Interplay of PI3K and cAMP/PKA signaling, and rapamycin-hypersensitivity in TGFβ1 enhancement of FSH-stimulated steroidogenesis in rat ovarian granulosa cells

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Abstract

Transforming growth factor (TGF)β1 facilitates FSH-induced differentiation of rat ovarian granulosa cells. The signaling crosstalk between follicle stimulating hormone (FSH) and TGFβ receptors remains unclear. This study was to investigate the interplay of cAMP/protein kinase A (PKA) and phosphatidylinositol-3-kinase (PI3K) signaling including mammalian target of rapamycin (mTOR)C1 dependence in FSH- and TGFβ1-stimulated steroidogenesis in rat granulosa cells. To achieve this aim, inhibitors of PKA (PKAI), PI3K (wortmannin), and mTORC1 (rapamycin) were employed. PKAI and wortmannin suppressions of the FSH-increased progesterone production were partly attributed to decreased level of 3β-HSD, and their suppression of the FSH plus TGFβ1 effect was attributed to the reduction of all the three key players, steroidogenic acute regulatory (StAR) protein, P450scc, and 3β-HSD. Further, FSH activated the PI3K pathway including increased integrin-linked kinase (ILK) activity and phosphorylation of Akt(S473), mTOR(S2481), S6K(T389), and transcription factors particularly FoxO1(S256) and FoxO3a(S253), which were reduced by wortmannin treatment but not by PKAI. Interestingly, PKAI suppression of FSH-induced phosphorylation of cAMP regulatory element-binding protein (CREB(S133)) disappeared in the presence of wortmannin, suggesting that wortmannin may affect intracellular compartmentalization of signaling molecule(s).

In addition, TGFβ1 had no effect on FSH-activated CREB and PI3K signaling mediators. We further found that rapamycin reduced the TGFβ1-enhancing effect of FSH-stimulated steroidogenesis, yet it exhibited no effect on FSH action. Surprisingly, rapamycin displayed a suppressive effect at concentrations that had no effect on mTORC1 activity. Together, this study demonstrates a delicate interplay between cAMP/PKA and PI3K signaling in FSH and TGFβ1 regulation of steroidogenesis in rat granulosa cells. Furthermore, we demonstrate for the first time that TGFβ1 acts in a rapamycin-hypersensitive and mTORC1-independent manner in augmenting FSH-stimulated steroidogenesis in rat granulosa cells.
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Introduction

Normal ovarian function is critical for successful reproduction. Pituitary follicle-stimulating hormone (FSH) is the major regulator of growth and development of antral follicles (Hirshfield 1991, Richards 2001). And local ovarian factor, transforming growth factor β (TGFβ) plays an important role in facilitating FSH-induced differentiation of ovarian granulosa cells, including progesterone and estrogen production, aromatase activity, and luteinizing hormone (LH) receptor expression (Dodson & Schomberg 1987, Dorrington *et al.* 1993, Gitay-Goren *et al.* 1993, Inoue *et al.* 2002). FSH induces the expression of steroidogenic acute regulatory (StAR) protein, cholesterol side-chain cleavage enzyme (P450scc), and 3β-hydroxysteroid dehydrogenase (3β-HSD), which are the key players in steroidogenesis (Eimerl & Orly 2002, Ke *et al.* 2004, 2005). StAR protein facilitates cholesterol transport into mitochondria where P450scc enzyme catalyzes the initial step of steroidogenesis, the conversion of cholesterol into pregnenolone, which is then converted to progesterone by 3β-HSD enzyme in the endoplasmic reticulum (Clarke *et al.* 1993). In addition, TGFβ has been shown to play important roles in ovarian functions (Ingman & Robertson 2002). TGFβ mainly acts as a positive regulator of granulosa cell differentiation as it enhances FSH-stimulated expression of LH receptor, inhibin, gap junction protein connexin 43, and steroidogenesis in rat and murine granulosa cells (Dodson & Schomberg 1987,

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Zhiwen *et al.* 1988, Gitay-Goren *et al.* 1993, Inoue *et al.* 2002, Ke *et al.* 2004, 2005).

Recent studies suggest that at least two cellular signaling pathways are intertwined and obligatory in FSH action. The prototype of FSH signaling is that FSH first binds to specific, cell-surface G-protein-coupled receptors (GPCRs) and activates adenylyl cyclase leading to the production of cAMP, which teams up with cAMP-dependent protein kinase (PKA) and then triggers signaling cascades to regulate transcription of specific genes via the cAMP regulatory element-binding protein (CREB)–CREB-binding protein (CBP) complex (Mayr & Montminy 2001, Conkright & Montminy 2005). In addition, FSH can also activate the phosphatidylinositol-3-OH kinase (PI3K) pathway. FSH activates PI3K in rat granulosa cells leading to phosphorylation of Akt and serum and glucocorticoid-induced kinase (Sgk; Gonzalez-Robayna *et al.* 2000, Richards *et al.* 2002). This may be a crucial mechanism that enhances progesterone production (Zeleznik *et al.* 2003) and the expression of genes, such as aromatase, LH receptor, inhibin-α, and P450scc enzyme (Gonzalez-Robayna *et al.* 2000, Richards *et al.* 2002, Park *et al.* 2005). In addition, FSH enhances hypoxia-inducible factor-1 (HIF-1) activity through PI3K/Akt-dependent activation of mammalian target of rapamycin (mTOR), and HIF-1 activity is necessary for upregulation of FSH target genes, such as vascular endothelial growth factor (VEGF), inhibin-α, and LH receptor (Alam *et al.* 2004). In addition to the typical Smad pathway (Derynck & Zhang 2003), TGFβ can also activate PI3K/Akt pathway and this is implicated in the regulation of cell migration (Bakin *et al.* 2000), survival (Chen *et al.* 1998, Ju *et al.* 2005, Zocchi *et al.* 2005), and epithelial–mesenchymal transition process (Nawshad *et al.* 2005, Lien *et al.* 2006).

Akt is a central player in signal transduction activated in response to growth factors and is thought to contribute to many important cellular functions, including nutrient metabolism, cell growth, apoptosis, and modulating the activity of transcription factors (Brazil *et al.* 2004, Hanada *et al.* 2004, Woodgett 2005). Akt is subjected to phosphorylation regulation by phosphoinositide-dependent kinase 1 (PDK1) at the activation loop site, Thr308. Furthermore, full activation of Akt also requires phosphorylation of its Ser473 at carboxyl-terminal hydrophobic motif by kinase(s) such as integrin-linked kinase (ILK) and mTOR complex 2 (mTORC2; Brazil *et al.* 2004, Hanada *et al.* 2004, Sarbassov *et al.* 2005). mTOR is a conserved serine/threonine kinase, and there are two known mTOR complexes within cells, mTORC1 containing mTOR, GβL and raptor and mTORC2 containing mTOR, GβL, and rictor (Inoki *et al.* 2005, Martin & Hall 2005, Wullschleger *et al.* 2006). mTORC1 regulates cell growth through modulating transcription, and translation in part by regulating p70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and mTORC2 is involved in actin polymerization and cell spreading. Additionally, mTORC1 is sensitive to rapamycin, and mTORC2 is not.

Several key observations suggest a potential link between Akt and transcriptional regulation. Akt directly phosphorylates FoxO transcription factors leading to nuclear export of FoxOs to cytoplasm and the release of their regulation of transcription (Burgering & Kops 2002, Tran *et al.* 2003). Ser256 of FoxO1 (forkhead homolog of rhabdomysarcoma, FKHR) and Ser253 of FoxO3a (forkhead-like protein-1, FKHRL1) are probably exclusively phosphorylated by Akt. FoxO family members participate in various cellular functions, including apoptosis, cell survival, stress detoxification, DNA repair, metabolism, and cell differentiation (Accili & Arden 2004). Three members of the forkhead family have been identified in the rodent ovary, FoxO1, FoxO3a, and FoxO4 (AFX; Kaestner *et al.* 2000, Brunet *et al.* 2001, Richards *et al.* 2002, Tran *et al.* 2003). Ablation of FoxO1 is embryonic lethal due to defective angiogenesis (Hosaka *et al.* 2004) and FoxO3a has a selective effect on ovarian function. Knockout of FoxO3a in mice causes a distinctive ovarian phenotype of global follicular activation leading to oocyte death, early depletion of functional ovarian follicles and secondary infertility, suggestive of premature ovarian failure (Castrillon *et al.* 2003, Hosaka *et al.* 2004). FSH through PI3K signaling induces rapid phosphorylation inactivation of FoxO1(Ser256), and this possibly leads to promotion of proliferation and differentiation of ovarian granulosa cells (Richards *et al.* 2002, Cunningham *et al.* 2003, Park *et al.* 2005). Together, these studies indicate that FoxOs are important regulators of follicular development, and that PI3K/Akt signaling is crucial for the upregulation of granulosa cell differentiation.

