

Crucial Role of Estrogen Receptor- α Interaction with Transcription Coregulators in Follicle-Stimulating Hormone and Transforming Growth Factor β 1 Up-Regulation of Steroidogenesis in Rat Ovarian Granulosa Cells

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This study was to explore estrogen receptor (ER) involvement in FSH and TGF β 1-stimulated steroidogenesis in rat ovarian granulosa cells. We first determined the specific involvement of ER α and ER β in the process, and then investigated the molecular interaction of ER α and transcription coregulators in FSH and TGF β 1 up-regulation of steroidogenic gene expression. Primary culture of ovarian granulosa cells from antral follicles of gonadotropin-primed immature rats was used. Interestingly, a selective ER α antagonist methyl-piperidino-pyrazole (MPP) [like ER antagonist ICI-182,780 (ICI)] decreased FSH \pm TGF β 1-stimulated progesterone production, whereas an androgen receptor antagonist hydroxyflutamide and particularly a selective ER β antagonist 4-[2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo [1,5-a] pyrimidin-3-yl] phenol had no significant effect. Consistent with this, a selective ER β agonist diarylpropionitrile (unlike 17 β -estradiol) also had no effect on FSH \pm TGF β 1-stimulated progesterone production. Furthermore, a selective ER α agonist 4,4',4"-({4-Propyl-[1H]-

pyrazole-1,3,5-triyl)trisphenol (like 17 β -estradiol) enhanced FSH-stimulated progesterone production, and this was abolished by pretreatment with MPP. Immunoblotting and chromatin immunoprecipitation analyses indicate that MPP/ICI suppression of FSH \pm TGF β 1 action is partly attributed to the reduced ER α -mediated expression of Hsd3b and Cyp11a1 genes, but not steroidogenic acute regulatory protein. Furthermore, FSH \pm TGF β 1 increased ER α association with histone acetylases (CBP and SRC-1) and coactivator of peroxisome proliferator-activated receptor γ (PGC-1 α), and MPP/ICI dramatically reduced these interactions. In addition, FSH \pm TGF β 1 increased CBP, SRC-1, and PGC-1 α binding to Hsd3b and Cyp11a1 genes. Together, we demonstrate for the first time that ER α interaction with transcription coregulators, histone acetylases (CBP/SRC-1), and PGC-1 α is crucial to FSH and TGF β 1-up-regulated expression of Hsd3b and Cyp11a1, and, thus, progesterone production in rat ovarian granulosa cells. (*Endocrinology* 149: 4658–4668, 2008)

OVARIAN GRANULOSA cell differentiation is critically regulated by pituitary hormones, FSH during follicle growth to preovulatory follicles, and LH during ovulation and luteinization (1, 2). In addition, an intraovarian factor, TGF β 1, *in vitro* facilitates FSH-induced granulosa cell differentiation with increased production of progesterone and estrogen, and increased expression of LH receptor, inhibin, and steroidogenic proteins [including steroidogenic acute regulatory (StAR) protein, cholesterol side-chain cleavage en-

zyme (P450scc), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), and aromatase] (3–9), as well as increased gap junction formation (8). StAR protein, P450scc enzyme, and 3 β -HSD enzyme are products of Star, Cyp11a1, and Hsd3b genes, respectively. The significance of TGF β 1 in female ovarian function *in vivo* has been implicated by TGF β 1-null female mice that display disrupted ovarian function (reduced serum progesterone level, ovulation rate, and the number of corpus luteum) and reduced fertility with early embryo arrest (10).

FSH stimulates synthesis of estrogen in granulosa cells, and TGF β 1 enhances FSH action (3). In addition, estrogen exerts local feedback regulation to augment FSH-induced granulosa cell differentiation with increased gap junction formation, progesterone synthesis, and LH receptor expression and LH responsiveness (11). Estrogen is currently known to act mainly through two members of the nuclear receptor superfamily of hormone-regulated transcription factors, namely estrogen receptor (ER) α and ER β (12). Both ER forms are present in granulosa cells of the rodent ovary, and ER β is suggested to be the predominant form (13). ER β is essential for granulosa cell proliferation, whereas ER α plays specific roles in ovulation, corpus luteum formation, and in-

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Abbreviations: AP-1, Activator protein-1; AR, androgen receptor; CHIP, chromatin immunoprecipitation; CREB, cAMP response element-binding protein; DPN, diarylpropionitrile; E₂, 17 β -estradiol; ER, estrogen receptor; ERE, estrogen response element; Fox, fork-head box protein; HF, hydroxyflutamide; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase; ICI, ICI-182,780; MPP, methyl-piperidino-pyrazole; P450scc, cholesterol side-chain cleavage enzyme; PGC-1 α , coactivator of PPAR γ ; PHTPP, 4-[2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo [1,5-a] pyrimidin-3-yl] phenol; PPAR γ , peroxisome proliferator-activated receptor; PPT, 4,4',4"-({4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol; StAR, steroidogenic acute regulatory.

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terstitial glandular cell development (12–14). Although ER α and ER β display certain functional redundancy, they are different in the ligand activation and transcriptional properties (12, 13). ER regulation of target gene expression is mediated through direct binding to estrogen response element (ERE), and/or indirect interaction with coregulators and other transcription factors [e.g. activator protein-1 (AP-1)] resulting in recruitment of RNA polymerase II-containing transcription initiation complex that enhances target gene transcription with a coordinated and timely cycling manner (15–19). Presently, a few interacting partners of ER α have been demonstrated. ER α could recruit histone acetylases (CBP, SRC-1, and RAC-3) that facilitate chromatin remodeling and, therefore, enhance transcription of ER-regulated genes (20, 21). Interestingly, the coactivator of peroxisome proliferator-activated receptor γ (PGC-1 α) also serves as a transcription coactivator of ER α in both a ligand-independent and ligand-dependent manner (22). In addition, our most recent study demonstrates that FSH increased phosphorylation of fork-head box protein (Fox) O1 and FoxO3a in rat ovarian granulosa cells (9). It has been reported that phosphorylation of FoxOs leads to their nuclear exit and, thus, the release of their suppression of target gene transcription (23, 24). In addition, FoxO1 interaction with ER α augmented ligand-dependent ER α transactivation (25). The transcriptional regulation activity of ER α could be modulated by coregulators in a cell context-specific manner (16, 26, 27).

ER α and ER β play differential roles in follicle growth and differentiation during rodent ovarian cycle (12–14). During luteinization, ER β is down-regulated (28, 29) in coincidence with up-regulation of ER α (30, 31). This inspires us to explore the role of ER α in FSH and TGF β 1-promoted differentiation of rat ovarian granulosa cells. There were two specific aims. The first was to determine the specific role of ER α and ER β in FSH and TGF β 1-stimulated progesterone production and the associated key steroidogenic proteins. The second aim was to investigate the molecular interaction of ER α and transcription coregulators in FSH and TGF β 1 up-regulation of steroidogenic gene expression.

