1* PII-promoter, and that overexpression of CRTC2 increased *Cyp19a1* PII-promoter activity (Brown *et al.* 2009). This study further demonstrates that pretreatment with the calcineurin auto-inhibitory peptide CNI abolished the FSH+TGFβ1-induced increase in *Cyp19a1* mRNA and aromatase activity at 24 and 48 h post-treatment respectively (Fig. 4). Together, these findings indicate that in ovarian granulosa cells of antral follicles FSH stimulates calcineurin-mediated activation of CRTC2 that may complex with CREB and perhaps other coregulator(s) to upregulate *Cyp19a1* expression, and that the concomitant presence of TGFβ1 increases the FSH-induced effect.

Besides direct effect on CRTC2-mediated *Cyp19a1* expression, calcineurin could act indirectly through LRH1/NR5A2 and SF1/NR5A1 that in turn further enhance

the transcription of *Cyp19a1* in granulosa cells in response to FSH and TGFβ1 stimulation. At 24-h post-treatment, while FSH treatment alone had no effect, FSH+TGFβ1 increased the mRNA levels of *Nr5a2* and *Nr5a1*, and this was completely diminished in the presence of CNI (Fig. 5). Furthermore, results of immunoblotting and ChIP analysis indicated that granulosa cells treated with FSH+TGFβ1 for 24 h had increased protein levels of LRH1 and SF1, and increased binding of these proteins to the *Cyp19a1* PII-promoter (Fig. 2). Moreover, CRTC2 also mediates FSH and TGFβ1 upregulation of *Nr5a2* expression as a CREB-binding site was identified in *Nr5a2* promoter, and treatment with FSH and TGFβ1 for 24 h increased CRTC2 binding to this region of *Nr5a2* the promoter (control < FSH < FSH+TGFβ1; Fig. 7). CRTC2 may enhance transcriptional activity of CREB together with other coregulator(s) on regulating *Nr5a2* expression. It has been shown that *Nr5a2* is also a target gene of estrogen receptor alpha (ERα (ESR1)) as has been revealed in the MCF7 breast cancer cell line (Annecotte *et al.* 2005). Also, we have previously demonstrated that ERα crucially mediates the induction by FSH and TGFβ1 of expression of steroidogenic genes, including *Hsd3b* and *Cyp11a1*, in granulosa

Figure 8

A proposed working model of FSH and TGFβ1 regulation of *Cyp19a1* expression through calcineurin and CRTC2. (A) FSH upregulates *Cyp19a1* expression moderately at 24 h post-treatment, and this may involve increased SF1 protein levels and CRTC2 binding to the *Cyp19a1* PII-promoter. (B) FSH acts synergistically with TGFβ1 to markedly upregulate *Cyp19a1* expression through a direct and an indirect mechanism. The former by inducing Ca²⁺/calcineurin-dependent activation of CRTC2, which binds to CREB-occupied *Cyp19a1* PII-promoter to directly activate *Cyp19a1* expression. The latter by facilitating calcineurin-dependent expression of *Nr5a2* and *Nr5a1* respectively through a CRTC2-mediated and a yet undiscovered mechanism. Also, the upregulated NR5A2/LRH1 and NR5A1/SF1 further bind and transactivate the *Cyp19a1* PII-promoter. Thus, acting through the NR5As in a calcineurin-dependent manner, FSH and TGFβ1 also indirectly facilitate *Cyp19a1* gene expression. R1, TGFβR. A full colour version of this figure is available via <http://dx.doi.org/10.1530/JME-14-0048>.

cells (Chen *et al.* 2008). In addition, in the MCF7 cancer cell line overexpression of GATA4 could increase *Nr5a2* promoter activity (Bouchard *et al.* 2005). While CRTC2–CREB participates in FSH and TGFβ1 upregulation of *Nr5a2* promoter activity, additional granulosa-cell-relevant transcription regulators such as ERα and GATA4 may also be engaged and this awaits further study. In addition, although CNi abolished FSH+TGFβ1-increased *Nr5a1* mRNA level (Fig. 4), no consensus CREB-binding site was found in the *Nr5a1* promoter; thus, how calcineurin regulates *Nr5a1* awaits further study.

Interestingly, our ChIP analyses disclosed that treatment with FSH±TGFβ1 for 24 h increased CRTC2 binding to the CRE-containing region (~400–1000 bp) of the *Cyp19a1* PII-promoter and the *Nr5a2* promoter (control<FSH<FSH+TGFβ1) (Figs 6 and 7, Table 1). In addition though the extent of CREB binding to these regions was similar in all groups, our recent study using coimmunoprecipitation analysis has revealed that treatment with FSH±TGFβ1 for 24 h increased the association of CRTC2 with CREB (control<FSH<FSH+TGFβ1) when phospho-CREB(S133) was barely detectable (Fang *et al.* 2012). Furthermore, results from previous studies indicated that CRTC could activate CREB transcriptional activity independent of CREB phosphorylation state (Conkright *et al.* 2003, Takemori *et al.* 2007). The results of these studies together indicate that despite the CREB(S133) phosphorylation state, FSH and TGFβ1 could promote CRTC2 binding with CREB and thus facilitate the transcriptional activity of CREB on *Cyp19a1* and *Nr5a2* genes in ovarian granulosa cells. In addition, we and others using primary rat granulosa cell cultures have demonstrated that TGFβ1 augments the FSH-induced advanced differentiation of granulosa cells, marked by increased production of progesterone and estrogen, and the expression of associated key steroidogenic proteins StAR, P450_{scc}, 3β-HSD, and aromatase and gap junction protein connexin 43 (Ying *et al.* 1986, Dodson & Schomberg 1987, Gitay-Goren *et al.* 1993, Inoue *et al.* 2002, Ke *et al.* 2004, 2005, Chen *et al.* 2007). Our previous and present studies have revealed that TGFβ1, acting through its type 1 receptor, acts as an important autocrine/paracrine factor, modulating granulosa cell function (Fang *et al.* 2012). The understanding of how TGFβ1 augments the action of FSH in granulosa cells remains limited. TGFβ1 may modulate the action of FSH at the receptor level. Results from a previous study indicated that TGFβ1 could increase FSH receptor mRNA level and FSH binding activity in granulosa cells (Gitay-Goren *et al.* 1993, Dunkel *et al.* 1994). Also, TGFβ1 increased the expression of transcription factor GATA4 in a granulosa tumor cell line KK1 (Anttonen

et al. 2006), and GATA4 has recently been reported to bind and stimulate FSH receptor promoter activity in mouse granulosa cells (Bennett *et al.* 2012).

This study carried out using primary cultures of ovarian granulosa cells retrieved from antral follicles reveals that FSH+TGFβ1 concomitantly upregulate aromatase and NR5As in a calcineurin-dependent manner, with CRTC2 similarly used to regulate both *Cyp19a1* and *Nr5a2* promoters. Our findings correspond to the *in vivo* work demonstrating parallel expression pattern of the NR5As and aromatase in granulosa cells of ovarian antral follicles (Mendelson & Kamat 2007), and further supports an important role of TGFβ1 acting synergistically with FSH to stimulate aromatase activity during antral follicle development. In all, this study reveals that calcineurin is critically involved in FSH and TGFβ1 stimulation of *Cyp19a1* expression in rat ovarian granulosa cells of antral follicles. FSH and TGFβ1 could induce calcineurin-dependent activation of CRTC2 that plays a permissive role in CREB activation of the *Cyp19a1* PII-promoter. Furthermore, FSH+TGFβ1 could also upregulate *Cyp19a1* through calcineurin-mediated induction of the NR5A members LRH1 and SF1.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0048>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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