Previous studies demonstrate that TGFβ1 augmented FSH-stimulated progesterone production (Dodson & Schomberg 1987, Ke *et al.* 2004, 2005), and increased key players in steroidogenesis, StAR protein, and P450scc enzyme (markers of differentiation) in rat ovarian granulosa cells (Ke *et al.* 2004, 2005). It is well established that FSH regulates granulosa cell functions mainly through cAMP–PKA pathway for induction of specific genes obligatory for differentiation events (Richards 2001) and, recent observations indicate that FSH can also activate PI3K pathway (Gonzalez-Robayna *et al.* 2000, Richards *et al.* 2002, Cunningham *et al.* 2003, Zeleznik *et al.* 2003, Alam *et al.* 2004, Park *et al.* 2005). The signaling crosstalk between FSH and TGFβ receptors remains unclear. Therefore, this study was to explore the interrelationship of cAMP/PKA and PI3K/Akt signaling in TGFβ1 and FSH-stimulated steroidogenesis in rat ovarian granulosa cells, and particularly the involvement of mTORC toward TGFβ1 enhancement of FSH action was determined.

Materials and Methods

Materials

Ovine FSH (oFSH-19-SIAFP) and equine chorionic gonadotropin (eCG) were purchased from the NHPP, NIDDK, and Dr A F Parlow (USA). Recombinant human TGFβ1 was

obtained from R&D System, Inc. (Minneapolis, MN, USA). Penicillin and streptomycin were from GIBCO Invitrogen Corporation. Antisera against progesterone (Lee & Sherwood 2005), StAR protein (Clark *et al.* 1994), and P450scc enzyme (Hu *et al.* 1991) were kindly provided by Dr O David Sherwood (University of Illinois, IL, USA), Dr Douglas M Stocco (Texas Tech University Health Sciences Center, Lubbock, TX, USA), and Dr Bon-Chu Chung (Academia Sinica, Taipei, Taiwan) respectively. Antibodies against phospho-Akt(Thr308), mTOR, phospho-mTOR (Ser2448), phospho-mTOR(Ser2481), S6K, phospho-S6K(Thr389), FoxO1, phospho-FoxO1(Ser256), and 4E-BP1 were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Wortmannin, Akt1-GST-agarose, and antibodies against ILK, Sgk, phospho-Sgk(Ser255/Thr256), Akt, phospho-Akt(Ser473), FoxO3a, phospho-FoxO3a(Ser253), phospho-CREB(Ser133), and CREB were from Upstate Biotechnology Co. (Lake Placid, NY, USA). Mouse monoclonal antibody against β-actin was from Sigma Chemical Co. PKAI (myristoylated protein kinase A inhibitor amide 14–22) and rapamycin were from Calbiochem (San Diego, CA, USA). 8-CPT-2′–O–Me-cAMP was from BioLog Life Science Institute (Bremen, Germany). Protein-A/G plus agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used were purchased from Sigma Chemical Co. unless otherwise stated.

Animals

Immature Sprague–Dawley rats (24–27 days) 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 (Lab Diet from PMI Feeds, Ins., 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 institutional guidelines.

Cell culture and treatment

Isolation and culture of ovarian granulosa cells from eCG-treated immature rats was performed as previously described (Hwang *et al.* 1996, Ke *et al.* 2005). Granulosa cells were plated into 24-well plates coated with matrigel (derived from Engelbreth–Holm–Swarm sarcoma tumors; Sigma Chemical Co.) at approximately 5×10^5 viable cells per well in 500 μl growth medium (DMEM/F12 medium containing 2 μg/ml bovine insulin, 0·1% fatty acid-free BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin) and allowed to attach for 24 h at 37 °C, 5% CO_2–95% air. Cultured cells were then washed twice and incubated in 500 μl incubation medium (DMEM/F12 containing 0·1% lactalbumin hydrolysate) for 24 h before the beginning of treatment. Cells were pretreated with PKAI, wortmannin or rapamycin for 1 h, and then treated with FSH, 8-Br-cAMP, and/or TGFβ1 for an additional 48 h. The 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 the performance of the progesterone enzyme-linked immunoassay. Cell number was determined using the crystal violet assay as previously described (Gillies *et al.* 1986).

Enzyme-linked immunoassay

Progesterone levels in conditioned media were measured using an enzyme-linked immunoassay. Progesterone standard and enzyme substrate 2,2′-azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) diammonium were purchased from Sigma Chemical Co. The protocol followed was that furnished in a commercial progesterone assay kit (Diagnostic Systems Laboratory, Webster, TX, USA). Progesterone–horseradish peroxidase conjugate was from Fitzgerald Industries International, Inc. (Concord, MA, USA). The absorbance of reaction products was measured at 410 nm using an ELISA reader (Dynatech MR50000, Worthing, West Sussex, UK).

Immunoblotting

Granulosa cells (approximately $5–6\times10^6$) were cultured in matrigel-coated 60 mm culture dishes, pretreated with PKAI, wortmannin, or rapamycin for 1 h, and then treated with 10 ng/ml FSH and/or 5 ng/ml TGFβ1 for 30 min or 1 h to determine their effects on the activation of the PI3K downstream signaling molecules, including Akt, Sgk, mTOR, S6K, FoxO1, FoxO3a, 4E-BP1, and PKA signaling including CREB, and for 48 h to determine their effects on protein levels of StAR protein (Clark *et al.* 1994), P450scc enzyme (Hu *et al.* 1991), and 3β-HSD enzyme (Thomas *et al.* 2002). Cell extracts were prepared in lysis buffer (50 mM Tris–HCl (pH 7·4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0·25% Na-deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and aprotinin, leupeptin, and pepstatin of 1 μg/ml each). Cell lysates (40–60 μg protein each) were analyzed by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% milk/0·05% TBST (Tris-buffered saline with 0·05% Tween 20) for 60 min, the membranes were incubated with primary antibody overnight at 4 °C. The primary antibody was visualized using horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies and enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech UK Limited). Relative quantification of ECL signals on X-ray film was analyzed using a two-dimensional laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

ILK kinase activity assay

Rat granulosa cells were cultured as previously described, and given control vehicle, FSH or FSH plus TGFβ1 for 30 min.

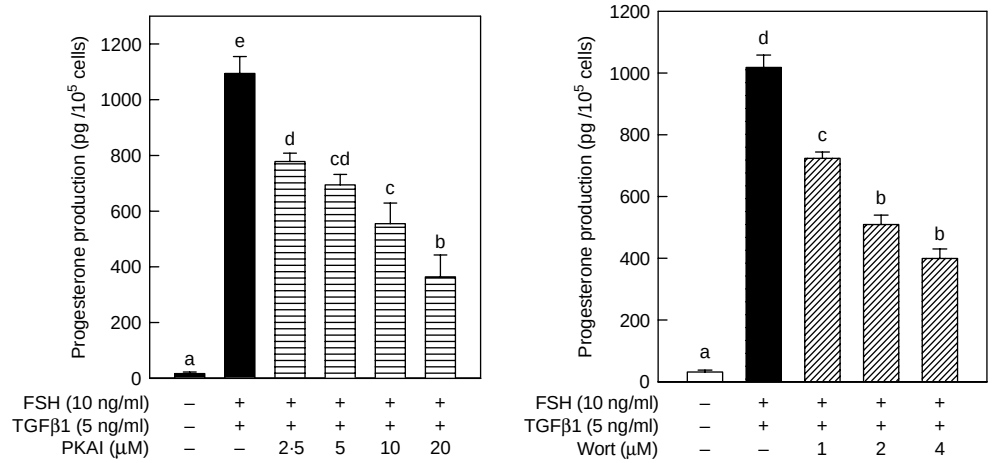


Figure 1 Dose-dependent effect of PKA and PI3K inhibitors on the FSH and TGFβ1-induced progesterone production in rat granulosa cells. Cells were pretreated with vehicle or various doses of PKAI or wortmannin (wort) for 1 h, and then treated with control vehicle or FSH (10 ng/ml) plus TGFβ1 (5 ng/ml) for an additional 48 h. Conditioned media were collected and analyzed for progesterone content using enzyme immunoassay. Each bar represents the mean (±S.E.M.) progesterone production ($n=6$). Different lowercase letters indicate significant differences among treatment groups ($P<0{\cdot}05$).