Materials and Methods

Materials

Ovine FSH (oFSH-19-SIAFP) and equine chorionic gonadotropin were purchased from the National Institute of Diabetes and Digestive and Kidney Diseases's National Hormone & Peptide Program and Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA). Recombinant human TGF β 1 was obtained from R&D System, Inc. (Minneapolis, MN). Penicillin and streptomycin were from GIBCO Invitrogen Corp. (Carlsbad, CA). ICI-182,780 (ICI), diarylpropionitrile (DPN), 4-[2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo [1,5-a] pyrimidin-3-yl] phenol (PHTPP), and 4,4',4"-4-(Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) were purchased from Tocris (Bristol, UK). Hydroxyflutamide (HF) was kindly provided by Schering-Plough Pharmaceutical (Kenilworth, NJ). Antisera against P450scc enzyme were a generous gift from Dr. Bon-Chu Chung (Academia Sinica, Taipei, Taiwan). Antibodies against ER α , CBP, PGC-1 α , RAC-3, β -catenin, phosphorylated Smad2 and phosphorylated Smad3, and Protein-A/G plus agarose bead were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against SRC-1, c-Jun, FoxO1, and FoxO3a were from Cell Signaling Technology, Inc. (Beverly, MA). Antibody against phosphorylated cAMP response element-binding protein (CREB) was from Upstate Biotechnology Inc. (Lake Placid, NY). All other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Animals

Immature Sprague Dawley rats (25–27 d) were obtained from the Animal Center at National Yang-Ming University (Taipei, Taiwan). Rats were maintained under controlled temperature (20–23 C) and light conditions (14-h light, 10-h darkness). Food (LabDiet; PMI Feeds, St. Louis, MO) and water were available *ad libitum*. This study was conducted in accordance with the U.S. National Research Council's Guide for the Care and Use of Laboratory Animals and the institutional guidelines.

Cell culture and treatment

Isolation and culture of ovarian granulosa cells from antral follicles of equine chorionic gonadotropin-treated immature rats were performed as previously described (7–9). In brief, granulosa cells (~5 × 10⁵) were inoculated into 24-well plates coated with matrigel (derived from Engelbreth-Holm-Swarm sarcoma tumors; Sigma) in DMEM/F12 medium containing 2 μ g/ml bovine insulin, 0.1% fatty acid-free BSA, 100 U/ml penicillin, and 100 μ g/ml streptomycin, and allowed to attach for 24 h at 37 C, 5% CO₂-95% air. Cultured cells were then incubated in DMEM/F12 medium containing 0.1% lactalbumin hydrolysate, 100 U/ml penicillin, and 100 μ g/ml streptomycin for 24 h before the beginning of treatment. Cells were pretreated for 1 h with ethanol vehicle or various doses of ER antagonist ICI (32), ER α antagonist methyl-piperidino-pyrazole (MPP) (33), androgen receptor (AR) antagonist HF (34), ER β antagonist PHTPP (35), or ER β agonist DPN (28), and then treated with FSH (10 ng/ml) and/or TGF β 1 (5 ng/ml) for an additional 48 h. To determine the involvement of ER α in estradiol augmentation of FSH-stimulated progesterone production, granulosa cells were pretreated for 1 h with ethanol vehicle or ER α antagonist MPP, and then treated with FSH and/or a selective ER α agonist PPT for an additional 48 h. All doses of drugs used throughout the study had no obvious cytotoxic effect. At the end of incubation, conditioned media were collected, cleared by centrifugation, and stored at -70 C until assayed for progesterone content by ELISA. Cell number was determined using the crystal violet assay as previously described (36).

ELISA for progesterone

Progesterone level in conditioned media was measured by ELISA as previously described (7–9). Antisera against progesterone (37) were kindly provided by Dr. O. David Sherwood (University of Illinois, Urbana, IL).

Immunoblotting

Granulosa cells (~6 × 10⁶) were cultured in Matrigel-coated 60-mm culture dishes, pretreated with ethanol vehicle, or various doses of MPP or ICI for 1 h, and then treated with FSH (10 ng/ml) and/or TGF β 1 (5 ng/ml) for an additional 48 h to determine their effects on the protein levels of StAR (38), P450scc (39), and 3 β -HSD enzymes (40). Cell lysates were prepared, and immunoblotting was performed as previously described (7–9). Relative quantification of chemiluminescent signals on x-ray film was analyzed using a two-dimensional laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

Coimmunoprecipitation

Granulosa cells (~15 × 10⁶) were cultured in Matrigel-coated 100-mm culture dishes, pretreated with ethanol vehicle, MPP, or ICI for 1 h, and then treated with FSH (10 ng/ml) and/or TGF β 1 (5 ng/ml) for 30 min to determine their effects on the ER α interaction with transcription coregulators. Cell lysates (500 μ g proteins) were precleared with 20 μ l protein A/G plus agarose, incubated for 2 h at 4 C. After centrifugation at 15,000 × g for 15 min, the supernatant was incubated with 1 μ g ER α antibody for 6 h at 4 C, followed by addition of 30 μ l protein A/G plus agarose and rotated overnight at 4 C. The immune precipitated products were separated by centrifugation, and analyzed by immunoblotting using antibodies of ER α , histone acetylases (CBP, SRC-1, and RAC-3), PGC-1 α , and transcription factors (c-Jun, β -catenin, phospho-Smad2, phospho-Smad3, phospho-CREB, FoxO1, and FoxO3a).

Chromatin immunoprecipitation (ChIP) assay

Granulosa cells ($\sim 15 \times 10^6$) were cultured in Matrigel-coated 100-mm culture dishes, pretreated with ethanol vehicle, MPP, or ICI for 1 h, and then treated with FSH (10 ng/ml) and/or TGF β 1 (5 ng/ml) for 3 h to determine their effects on the binding of ER α , CBP, SRC-1, and PGC-1 α to DNA using ChIP assay as previously described (41, 42). In brief, cells were first fixed in 1% formaldehyde in PBS (10 min, 37°C) to cross-link DNA and proteins, and then 125 mM glycine was added (5 min, 37°C) to stop the reaction. Cells were then lysed in Nonidet P-40 lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40] by incubating on ice for 10 min, and centrifuged at 1500 $\times g$ for 5 min. The nuclear pellet was further sonicated in lysis buffer [10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 2% Triton X-100], and centrifuged at 15,000 $\times g$ for 20 min. The DNA fragment sizes were at the range of approximately 400–1000 bp. Small aliquots of the supernatants were kept and served as input to normalize PCR results. Supernatants were then precleared by protein A/G-agarose followed by centrifugation, and the supernatants were immune precipitated as described previously in the Coimmunoprecipitation section. Normal IgG in place of antibody of interest was used as the negative control. To remove RNAs and proteins, the immune complexes were incubated sequentially in 200 mM NaCl containing 2 μ g/ml ribonuclease A for 2 h at 65°C, and then with 300 μ g/ml proteinase K for an additional 2 h at 65°C. DNA was then extracted by phenol/chloroform (1:1, vol/vol), followed by isopropanol precipitation, and then dissolved in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. PCR was performed using PerkinElmer GeneAmp PCR system 2400 (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA). For Star, the DNA was amplified for 30 cycles (denaturation: 95°C, 1 min; annealing: 64°C, 1 min; elongation: 72°C, 1 min) using antisense and sense primers (5'-CATCCAGCAAGGAGAGGAAG-3' and 5'-CGTGGAGTT-GGTCTTGAGG-3') (−853 to −358, accession no. NC005115). For Cyp11a1, the DNA was amplified for 25 cycles (denaturation: 95°C, 1 min; annealing: 45°C, 1 min; elongation: 72°C, 1 min) using antisense and sense primers (5'-ATCACAGAGATGCTGGCAGGA-3' and 5'-GCACGTTGATGAGGAAGATGG-3') (−801 to −321, accession no. NC005107). For Hsd3b, the DNA was amplified for 25 cycles (denaturation: 95°C, 1 min; annealing: 58°C, 70 sec; elongation: 72°C, 70 sec) using antisense and sense primers (5'-ACTGGCAAATTCTCCATAG-3' and 5'-TTCCCTCCCAGCTGACAAGTGG-3') (−552 to −151, accession no. L17138). All of these regions contain ERE half-site. The primer pairs used correspond to the rat nucleotide sequences (43, 44). The PCR cycle number chosen for each gene was at linearity range of amplification. PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining.