Cells lysates were prepared as previously described, and ILK kinase activity assay was performed. Cell lysates (300 μg each) were immunoprecipitated with 4 μg mouse monoclonal anti-ILK antibody, overnight at 4 °C. The immune complexes were isolated with protein A/G plus agarose beads overnight at 4 °C, and washed thrice with washing buffer (50 mM HEPES (pH 7), 2 mM $MgCl_2$, 2 mM $MnCl_2$, 200 mM Na_3VO_4, and aprotinin, leupeptin, and pepstatin of 1 μg/ml each). The kinase activity assay was performed using 2 μg Akt1-GST-agarose as the substrate, and 200 μM ATP in the

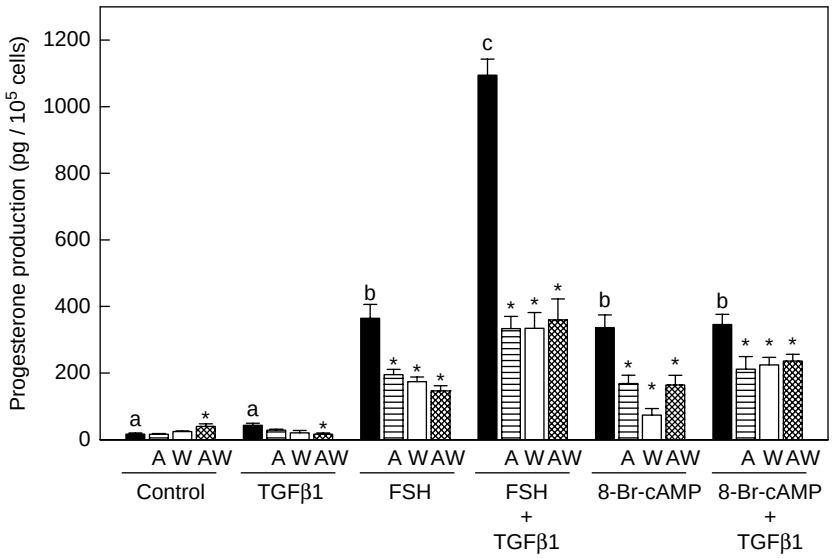


Figure 2 Effect of PKA and PI3K inhibitors on the FSH or 8-Br-cAMP (±TGFβ1)-regulated progesterone production in rat granulosa cells. Cells were pretreated with vehicle, PKAI (20 μM) and/or wortmannin (4 μM) for 1 h, and then treated with control vehicle, FSH (10 ng/ml) or 8-Br-cAMP (1 mM) in the absence or presence of TGFβ1 (5 ng/ml) for an additional 48 h. Conditioned media were collected and analyzed for progesterone content using enzyme immunoassay. Each bar represents the mean (±S.E.M.) progesterone production ($n=6$). Different lowercase letters indicate significant differences among all treatment groups in the absence of inhibitors ($P<0{\cdot}05$). Asterisk indicates a significant difference when compared with the respective control without inhibitors ($P<0{\cdot}05$). A, PKAI; W, wortmannin.

reaction buffer (50 mM HEPES (pH 7), 2 mM $MgCl_2$, 2 mM $MnCl_2$, 200 mM Na_3VO_4, and 200 mM NaF), and allowed to react for 45 min at 37 °C. Phosphorylation of the substrate was detected by immunoblotting using anti-phospho-Akt(Ser473) antibody.

Statistical analysis

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, USA). Also, Student's *t*-test was used to identify significant differences between two treatment groups.

Results

Effects of PKA and PI3K inhibitors on the FSH and TGFβ1-regulated steroidogenesis

PKAI (a PKA inhibitor, 2·5–20 μM) and wortmannin (a PI3K inhibitor, 1–4 μM) each dose dependently suppressed the FSH plus TGFβ1-stimulated progesterone production in rat granulosa cells (Fig. 1). Also, both PKAI (20 μM) and wortmannin (4 μM) alone decreased the FSH- and FSH plus TGFβ1-stimulated progesterone production (Fig. 2). Interestingly, the combined treatment of PKAI and wortmannin exhibited similar inhibitory effects to those of either treatment alone (Fig. 2). It is worth noting that though 8-Br-cAMP (1 mM) mimicked the FSH effect in stimulating progesterone production, TGFβ1 did not augment 8-Br-cAMP effect as it did on the FSH effect (Fig. 2). PKAI and wortmannin decreased the 8-Br-cAMP-stimulated progesterone production (potency, PKAI ≈ PKAI + wortmannin < wortmannin; Fig. 2). Additionally, the involvement of cAMP–GEF signaling pathway in progesterone secretion was examined by employing a cAMP–GEF activator, 8CPT-2Me-cAMP, which effectively discriminates between the cAMP–GEF and the PKA signaling pathways (Enserink *et al.* 2002). Unlike 8-Br-cAMP, 8CPT-2Me-cAMP (10–1000 μM) had no effect on progesterone production either in the absence or presence of TGFβ1 (data not shown).

We next investigated whether PKAI and wortmannin inhibition of FSH- and TGFβ1-stimulated progesterone production in rat granulosa cells may involve the regulation of three key players in steroidogenesis, namely StAR protein, P450scc, and 3β-HSD enzymes. Consistent with our recent studies (Ke *et al.* 2004, 2005), FSH alone increased StAR protein level and had no effect on P450scc enzyme level, and

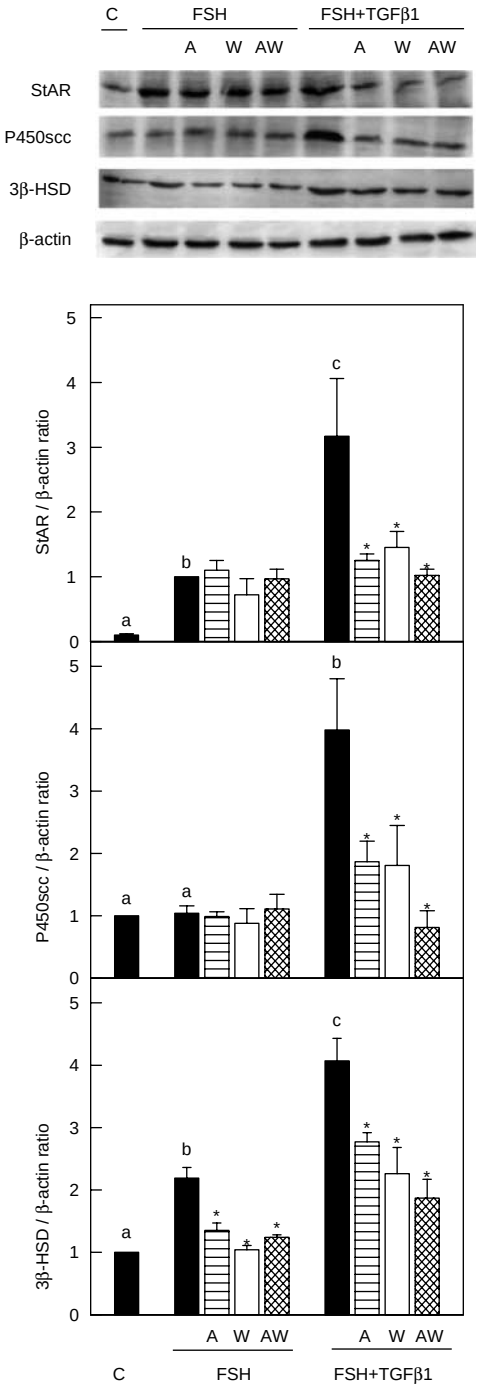


Figure 3 Effect of PKA and PI3K inhibitors on FSH and TGFβ1-regulated StAR protein, P450scc, and 3β-HSD enzyme levels in rat granulosa cells. Cells were cultured as described in Fig. 2. Cell lysates were analyzed by immunoblotting for StAR protein, P450scc, and 3β-HSD enzymes with β-actin used as an internal control. Quantitative analysis of StAR, P450scc, and 3β-HSD in reference to β-actin was 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 (±S.E.M.) relative density (n=3–5). Different lowercase letters indicate significant differences among treatment groups in the absence of inhibitors (P<0·05). Asterisk indicates a significant difference when compared with the respective control without inhibitors (P<0·05). C, control; A, PKAI; W, wortmannin.

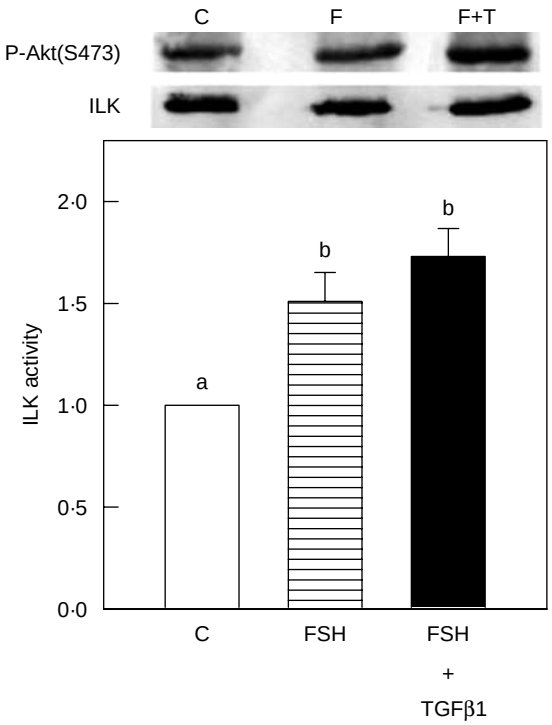


Figure 4 Regulatory effect of FSH and TGFβ1 on ILK kinase activity in rat granulosa cells. Cells were treated with vehicle, FSH (10 ng/ml) or FSH plus TGFβ1 (5 ng/ml) for 30 min. Cell lysates were immunoprecipitated using ILK antibody, this was then subjected to the ILK kinase activity assay using Akt1 as substrate, and phospho-Akt(S473) was detected by immunoblotting. Each bar represents the mean (±S.E.M.) relative density ($n=3$). Different lowercase letters indicate significant differences among all groups ($P<0{\cdot}05$). C, control; F, FSH; T, TGFβ1.

the combined treatment with TGFβ1 further increased the levels of StAR and P450scc enzyme (Fig. 3). Here, for the first time, we demonstrate that TGFβ1 enhanced the FSH-increased 3β-HSD enzyme level (Fig. 3) and PKAI (20 μM) and wortmannin (4 μM) alone suppressed the FSH plus TGFβ1-stimulated increases in the protein level of all the three players (Fig. 3). We also noticed that PKAI and/or wortmannin suppressed the FSH-increased 3β-HSD enzyme level, yet they had no effect on the FSH-increased StAR protein level (Fig. 3). TGFβ1 alone did not affect the content

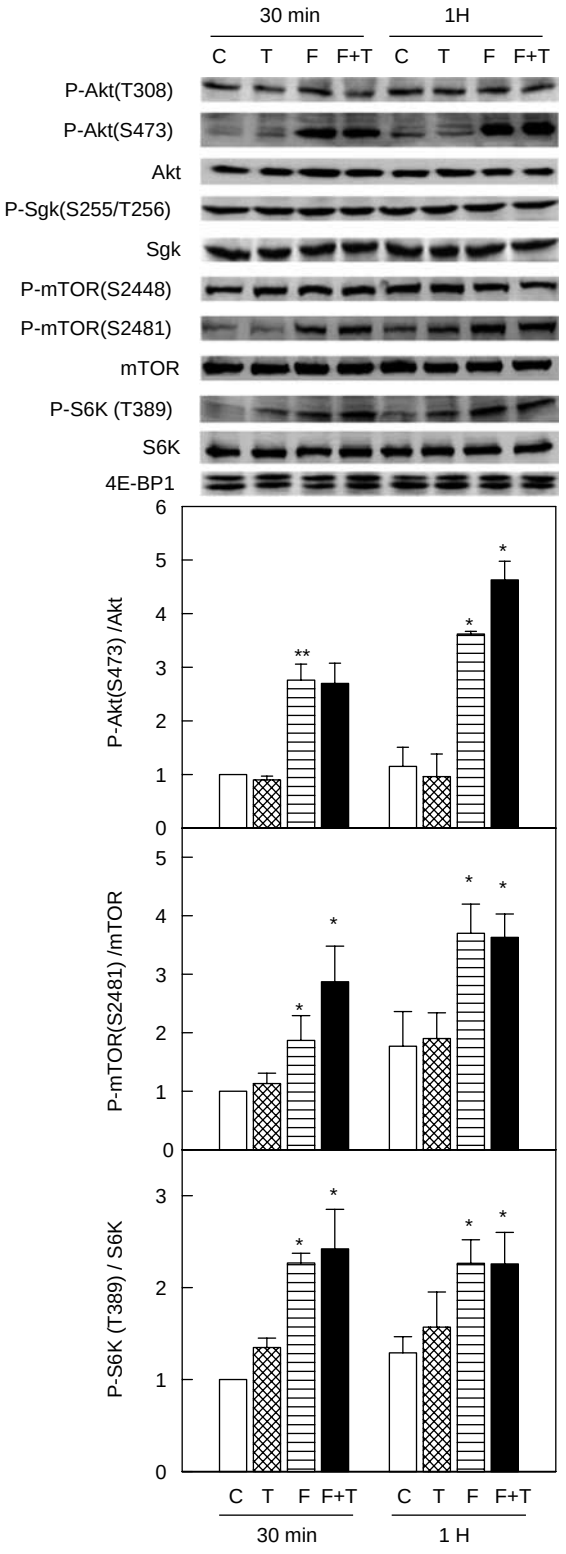
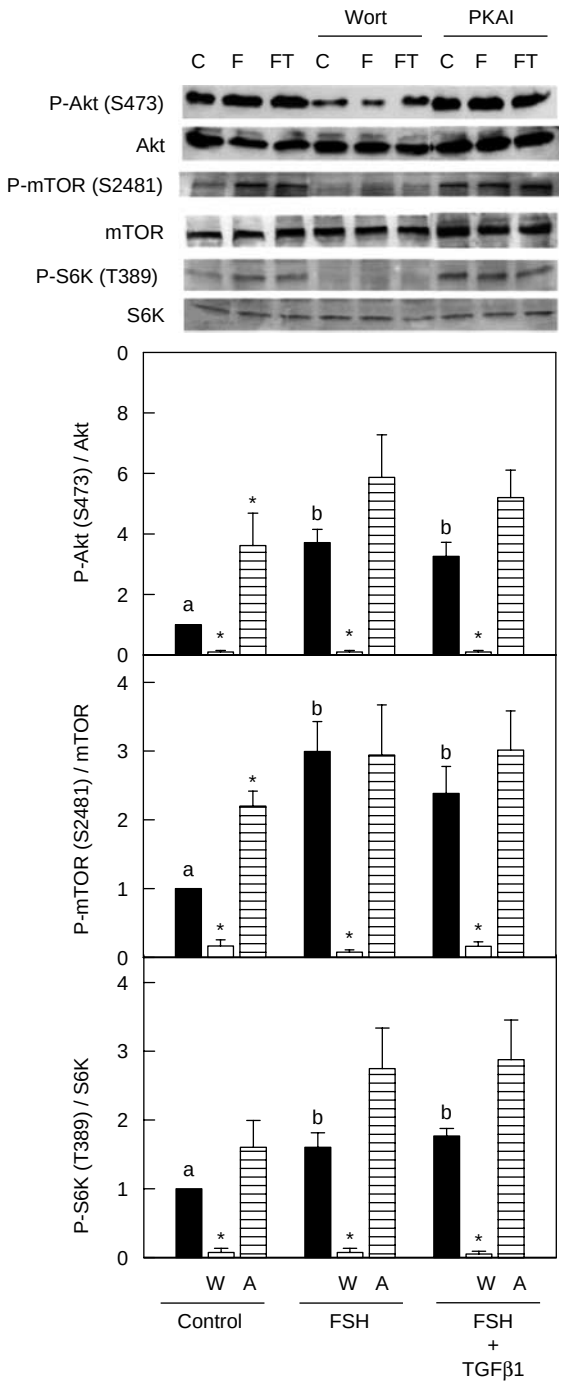


Figure 5 Effect of FSH and TGFβ1 on PI3K signaling pathway mediators in rat granulosa cells. Cells were treated with control vehicle, FSH (10 ng/ml), TGFβ1 (5 ng/ml), or FSH plus TGFβ1 for 30 min or 1 h. Cell lysates were then analyzed by immunoblotting. For the immunoblot of 4E-BP1, the lower band (fast migrating) generally represents the hypo-phosphorylated form, whereas the upper (slower migrating) band represents the hyper-phosphorylated form. Quantitative analysis was performed using two-dimensional scanning densitometry. Relative density ratios were calculated using the 30-min control group value as one. Each bar represents the mean (±S.E.M.) relative density ($n=4$). Asterisk indicates a significant difference when compared with the respective control ($P<0{\cdot}05$). C, control; F, FSH; T, TGFβ1.

of all the three players, and PKAI and wortmannin did not alter their basal levels (data not shown). Consistent with the inhibitory effect on progesterone production, the combined treatment of PKAI and wortmannin exhibited similar suppression on FSH-increased 3β-HSD enzyme level and FSH plus TGFβ1-increased StAR protein, P450scc, and 3β-HSD enzyme levels as those of either treatment alone (Fig. 3).