Statistics

Quantitative data were analyzed by ANOVA and Duncan's multiple range tests at a significance level of 0.05 using the general linear model of the SAS program (SAS Institute Inc., Cary, NC). In addition, the Student's *t* test was used to identify significant differences between the two treatment groups.

Results

Specific involvement of ER α in FSH and TGF β 1-stimulated steroidogenesis

We first determine the specific involvement of ER in FSH and TGF β 1-stimulated steroidogenesis in rat ovarian granulosa cells. ER antagonist ICI dose dependently (10^{−10} to 10^{−6} M) decreased FSH plus TGF β 1-induced progesterone production, whereas AR antagonist HF (10^{−6} and 10^{−5} M) had no effect (Fig. 1). Therefore, because both ER α and ER β are present in granulosa cells, we further investigated their specific involvement by using selective modulators. Interestingly, a selective ER α antagonist MPP, like ICI, dose dependently decreased FSH ± TGF β 1-stimulated progesterone production, and the inhibitory effect of MPP at the dose of 5 \times 10^{−6} M was similar to that of ICI at 10^{−6} M (Fig. 2).

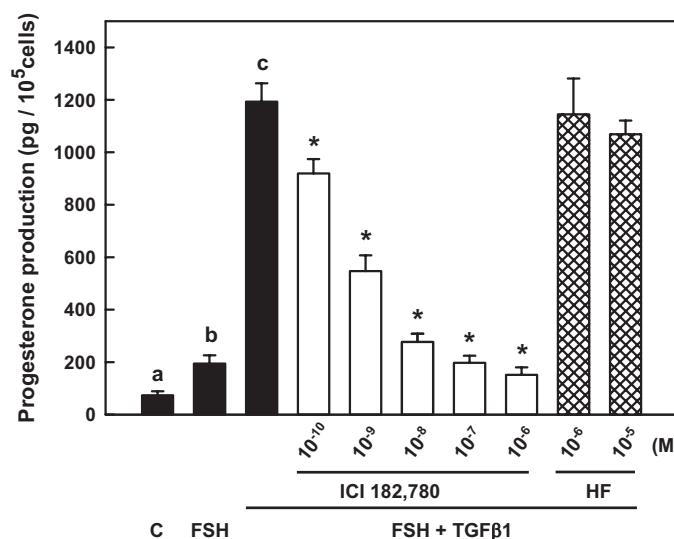


FIG. 1. Effect of ER and AR antagonists on FSH and TGF β 1-induced progesterone production in rat ovarian granulosa cells. Cells were pretreated with ethanol vehicle or various doses of an ER antagonist ICI-182780 and an AR antagonist HF for 1 h, and then treated with vehicle control (C), 10 ng/ml FSH, and/or 5 ng/ml TGF β 1 for an additional 48 h. Conditioned media were collected and analyzed for progesterone content by ELISA. Each bar represents the mean (\pm SE) progesterone production ($n = 9$). Different lowercase letters indicate significant differences among treatment groups in the absence of antagonists ($P < 0.05$). Asterisk indicates a significant difference compared with the FSH plus TGF β 1-treated group ($P < 0.05$).

Whereas a selective ER β antagonist PHTPP had no obvious effect on FSH ± TGF β 1-stimulated progesterone production in rat granulosa cells (Fig. 3). In addition, 17 β -estradiol (E₂) is known to enhance FSH-induced progesterone secretion in

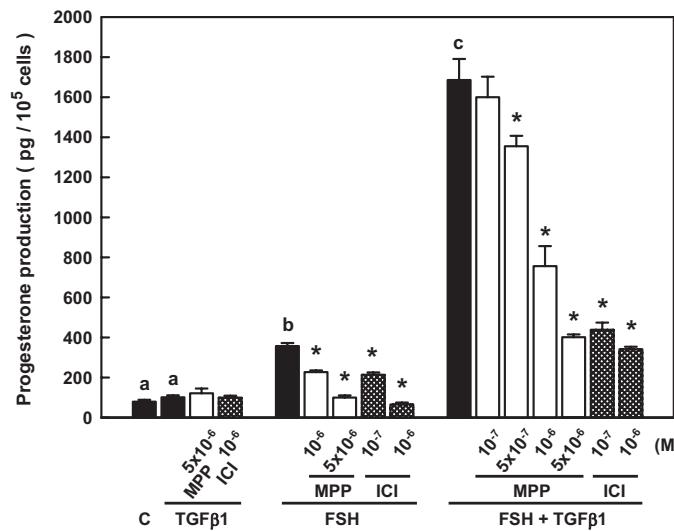


FIG. 2. Effect of selective ER α antagonist on FSH and TGF β 1-induced progesterone production in rat ovarian granulosa cells. Cells were pretreated with ethanol vehicle, or various doses of ICI or an ER α antagonist MPP for 1 h, and then treated with FSH and/or TGF β 1 for an additional 48 h. Each bar represents the mean (\pm SE) progesterone production ($n = 9$). Different lowercase letters indicate significant differences among treatment groups in the absence of antagonists ($P < 0.05$). Asterisk indicates a significant difference compared with the respective control (C) without antagonists ($P < 0.05$).