Regulatory effects of FSH and TGFβ1 on PI3K signaling molecules

To determine the involvement of PI3K signaling pathway in FSH and TGFβ1-stimulated steroidogenesis in rat ovarian granulosa cells, we examined the ILK kinase activity and phosphorylation activation of the PI3K downstream signaling mediators. FSH stimulated ILK kinase activity within 30-min treatment, and the stimulatory effect is similar to that of FSH plus TGFβ1 treatment (Fig. 4). Administration of FSH for 30 min to 1 h increased the phosphorylation of Akt(S473), mTOR(S2481), and S6K(T389), but not that of Akt(T308), Sgk(S255/T256), mTOR(S2448), and 4E-BP1 (Figs 5 and 6). We then chose 1-h treatment for the following experiments. FSH treatment for 1 h also increased the transcription factor phosphorylation of FoxO1(S256), FoxO3a(S253), and CREB(S133) (Fig. 7). The extent of FSH-induced phosphorylation of Akt(S473), mTOR(S2481), and S6K(T389), and transcription factors including FoxO1(S256), FoxO3a(S253), and CREB(S133) was similar to that of FSH plus TGFβ1 treatment (Figs 5–7). The acute induction of FSH (±TGFβ1) on the phosphorylation of PI3K pathway mediators (Akt, mTOR, S6K, FoxO1, and FoxO3a) disappeared at 24-h post-treatment (data not shown).

We further administered wortmannin and PKAI in an attempt to determine the relationship between the PI3K/PKA signaling. Wortmannin (4 μM) dramatically suppressed the basal and FSH (±TGFβ1)-stimulated phosphorylation of Akt(S473), mTOR(S2481), S6K(T389), FoxO1(S256), and FoxO3a(S253) (Figs 6 and 7A). On the other hand, PKAI (20 μM) increased the basal phosphorylation of Akt(S473), mTOR(S2481), FoxO1(S256), and FoxO3a(S253) (Figs 6 and 7A). We also noticed an interesting observation that PKAI only reduced the FSH (±TGFβ1)-stimulated phosphorylation of FoxO3a, and it had no significant effect on that of Akt(S473), mTOR(S2481), S6K(T389), and FoxO1(S256) (Figs 6 and 7A). Consistent with earlier studies (Richards 2001, Enserink *et al.* 2002), administration of FSH activated the PKA pathway as indicated by the increase in the phosphorylation of CREB(S133) and its suppression by PKAI (Fig. 7B). Wortmannin alone had no effect on the phosphorylation of CREB(S133), and surprisingly, PKAI suppressive effect

Figure 6 Effect of PKA and PI3K inhibitors on FSH and TGFβ1-activated PI3K signaling mediators in rat granulosa cells. Cells were pretreated with vehicle, PKAI (20 μM), or wortmannin (4 μM) for 1 h, and then treated with control vehicle, FSH (10 ng/ml) or FSH plus TGFβ1 (5 ng/ml) for an additional 1 h. Cell lysates were then analyzed by immunoblotting. Quantitative analysis was performed using two-dimensional scanning densitometry. Relative density ratios were calculated using the control group value as one. Each bar represents the mean (±S.E.M.) relative density ($n=3$–4). Different lowercase letters indicate significant differences among treatment groups in the absence of inhibitors ($P<0{\cdot}05$). Asterisk indicates a significant difference when compared with the respective control ($P<0{\cdot}05$). C, control; F, FSH; FT, FSH+TGFβ1; W/Wort, wortmannin; A, PKAI.

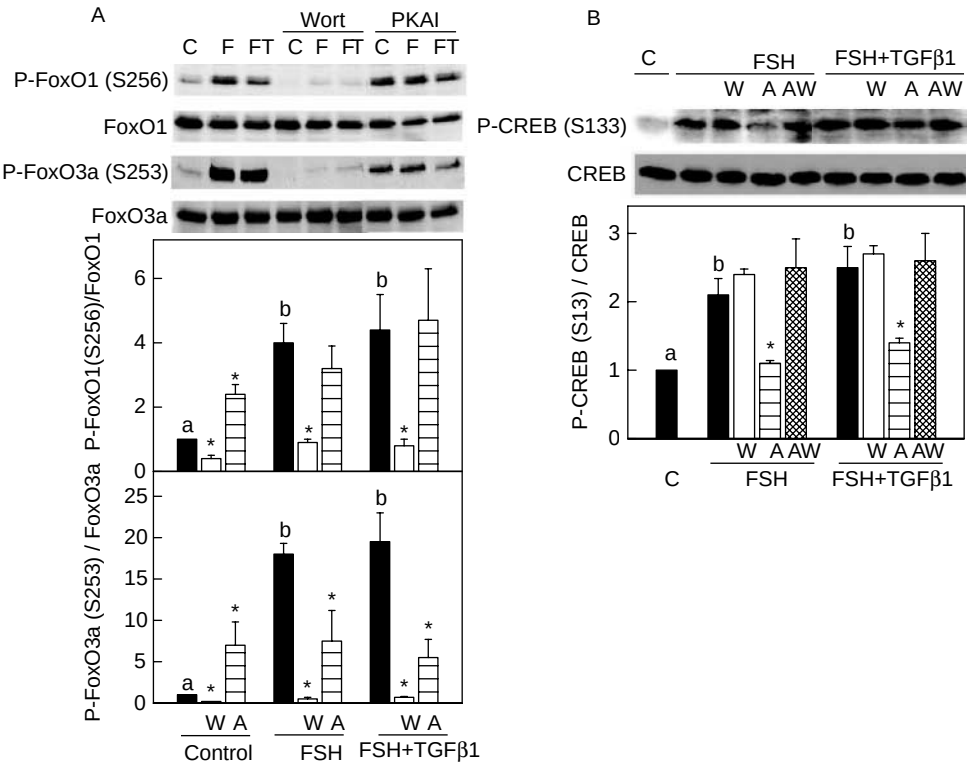


Figure 7 Effect of PKA and PI3K signaling inhibitors on FSH and TGFβ1-regulated transcription factors of PI3K and PKA downstream in rat granulosa cells. Cells were pretreated with vehicle, PKAI (20 μM), and/or wortmannin (4 μM) for 1 h, and then treated with control vehicle, FSH (10 ng/ml) or FSH plus TGFβ1 (5 ng/ml) for an additional 1 h. Cell lysates were analyzed by immunoblotting for (A) phospho-FoxO1(S256):FoxO1, phospho-FoxO3a(S253):FoxO3a and (B) phospho-CREB(S133):CREB ratios. Quantitative analysis was performed using two-dimensional scanning densitometry. Relative density ratio was calculated using the control group value as one. Each bar represents the mean (±S.E.M.) relative density ($n=3$). Different lowercase letters indicate significant differences among treatment groups in the absence of inhibitors ($P<0{\cdot}05$). Asterisk indicates a significant difference when compared with the respective control ($P<0{\cdot}05$). C, control; W, wortmannin; A, PKAI.

disappeared in the presence of wortmannin (Fig. 7B). These results indicate a delicate intertwined regulation of FSH-induced PI3K and PKA signaling in rat granulosa cells.

Involvement of mTOR complex (mTORC) in TGFβ1 enhancement of FSH action

To determine the critical role of mTORC in FSH and TGFβ1-stimulated steroidogenesis in rat granulosa cells, rapamycin (an mTORC1 inhibitor) was used. We demonstrate for the first time that rapamycin (10^{-20} to 10^{-9} M) significantly suppressed the FSH plus TGFβ1-stimulated progesterone production, and rapamycin had no significant effect on FSH action (Fig. 8). Rapamycin at 10^{-9} M, but not 10^{-15} or 10^{-12} M, reduced the FSH (±TGFβ1)-stimulated phosphorylation of S6K(T389) (Fig. 9A). This indicates that only 10^{-9} M rapamycin suppressed the mTORC1 activity. Consistent with progesterone production, rapamycin at doses of 10^{-15} and 10^{-9} M exhibited similar suppressive effect on FSH plus TGFβ1-stimulated increases in the level of StAR protein, P450scc, and 3β-HSD enzymes (Fig. 9B). Conversely, rapamycin exhibited no significant effects on FSH-increased progesterone production and the levels of StAR and 3β-HSD (Figs 8 and 9B).

We then determined the effect of rapamycin on the activation of PI3K and PKA downstream transcription factors, FoxO1, FoxO3a, and CREB. Rapamycin at doses of 10^{-15} to 10^{-9} M had no effect on the phosphorylation of FoxO1(S256) and FoxO3a(S253) (Fig. 10A). Interestingly, rapamycin (10^{-15} to 10^{-9} M) moderately decreased the FSH (±TGFβ1)-stimulated phosphorylation of CREB(S133) (Fig. 10B).

Discussion

The present study demonstrates several original findings regarding FSH and TGFβ1 promotion of ovarian granulosa

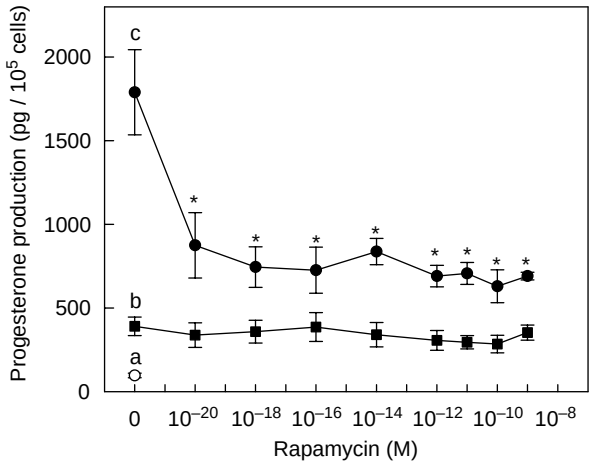


Figure 8 Dose-dependent effect of mTORC1 inhibitor on the FSH and TGFβ1-induced progesterone production in rat granulosa cells. Cells were pretreated with vehicle or various doses of rapamycin for 1 h, and then treated with control vehicle, FSH (10 ng/ml) or FSH plus TGFβ1 (5 ng/ml) for an additional 48 h. Conditioned media were collected and analyzed for progesterone content using enzyme immunoassay. Each point represents the mean (±S.E.M.) progesterone production ($n=6$). Different lowercase letters indicate significant differences among treatment groups in the absence of rapamycin ($P<0{\cdot}05$). Asterisk indicates a significant difference when compared with the respective control ($P<0{\cdot}05$). ○, Control; ■, FSH; ●, FSH+TGFβ1.

cell differentiation. First, TGFβ1 facilitation of FSH-stimulated progesterone production in rat ovarian granulosa cells is mainly attributed to increased protein level of StAR, P450scc, and 3β-HSD enzymes, and the enhancing effects of TGFβ1 were blocked by either PKA inhibitor (PKAI) or PI3K inhibitor (wortmannin; Figs 1–3). And, though FSH increased progesterone production and protein levels of 3β-HSD enzyme and StAR, only the former two FSH effects were sensitive to PKAI and wortmannin (Figs 2 and 3). Furthermore, the combined treatment of PKAI and wortmannin exhibited a similar extent of suppression to that of either treatment alone in FSH- and TGFβ1 plus FSH-stimulated steroidogenesis (Figs 2 and 3). In addition to PKAI, wortmannin also suppressed 8-Br-cAMP-induced progesterone production (Fig. 2). These results suggest a delicate intertwined regulation between cAMP/PKA and PI3K signaling mediators in FSH and TGFβ1 regulation of steroidogenesis in rat granulosa cells. In addition, FSH may act through PI3K- and PKA-independent signaling in regulation of StAR protein level, and this awaits further study. Secondly, we made an interesting observation that PKAI reduced the FSH (±TGFβ1)-increased phosphorylation of FoxO3a(S253) but not FoxO1(S256) (Fig. 7A), and this appears to be independent of Akt activity as FSH (±TGFβ1)-increased Akt(S473) phosphorylation was not blocked by PKAI (Fig. 6). In addition, wortmannin suppressed the FSH (±TGFβ1)-increased phosphorylation of Akt(S473), FoxO3a(S253), and FoxO1(S256) (Figs 6 and 7A). FoxO3a was recently reported to play a critical role in murine ovarian follicle development (Castrillon *et al.* 2003). These results suggest that FSH regulation of granulosa cell function may in part act through PI3K/Akt pathway to modulate the transcription factor activity of FoxO3a and FoxO1. Moreover, FSH may also act through PKA-dependent and Akt-independent signaling to regulate the activity of FoxO3a but not FoxO1. Thirdly, PKAI treatment suppressed the FSH-increased CREB(S133) phosphorylation as expected, but most interesting is that although wortmannin did not alter FSH-increased phospho-CREB(S133) levels, the PKAI suppressive effect disappeared in the presence of wortmannin (Fig. 7B). Since wortmannin targets all classes of PI3K including class III PI3K which is involved in the membrane–vesicle-trafficking system (Wymann *et al.* 2003) and GPCR-coupled PI3Kγ which participates in the receptor endocytosis (Naga Prasad *et al.* 2001), wortmannin may work through multi-mechanisms to modulate cAMP/PKA signaling, and yet enhance the non-cAMP/PKA signal induction of CREB(S133) phosphorylation. The signal discrimination on CREB(S133) phosphorylation between cAMP/PKA and non-cAMP/PKA stimuli may affect the specification of *CREB* target genes (Mayr & Montminy 2001). Also consistent with the previous study (Hillier *et al.* 1994), we have shown that FSH increased estradiol secretion in cultured rat ovarian granulosa cells, and TGFβ1 augmented such action of FSH while TGFβ1 alone had no effect (unpublished data). Figure 11 is a diagram of a proposed model regarding the molecular signaling of FSH and TGFβ1-stimulated steroidogenesis in ovarian granulosa cells.

Akt signaling is a well-established PI3K effector that controls diverse cellular processes, such as survival, metabolism, growth, and localization of transcriptional regulators (Brazil *et al.* 2004, Hanada *et al.* 2004, Woodgett 2005) and the role of Akt in the differentiation of ovarian granulosa cells has also been documented (Gonzalez-Robayna *et al.* 2000, Zeleznik *et al.* 2003, Alam *et al.* 2004). The present study clearly shows that FSH stimulates the phosphorylation of Akt(S473), mTOR(S2481), S6K1(T389), FoxO1(S256), and FoxO3a(S253), but not that of Akt(T308), Sgk(S255/T256), mTOR(S2448), and 4E-BP1 in rat ovarian granulosa cells (Figs 6 and 7A). This indicates that FSH may not affect PDK1 activity in our system as the levels of phospho-Akt(T308) and phospho-Sgk(S255/T256) remained unchanged after FSH treatment. FSH-induced increases of Akt(S473) phosphorylation and its activity as indicated by FoxO1(S256) and FoxO3a(S253) phosphorylation may be mediated by the integrin signal mediator ILK. This is supported by the following evidence. First, this study shows that FSH increased ILK activity in rat granulosa cells (Fig. 4). Secondly, in spite of the controversial role of ILK to Akt(S473) phosphorylation (Brazil *et al.* 2004, Hanada *et al.* 2004, Woodgett 2005), our data agree with ILK conditional knockout and siRNA knockdown studies showing only Akt(S473) phosphorylation but not Akt(T308) phosphorylation was affected (Troussard *et al.* 2003). Finally, a recent report showed that mTORC2 (Rictor–mTOR complex) mediates PI3K-activated