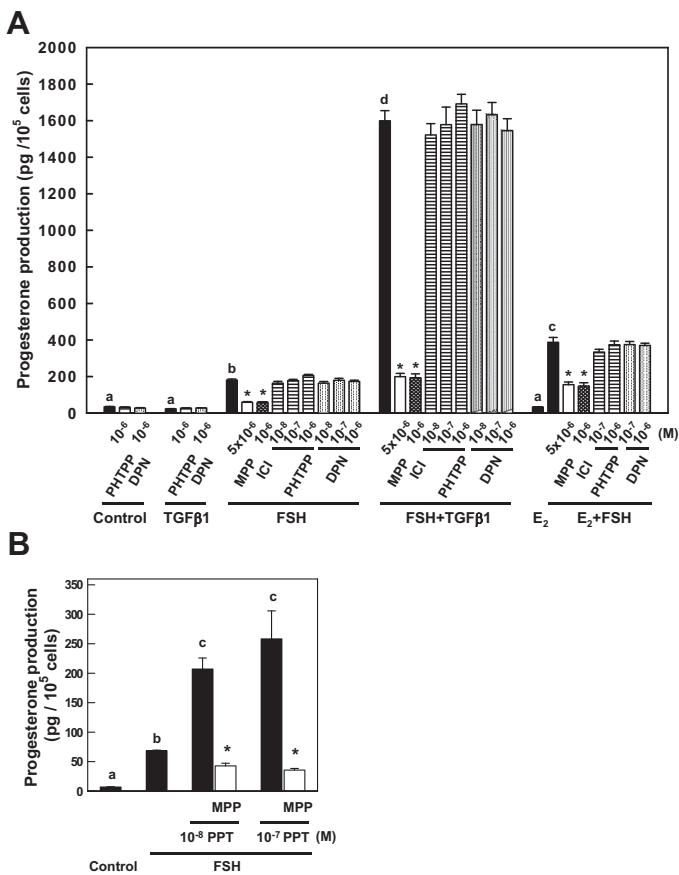


FIG. 3. Effect of selective ER β modulators and ER α agonist on FSH and TGF β 1-induced progesterone production in rat ovarian granulosa cells. A, Cells were pretreated with vehicle, various doses of an ER β antagonist PHTPP or agonist DPN for 1 h, and then treated with FSH and/or TGF β 1, or 10^{-9} M E₂ for an additional 48 h. Pretreatment with 5×10^{-6} M MPP and 10^{-6} M ICI was included as reference. B, Cells were pretreated with vehicle or an ER α antagonist MPP (5×10^{-6} M) for 1 h, and then treated with FSH in the absence or presence of an ER α agonist PPT (10^{-8} and 10^{-7} M) for an additional 48 h. Each bar represents the mean (\pm SE) progesterone production ($n = 6$). Different lowercase letters indicate significant differences among treatment groups in the absence of antagonists ($P < 0.05$). Asterisk indicates a significant difference compared with the respective control without antagonists ($P < 0.05$).

granulosa cells (45). Here, we show that MPP and ICI reduced this action of estradiol, whereas PHTPP had no effect (Fig. 3). Also unlike estradiol, a selective ER β agonist DPN exerted no significant effect on FSH ± TGF β 1-induced progesterone production (Fig. 3A). In addition, a selective ER α agonist PPT (like E₂) augmented FSH-stimulated progesterone production, and pretreatment with ER α antagonist MPP abolished this action of PPT (Fig. 3B). Together, these results implicate for the first time the crucial role of ER α mediation of FSH and TGF β 1-stimulated progesterone production in rat ovarian granulosa cells.

We next investigated the role of ER α in FSH and TGF β 1 regulation of the three key players in progesterone production (StAR protein, P450scc, and 3 β -HSD enzymes) at the level of protein and gene expression, respectively, using immunoblotting and ChIP assays, respectively. Consistent with our recent studies (7–9), FSH alone at the dose of 10 ng/ml

increased StAR protein and 3 β -HSD enzyme levels and had no significant effect on P450scc enzyme level, and the combined treatment with TGF β 1 further increased the levels of all three key molecules (Fig. 4). TGF β 1 alone did not affect the content of all three players (data not shown). Here, we demonstrate that MPP and ICI reduced the protein levels of FSH-increased 3 β -HSD enzyme and FSH plus TGF β 1-increased 3 β -HSD and P450scc, but not the StAR protein level (Fig. 4). ChIP assays further support that ER α exerts a critical role in FSH and TGF β 1-induced gene expression of Hsd3b and Cyp11a1, but not the Star. FSH significantly increased ER α binding (directly and/or indirectly) to Hsd3b gene, and FSH plus TGF β 1 further increased ER α binding to Hsd3b and Cyp11a1 genes (Fig. 5). Furthermore, MPP and ICI significantly reduced these interactions. In addition, we did not detect any significant association of ER α with Star gene in all groups (Fig. 5). These results indicate ER α crucial mediation of FSH and TGF β 1-stimulated progesterone production in rat ovarian granulosa cells is attributed at least partly through up-regulation of the expression of Hsd3b and Cyp11a1 genes.

Molecular interaction of ER α with transcription coregulators in FSH and TGF β 1 up-regulation of steroidogenic gene expression

Stimulation of target gene expression in response to ER is mediated by its direct binding to ERE and/or indirect interaction with other transcription coregulators (15). Therefore, we further explored ER α interaction with transcription coregulators in FSH and TGF β 1-stimulated expression of Hsd3b and Cyp11a1 genes in rat ovarian granulosa cells using coimmunoprecipitation with ER α antibody. Interestingly, FSH increased ER α interaction with histone acetylases CBP and SRC-1, but not RAC-3, and TGF β 1 significantly enhanced FSH-induced ER α interaction with CBP (Fig. 6). Surprisingly, TGF β 1 in the presence of FSH increased ER α association with PGC-1 α , whereas FSH alone had no effect. In addition, TGF β 1 alone had no significant effect on ER α association with CBP, SRC-1, and PGC-1 α (Fig. 6). Furthermore, MPP and ICI dramatically suppressed the FSH ± TGF β 1-induced ER α interaction with CBP and SRC-1, as well as PGC-1 α . Both FSH and TGF β 1 had no obvious effect on the ER α association with transcription factors c-Jun and β -catenin, and this is not affected by pretreatment with either MPP or ICI (Fig. 6). In addition, FSH and TGF β 1 had no obvious effect on the ER α association with TGF β 1 signaling transcription factors, phosphorylated Smad2 and Smad3. Our most recent work shows that FSH increased the phosphorylation of CREB and FoxOs (FoxO1 and FoxO3a) in rat granulosa cells (9). Here, we detected no clear association of ER α with phosphorylated CREB, as well as FoxO1 and FoxO3a in all groups (data not shown).