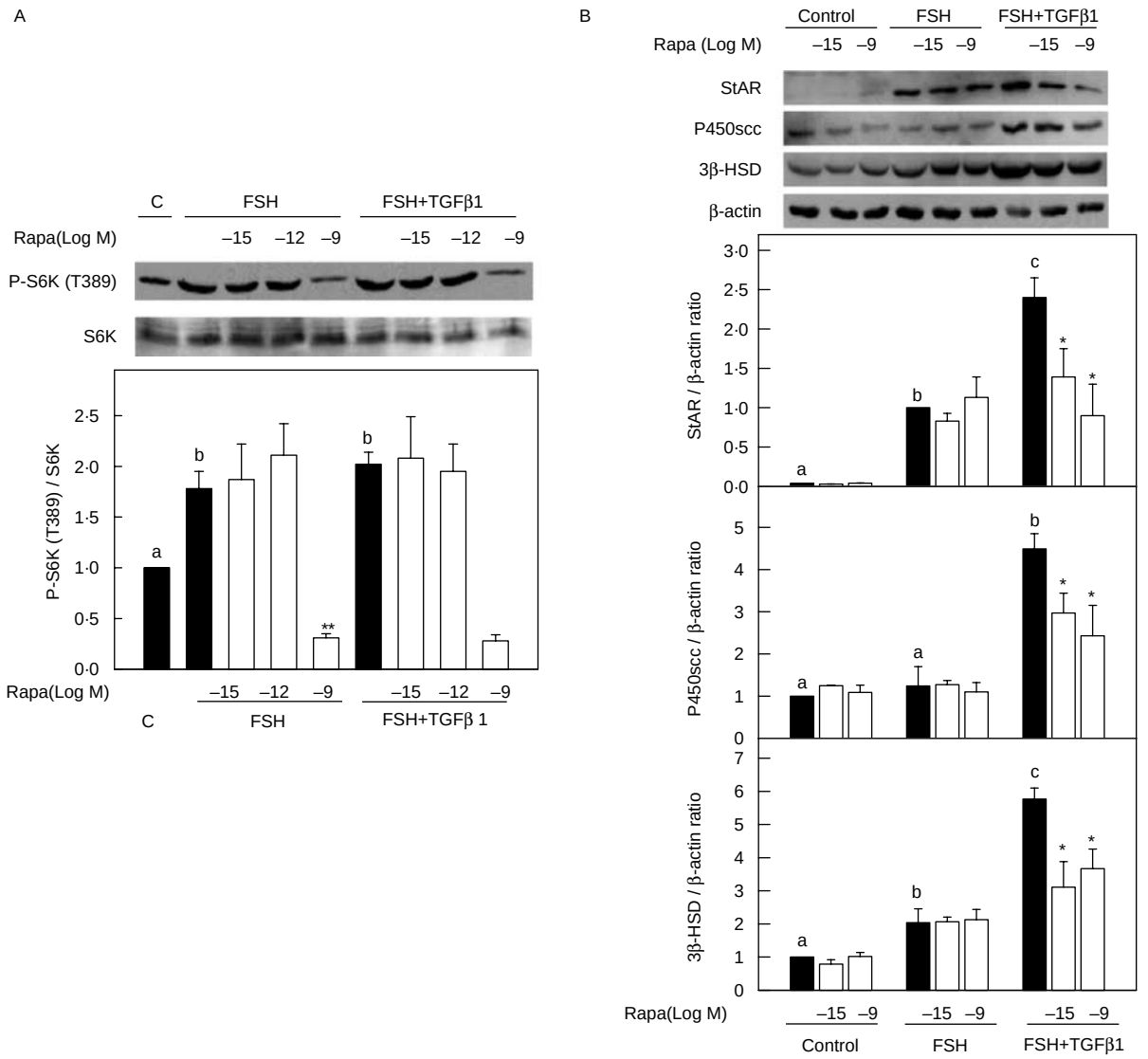


Figure 9 Effect of mTORC1 inhibitor on FSH and TGFβ1-regulated mTORC1 activity and StAR protein, P450scc, 3β-HSD enzyme levels in rat granulosa cells. Cells were pretreated with vehicle, or rapamycin (10^{-15} to 10^{-9} M) for 1 h, and then treated with control vehicle, FSH (10 ng/ml) or FSH plus TGFβ1 (5 ng/ml) for an additional 1 h. (A) Cell lysates were analyzed for P-S6K(T389):S6K ratio, mTORC1 activity. For an additional 48 h, (B) cell lysates were analyzed for StAR protein, P450scc, and 3β-HSD enzymes with β-actin used as an internal control. Quantitative analysis was performed using two-dimensional scanning densitometry. Relative density ratios were calculated using the FSH-treated group value or the control group value as one. Each bar represents the mean (±S.E.M.) relative density ($n=3$). Different lowercase letters indicate significant differences among treatment groups in the absence of rapamycin ($P<0{\cdot}05$). Asterisk indicates a significant difference when compared with the respective control ($P<0{\cdot}05$). Rapa, rapamycin.

Akt(S473) phosphorylation. However, in contrast to ILK, Rictor siRNA knockdown affected both Akt(S473) and Akt(T308) phosphorylation (Sarbassov *et al.* 2005). Another PDK1 target is S6K1. Following S6K1(T389) phosphorylation, S6K1 requires T207 phosphorylation by PDK1 to have full activity (Pullen *et al.* 1998). S6K1(T389) was documented to be phosphorylated by mTORC1 (Raptor–mTOR complex). Phosphorylation of mTOR(S2448) as an *in vivo* target of S6K1 (Chiang & Abraham 2005, Holz & Blenis 2005) remained unchanged by FSH treatment in rat granulosa cells. Our results demonstrate a lack of stimulated-PDK1 activity by FSH treatment in rat granulosa cells, and this may implicate a distinct signal repertoire of different classes of PI3K in activation of downstream target, such as Akt and S6K1 (Vanhaesebroeck *et al.* 2005, Wymann & Marone 2005).

FSH has been reported to stimulate the expression of differentiation markers of rat granulosa cells (LH receptor, inhibin-α, microtubule-associated protein 2D, and PKA type IIβ regulatory subunit) via Akt-mTORC1 signaling that

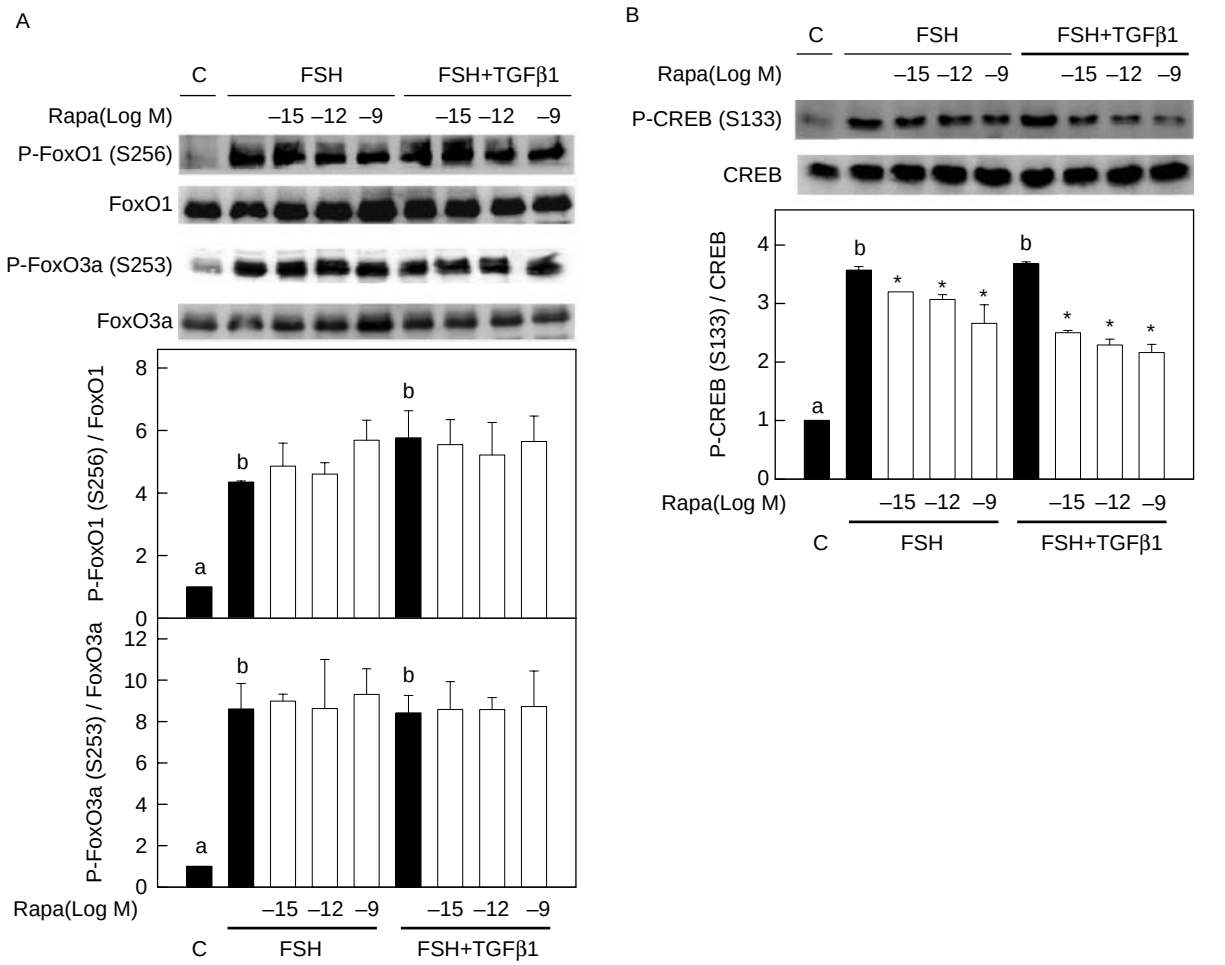


Figure 10 Dose-dependent effect of mTORC1 inhibitor on FSH and TGFβ1-activated PI3K and PKA signaling transcription factors in rat granulosa cells. Cells were pretreated with vehicle or various doses rapamycin (10^{-15} to 10^{-9} M) for 1 h, and then treated with control vehicle, FSH (10 ng/ml) or FSH plus TGFβ1 (5 ng/ml) for an additional 1 h. (A) Cell lysates were analyzed for P-FoxO1(S256):FoxO1 and P-FoxO3a(S253):FoxO3a; (B) cell lysates were analyzed for P-CREB(S133):CREB by immmunoblotting. Quantitative analysis was performed using two-dimensional scanning densitometry. Relative density ratios were calculated using the control group value as one. Each bar represents the mean (±S.E.M.) relative density (n=3–4). Different lowercase letters indicate significant differences among treatment groups in the absence of inhibitors ($P<0{\cdot}05$). Asterisk indicates a significant difference when compared with the respective control ($P<0{\cdot}05$). C, control; Rapa, rapamycin.

regulates translation and activation of HIF1α (Alam *et al.* 2004). In order to specify the role of mTORC1 in FSH-stimulated progesterone production in rat granulosa cells, rapamycin (a well-characterized inhibitor of mTORC1) was used in our system. Surprisingly, we found that rapamycin over a broad range of concentrations (10^{-20} to 10^{-9} M) exhibited no effect on FSH-stimulated steroidogenesis, and that rapamycin only suppressed the TGFβ1 enhancing effect of FSH-stimulated steroidogenesis as indicated by protein levels of StAR, P450scc, and 3β-HSD enzymes, and progesterone production (Figs 8 and 9B). Rapamycin at the concentration of 10^{-9} M did effectively block S6K1(T389) phosphorylation (Fig. 9A), yet it was without effect on FSH-increased progesterone production and protein levels of StAR and 3β-HSD enzyme. Conversely, the rapamycin suppressive effect on TGFβ1 enhancement of FSH action displayed an unusual broad effective range even at the concentrations that have no effect on S6K1(T389) phosphorylation (Figs 8 and 9). Therefore, this study indicates that TGFβ1 enhancement of FSH action is specific and hypersensitive to rapamycin blockade and is independent of mTORC1 signaling.