To study further the critical association of ER α and transcription regulator complex in FSH and TGF β 1 induction of steroidogenic gene expression in rat ovarian granulosa cells, ChIP assays with antibodies of CBP, SRC-1, and PGC-1 α were used. Our results clearly demonstrate CBP, SRC-1, and PGC-1 α binding to Hsd3b and Cyp11a1 genes, and CBP could also bind to Star gene (Fig. 7). FSH increased CBP,

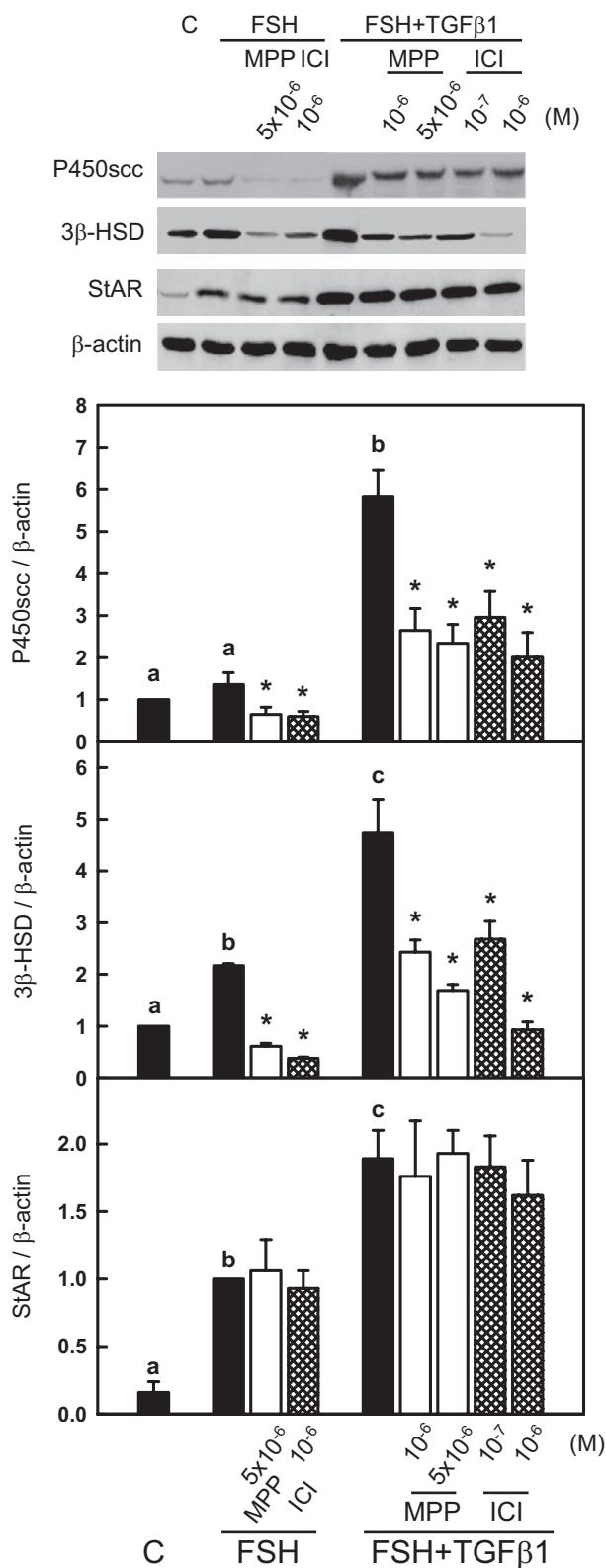


FIG. 4. Effects of selective ER α antagonists on FSH and TGF β 1-regulated key players of steroidogenesis in rat ovarian granulosa cells. Cells were treated as described in Fig. 2. Cell lysates were analyzed by immunoblotting for StAR protein, P450scc and 3 β -HSD enzymes with β -actin used as an internal control (C). Quantitative analysis of StAR, P450scc, and 3 β -HSD in reference to β -actin was

SRC-1, and PGC-1 α binding to Hsd3b gene, and had no significant effect on Cyp11a1 gene. TGF β 1 plus FSH further increased the binding of these coregulators to Hsd3b and Cyp11a1 genes (Fig. 7). Furthermore, MPP and ICI dramatically suppressed the FSH and TGF β 1-induced CBP, SRC-1, and PGC-1 α binding to Hsd3b and Cyp11a1 genes. We also noticed that FSH, in the absence or presence of TGF β 1, similarly increased CBP binding to Star gene, and this was not affected by the pretreatment of MPP or ICI (Fig. 7A).

Together, these results suggest that FSH-induced expression of Hsd3b gene in rat granulosa cells is partly through recruitment of ER α and histone acetylases (CBP/SRC-1). Furthermore, TGF β 1 enhancement of Hsd3b and Cyp11a1 expression in the presence of FSH is partly through recruitment of ER α together with CBP/SRC-1 and PGC-1 α . In addition, FSH and TGF β 1 act in an ER α -independent manner to increase Star expression in rat granulosa cells.

Discussion

Specific involvement of ER α in FSH and TGF β 1-stimulated steroidogenesis

The ovarian endocrine is very complicated and interesting. In our laboratory we are devoted to study the mechanism(s) of pituitary FSH and intraovarian TGF β 1-induced granulosa cell differentiation characterized by increased steroidogenic activity, production of both progesterone and estrogen. Our recent studies show that TGF β 1 augmentation of FSH-induced increase in progesterone production in rat granulosa cells of antral follicles is partly attributed to increased protein levels of StAR protein, and P450scc and 3 β -HSD enzymes (7–9). This study further implicates for the first time that ER α is a critical mediator in FSH and TGF β 1-induced progesterone production, partly acting through modulating the expression of Hsd3b and Cyp11a1 genes, but not Star gene in rat ovarian granulosa cells, and interestingly ER β appears not to be significantly involved in the process as evidenced by the following. A selective ER α antagonist MPP, like ER antagonist ICI, suppressed the FSH ± TGF β 1-stimulated progesterone production in rat granulosa cells, whereas AR antagonist HF as well as selective ER β antagonist PHTPP and agonist DPN all had no significant effect (Figs. 1–3). This is partly attributed to the suppressive effects of MPP and ICI on the FSH ± TGF β 1-induced expression of Hsd3b and Cyp11a1, but not the Star, as indicated by the results of immunoblotting and ChIP analyses (Figs. 4 and 5). Furthermore, a selective ER α agonist PPT (like E₂) enhanced FSH-stimulated progesterone, and this was blocked by pretreatment with ER α antagonist MPP (Fig. 3B). Therefore, because FSH and TGF β 1 increased ER α binding directly and/or indirectly to Hsd3b and Cyp11a1 genes (Fig. 5), we explored the molecular mechanism of ER α mediation of FSH and TGF β 1-regulated expression of Hsd3b and Cyp11a1 in rat ovarian granulosa cells.

performed using two-dimensional scanning densitometry. Relative density ratios were calculated using the FSH-treated group value or control group value as one. Each bar represents the mean (\pm SE) relative density ($n = 3$). Different lowercase letters indicate significant differences among treatment groups in the absence of inhibitors ($P < 0.05$). Asterisk indicates a significant difference compared with the respective control without inhibitors ($P < 0.05$).

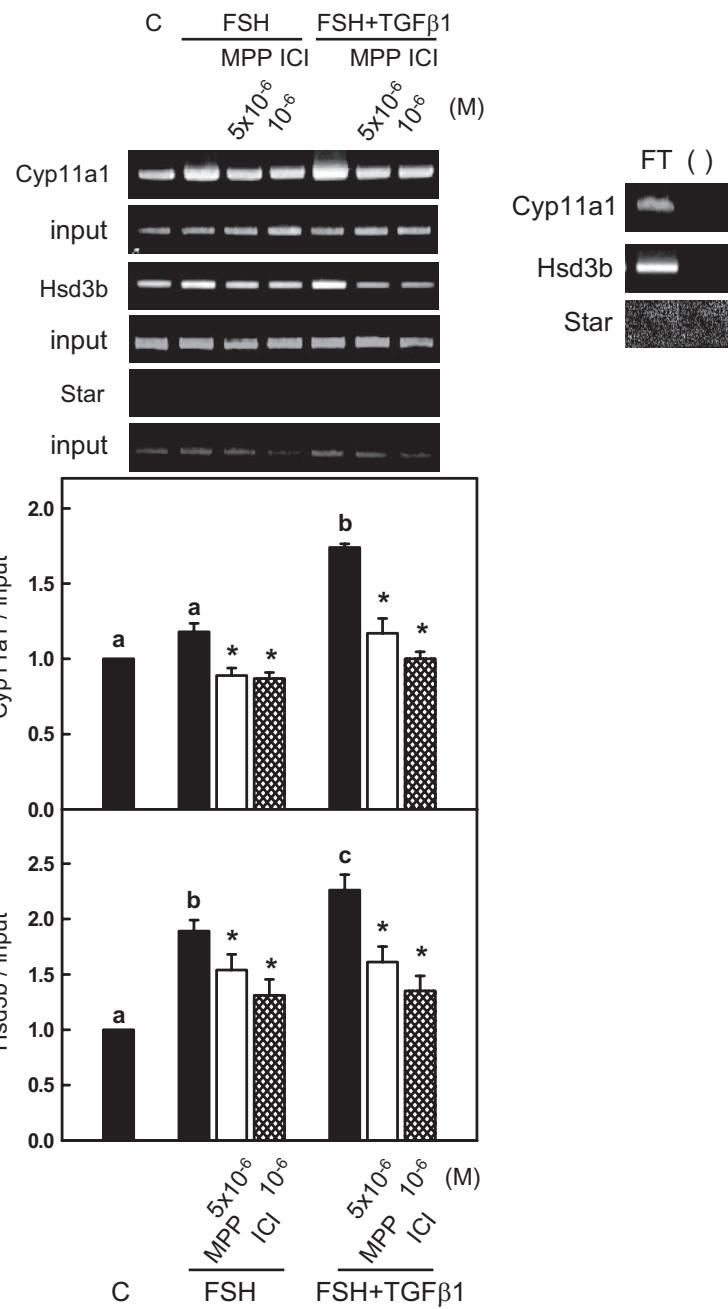
ChIP: ER α 

FIG. 5. Effect of selective ER α antagonists on FSH and TGF β 1-induced ER α binding to gene of key players of steroidogenesis in rat ovarian granulosa cells. Cells were pretreated with ethanol vehicle, 5 \times 10 $^{-6}$ M PMP or 10 $^{-6}$ M ICI for 1 h, and then treated with vehicle control (C), 10 ng/ml FSH and/or 5 ng/ml TGF β 1 for an additional 3 h. Nuclear extracts were analyzed by ChIP assay with ER α antibody or normal IgG as a negative control (−), and input DNA served as an internal control. Ethidium bromide-stained intensity of the PCR products was determined and normalized to the input DNA. Relative density ratios were calculated using the control group value as one. Each bar represents the mean (\pm SE) relative density (n = 3). Different lowercase letters indicate significant differences among treatment groups in the absence of inhibitors (P < 0.05). Asterisk indicates a significant difference compared with the respective control without inhibitors (P < 0.05). FT, FSH plus TGF β 1.

Molecular interaction of ER α with transcription coregulators in FSH and TGF β 1 up-regulation of steroidogenic gene expression

The molecular mechanisms of transcription regulation of steroidogenic genes Hsd3b, Cyp11a1, and Star remain largely unclear. There are regulators reported to be involved in transcription regulation of human Cyp11a1 and mouse Star genes, including CREB, CBP, and AP-1 (heterodimer of c-Jun and c-fos) (46). According to nucleotide sequence analysis, promoters of rat Hsd3b, Cyp11a1, and Star genes all lack a consensus cAMP response element site (TGACGTCA) (47, 48), and all have consensus AP-1 site (GTCGTCA) (47, 49). In

addition, all three rat gene promoters only have ERE half-site (AGGTCA) and not full palindrome ERE (50, 51). Up to date, no report has clearly documented ER α involvement in transcription regulation of Hsd3b, Cyp11a1, and Star genes. Several coregulators have been reported to activate ER α transcription activity, including histone acetylases (CBP, SRC-1, and RAC-3), and transcription factors (Smad3 and FoxO1) in cancer cells (21, 25, 52). Histone acetylases facilitate histone acetylation, leading to chromatin remodeling and transcription initiation by formation of stable RNA polymerase II complex. Moreover, the peroxisome proliferator-activated receptor (PPAR) γ PGC-1 α and β -catenin have also acted as