The present study further suggests that TGFβ1 enhancement of FSH-stimulated steroidogenesis extends beyond FSH-activated PKA and PI3K signaling as supported by the following evidence. First, TGFβ1 did not augment 8-Br-cAMP stimulation of progesterone production as it did on FSH effect in rat granulosa cells (Fig. 2). Secondly, in

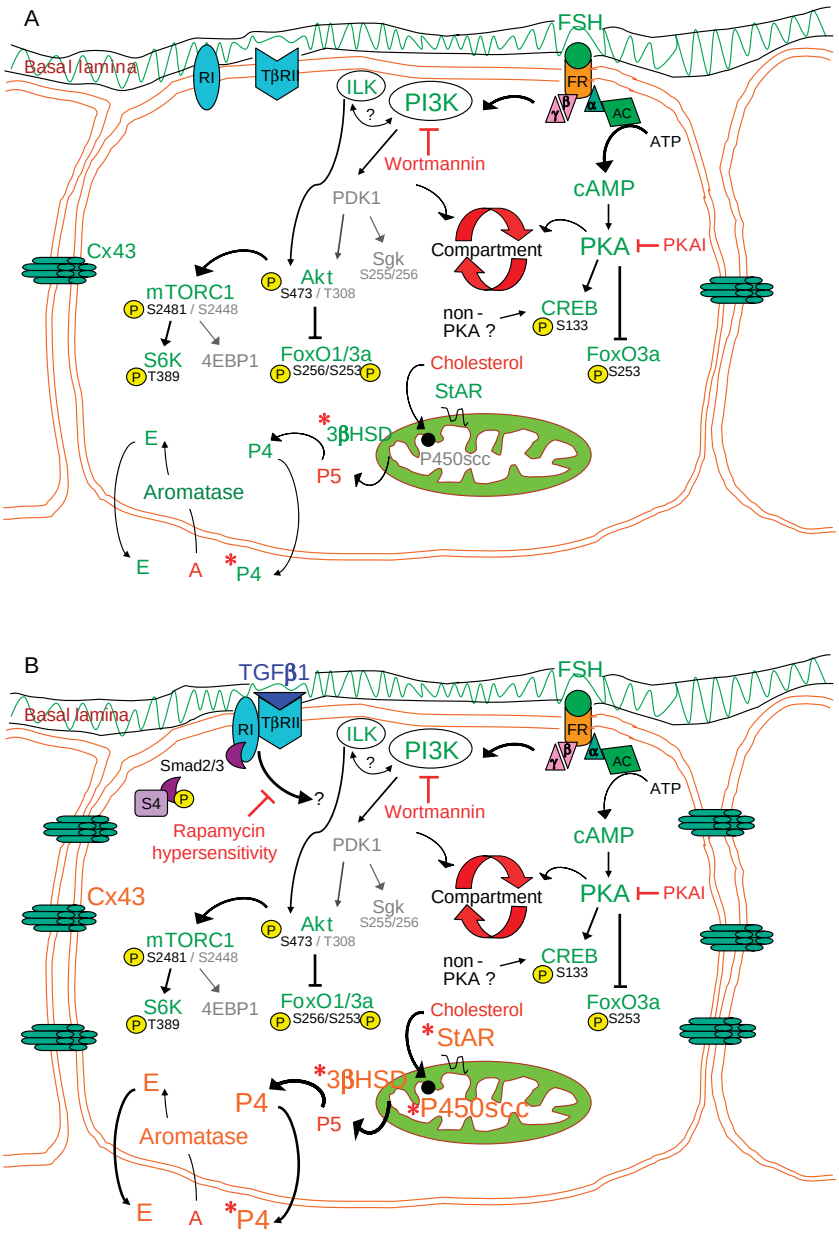


Figure 11 A proposed model regarding the molecular signaling of FSH and TGFβ1-stimulated steroidogenesis in rat ovarian granulosa cells. (A) FSH action alone, and (B) FSH plus TGFβ1 action. FSH can activate cAMP/PKA and PI3K/ILK pathways, and we propose that both the pathways may mutually interact through compartmentalization, and PI3K and ILK may interact and act in parallel via close proximity near membrane structure. Furthermore, TGFβ1 enhancement effect of FSH-stimulated steroidogenesis exhibits rapamycin hypersensitivity. Molecules in green indicate FSH induction of cAMP/PKA and PI3K/ILK signaling molecules and transcription factors (activation or phosphorylation), and FSH-increased levels of steroidogenic molecules. Molecules in gray indicate those not induced by FSH treatment alone. Molecules in orange indicate TGFβ1 enhancement of FSH-increased levels. Asterisks (*) in red indicate molecules suppressed by inhibitors (PKAI, wortmannin, and rapamycin). AC, adenylate cyclase; α/β/γ, G-protein subunits; FR, FSH receptor; TβRI, TGFβ type I receptor; RII, TGFβ type II receptor; Cx43, connexin43; P, phosphate; P5, pregnenolone; P4, progesterone; E, estrogen.

contrast to the TGFβ effect on IGF-I signaling (Danielpour & Song 2006), our study shows that TGFβ1 did not alter FSH-induced phosphorylation activation of cAMP–PKA signaling mediator (CREB) and PI3K signaling mediators (ILK, Akt, mTOR, S6K, and FoxOs) in rat granulosa cells (Figs 5–7). In addition, TGFβ1 was reported not to alter FSH-increased cAMP levels in rat granulosa cells (Inoue *et al.* 2002). TGFβ1 augmentation of FSH-stimulated steroidogenesis may signal

through site(s) close to the level of FSH receptor activation other than downstream signal crosstalking, such as an interaction of Smad3–Akt (Conery *et al.* 2004, Remy *et al.* 2004, Song *et al.* 2006) or Smad3–PKA regulatory subunit (Zhang *et al.* 2004). Early reports have shown that TGFβ1 attenuated FSH-induced downregulation of FSH receptors, and increased the expression of FSH receptors in granulosa cells (Gitay-Goren *et al.* 1993, Dunkel *et al.* 1994). In addition, recent progress indicates that the molecular mechanism of receptor endocytosis is pivotal on signal transduction (Miaczynska *et al.* 2004, Le Roy & Wrana 2005). The ratio of two main endocytic routes, clathrin-mediated endocytosis and raft/caveolar endocytosis, has been proposed to organize and coordinate the duration, intensity, integration, and compartmentalization of the core variable in cell signaling to determine the net outcome of signaling events (Polo & Di Fiore 2006). TGFβ signaling has been demonstrated to be regulated through clathrin-mediated endocytosis (signaling) and raft/caveolar endocytosis (degradation) in a ligand-independent manner (Di Guglielmo *et al.* 2003). Also, rapamycin is known to bind to FKBP12 leading to the release of its inhibition on TGFβ receptor type I in a ligand-independent manner (Chen *et al.* 1997). Whether rapamycin-induced activation of TGFβ receptor type I affects the ratio of clathrin-mediated endocytosis to raft/caveolar endocytosis is unknown. The hypersensitive and mTORC1-independent effect of rapamycin on the TGFβ1 facilitation of FSH-stimulated steroidogenesis in rat granulosa cells is worthy of further investigation. Clathrin-mediated endocytosis is also critical for GPCR signaling (Marchese *et al.* 2003); therefore, the cross-modulation between distinct receptors in trafficking routes may provide a possible mechanism for TGFβ1 facilitation of FSH-stimulated steroidogenesis in rat granulosa cells.

Altogether, this study demonstrates a delicate interplay between cAMP/PKA and PI3K signaling in FSH and TGFβ1 regulation of steroidogenesis in rat ovarian granulosa cells. Furthermore, we demonstrate for the first time that TGFβ1 acts in a rapamycin-hypersensitive and mTORC1-independent manner in augmenting FSH-stimulated steroidogenesis in rat granulosa cells.

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