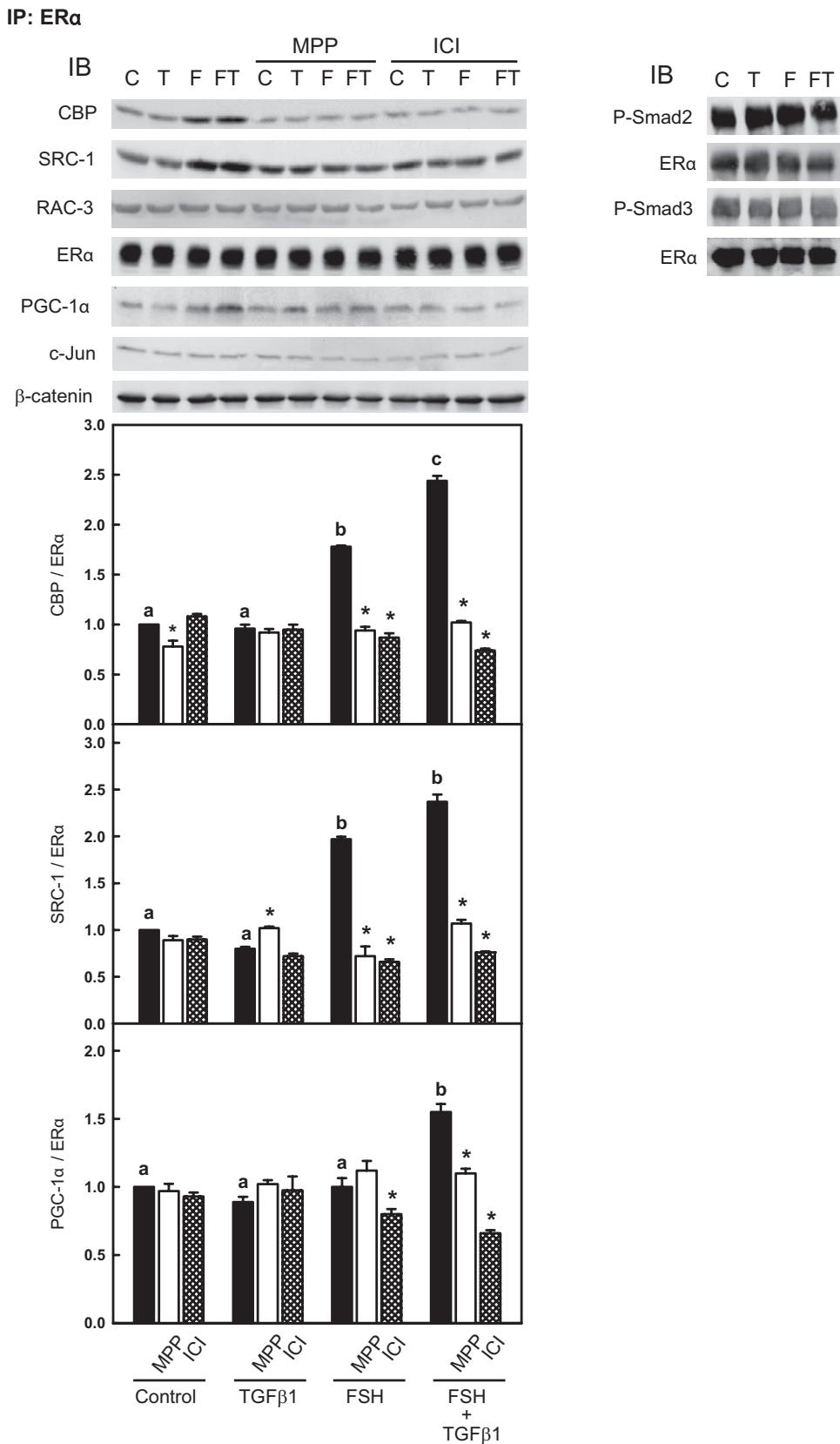


FIG. 6. Effect of selective ER α antagonists on FSH (F) and TGF β 1 (T)-regulated ER α interaction with transcription coregulators in rat ovarian granulosa cells. Cells were pretreated with ethanol vehicle, 5×10^{-6} M MPP or 10^{-6} M ICI for 1 h, and then treated with vehicle control (C), 10 ng/ml FSH, and/or 5 ng/ml TGF β 1 for an additional 30 min. Cell lysates were immunoprecipitated with ER α antibody, and the immune complexes were analyzed by immunoblotting for ER α and transcription co-regulators (CBP, SRC-1, RAC-3, PGC-1 α , c-Jun, β -catenin, phospho-Smad2 and Smad3). Quantitative analysis was performed in reference to ER α using two-dimensional scanning densitometry. Relative density ratios were calculated using the control group value as one. Each bar represents the mean (\pm SE) relative density ($n = 3$). Different lowercase letters indicate significant differences among treatment groups in the absence of antagonists ($P < 0.05$). Asterisk indicates a significant difference compared with the respective control without antagonists ($P < 0.05$). FT, FSH plus TGF β 1.

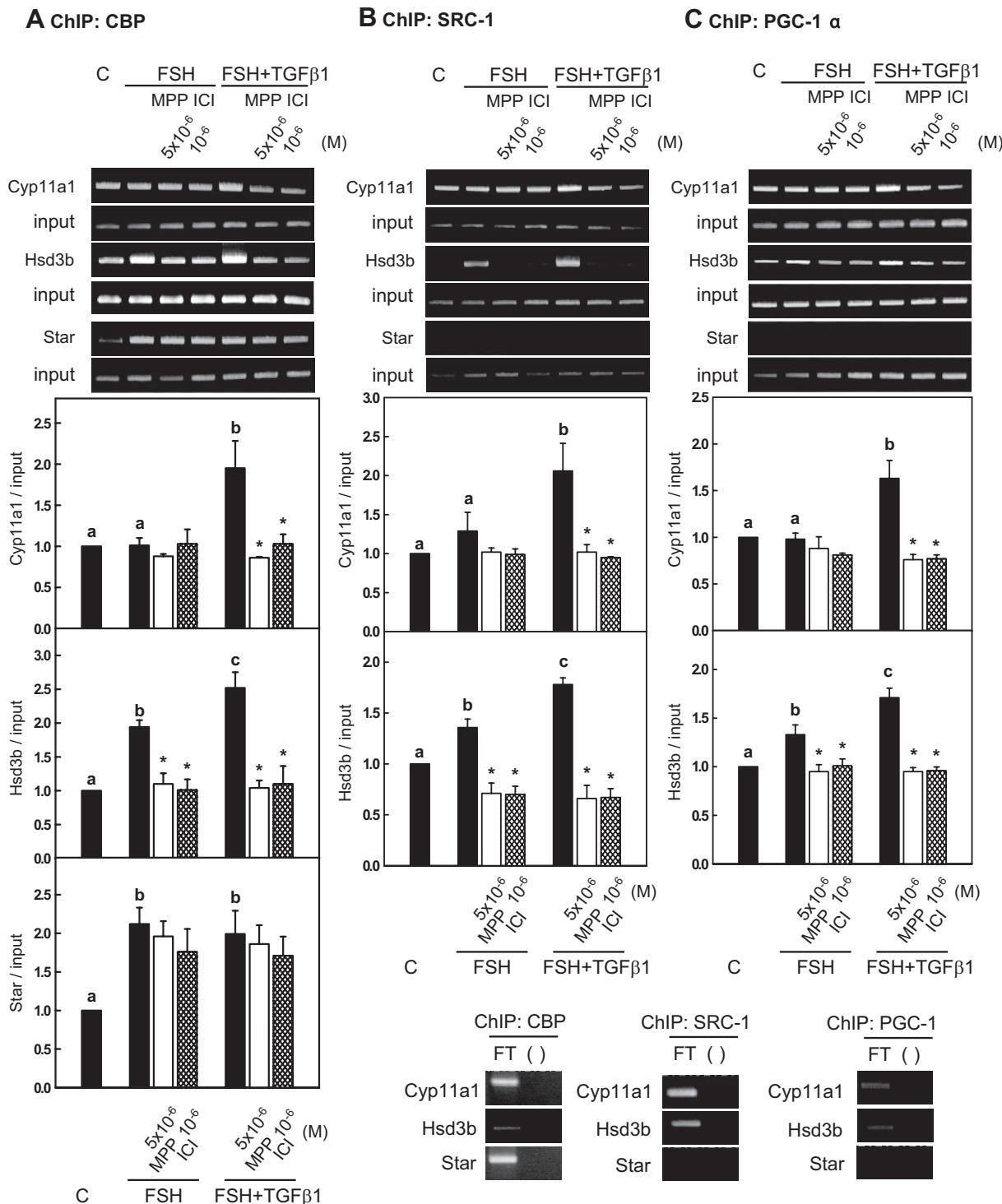


FIG. 7. Effect of selective ER α antagonists on FSH and TGF β 1-induced binding of histone acetylases and PGC-1 α to key steroidogenic genes in rat ovarian granulosa cells. Cells were treated, and nuclear extracts were analyzed by ChIP assay as described in Fig. 5. Nuclear extracts were immunoprecipitated with antibodies for CBP (A), SRC-1 (B), and PGC-1 α (C), or normal IgG as negative control (–). Ethidium bromide-stained intensity of the PCR products was determined and normalized to the input DNA. Relative density ratios were calculated using the control group value as one. Each bar represents the mean (\pm SE) relative density (n = 3). Different lowercase letters indicate significant differences among treatment groups in the absence of antagonists (P < 0.05). Asterisk indicates a significant difference compared with the respective control without antagonists (P < 0.05). C, Control; FT, FSH plus TGF β 1.

coactivators of ER α that enhanced SRC-1 and CBP binding to target genes and increased ER α transcription activity (53–56). In addition, ER α interaction with histone acetylases enhanced AP-1 transcription activity (19).

Our present study indicates for the first time that FSH-increased expression of Hsd3b gene in rat granulosa cells is partly through recruitment of ER α and histone acetylases (CBP/SRC-1), as indicated by results of coimmunoprecipitation (Fig. 6) and ChIP assays (Figs. 5 and 7). Moreover, this study implicates for the first time that TGF β 1 enhancement of FSH-induced expression of Hsd3b and Cyp11a1 genes in rat granulosa cells is through recruitment of ER α together with histone acetylases (CBP/SRC-1) and PGC-1 α , as indicated by results of coimmunoprecipitation (Fig. 6) and ChIP assays (Figs. 5 and 7). All these FSH and TGF β 1-induced effects were suppressed by MPP and ICI (Figs. 5–7). ER α , in the presence of antagonists, may fail to establish a proper conformation for recruitment of coregulators to activate transcription. The PGC-1 α involvement in stimulation of steroidogenesis is partially supported by a previous study reporting that PPAR γ activator increased estradiol and progesterone production in rat granulosa cells (57). Because PGC-1 α is known to play a critical role in mitochondria biogenesis (58), we think TGF β 1 may enhance FSH effect via increasing the capacity of steroidogenesis that takes place inside the mitochondria. Therefore, we suspect that PGC-1 α involvement in FSH and TGF β 1-stimulated steroidogenesis may manifest the coordinated transcription program between enzymes for steroidogenesis and mitochondria biogenesis. In addition, TGF β 1 involvement in enhancing FSH-induced Hsd3b gene expression is partially supported by a recent study reporting a reduction of Hsd3b mRNA level in the ovary of gonadotropin-primed TGF β 1-null prepubertal female mice (10).

ER transcription regulation could also act indirectly through non-ERE (such as AP-1-responsive elements) (19). The promoters of Hsd3b, Cyp11a1, and Star gene all have a consensus AP-1 site (47, 50). And our results demonstrate ER α association with c-Jun in rat granulosa cells; however, this interaction was not altered by the presence of FSH and/or TGF β 1 (Fig. 6). In addition, β -catenin plays a pivotal role in the Wnt-signaling pathway and cell adherens junction formation (56). β -Catenin has also been reported to interact with many nuclear receptors, including AR, ER, glucocorticoid receptor and thyroid hormone receptor, and PPAR. In prostate cancer cells, β -catenin specifically interacted with AR, but not ER α , progesterone receptor β and glucocorticoid receptor (59). The present study demonstrates ER α association with β -catenin in rat granulosa cells; however, like c-Jun-ER α , this interaction was not altered by the presence of FSH and/or TGF β 1 (Fig. 6).

A previous study reported that FoxO1 interaction with ER α results in increased ER α transactivation activity (25). Our most recent study demonstrated that FSH increases the phosphorylation of FoxO1 and FoxO3a in rat ovarian granulosa cells (9). Phosphorylation of FoxOs is known to promote their exit from the nucleus (23, 24), and consistent with this concept, we did not detect any significant ER α interaction with the phosphorylated form or nonphosphorylated form of FoxO1 and FoxO3a in rat granulosa cells, regardless of FSH and/or TGF β 1 treatment (data not shown). This

suggests that ER α may act through FoxOs independent manner in mediation of FSH and TGF β 1-stimulated steroidogenesis in rat ovarian granulosa cells. In addition, TGF β 1 has enhanced ER α -mediated transcription activity through direct physical interactions between Smad2/Smad3 and ER α , resulting in translocation into the nucleus and recruitment of transcription coregulators such as CBP, c-Jun, and β -catenin (52, 60). Our current study demonstrates that TGF β 1 and/or FSH did not significantly affect ER α association with phosphorylated Smad2 and Smad3 (Fig. 6), suggesting that FSH and TGF β 1 up-regulation of Hsd3b and Cyp11a1 expression is not attributed to ER α interaction with Smad2/3 in rat granulosa cells.

Although the promoter of rat Star gene has an ERE half-site (AGGTCA) (49), we did not detect any association of ER α with Star gene in rat granulosa cells using ChIP assay (Fig. 5). And consistent with this, we also did not observe any significant effect of ER α antagonist MPP on FSH ± TGF β 1-increased StAR protein level (Fig. 4). Our study suggests that FSH and TGF β 1 up-regulation of Star gene expression in rat granulosa cells may be through an ER α -independent manner. In addition, the Star gene can be regulated by FSH-induced cAMP signaling (61, 62), yet it has no consensus sequence of cAMP response element (TGACGTCA) (48). Here, we show that FSH significantly enhanced CBP binding to Star gene in rat granulosa cells, and this was not altered by pretreatment with ER antagonists MPP and ICI (Fig. 7A), or by the presence of TGF β 1 (data not shown). Together, our previous (9) and present studies suggest that FSH up-regulates Star gene expression in rat granulosa cells through cAMP/CBP-dependent, and PKA-/PI3K-independent and ER α -independent pathway, and TGF β 1 augmentation of this FSH effect is through PKA-/PI3K-/rapamycin-dependent and ER α -independent pathway. In addition, several signaling pathways have played a permissive role in regulation of StAR protein, including protein kinase C, MAPK/ERKs, calcium messenger systems (63, 64). The molecular mechanism whereby FSH and TGF β 1 regulate Star gene expression awaits further study.

In conclusion, the present study demonstrates for the first time that ER α , but not ER β , plays a critical role in FSH and TGF β 1-induced steroidogenesis in rat ovarian granulosa cells through up-regulation of gene expression of Hsd3b and Cyp11a1, but not Star. This is partly attributed to FSH and TGF β 1-induced recruitment of ER α together with histone acetylases (CBP/SRC-1) and PGC-1 α . Our work furthers the understanding of the mechanism of FSH and TGF β 1 induction of ovarian granulosa cell differentiation that is critically linked to oocyte maturation function and, thus, vital to fertility control.

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