Follicle-stimulating Hormone Activation of Hypoxia-inducible Factor-1 by the Phosphatidylinositol 3-Kinase/AKT/Ras Homolog Enriched in Brain (Rheb)/Mammalian Target of Rapamycin (mTOR) Pathway Is Necessary for Induction of Select Protein Markers of Follicular Differentiation*

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We sought to elucidate the role of AKT in follicle-stimulating hormone (FSH)-mediated granulosa cell (GC) differentiation. Our results define a signaling pathway in GCs whereby the inactivating phosphorylation of tuberin downstream of phosphatidylinositol (PI) 3-kinase/AKT activity leads to Rheb (Ras homolog enriched in brain) and subsequent mTOR (mammalian target of rapamycin) activation. mTOR then stimulates translation by phosphorylating p70 S6 kinase and, consequently, the 40 S ribosomal protein S6. Activation of this pathway is required for FSH-mediated induction of several follicular differentiation markers, including luteinizing-hormone receptor (LHR), inhibin-α, microtubule-associated protein 2D, and the PKA type IIβ regulatory subunit. FSH also promotes activation of the transcription factor hypoxia-inducible factor-1 (HIF-1). FSH-stimulated HIF-1 activity is inhibited by the PI 3-kinase inhibitor LY294002, the Rheb inhibitor FTI-277 (farnesyltransferase inhibitor-277), and the mTOR inhibitor rapamycin. Finally, we find that the FSH-mediated up-regulation of reporter activities for LHR, inhibin-α, and vascular endothelial growth factor is dependent upon HIF-1 activity, because a dominant negative form of HIF-1α interferes with the up-regulation of these genes. These results show that FSH enhances HIF-1 activity downstream of the PI 3-kinase/AKT/Rheb/mTOR pathway in GCs and that HIF-1 activity is necessary for FSH to induce multiple follicular differentiation markers.

The anterior pituitary hormone follicle-stimulating hormone (FSH)1 provides the key stimulus that promotes proliferation and differentiation of the ovarian follicle to the pre-ovulatory phenotype (1, 2). FSH binding to the FSH receptor, a G protein-coupled receptor found in females exclusively on the surface of granulosa cells (GCs), leads to Gα-stimulated activation of adenylyl cyclase and increased production of the second messenger, cAMP (3). FSH has been shown to activate multiple signaling pathways in GCs. We have demonstrated that FSH stimulates the activation of protein kinase A (PKA) and have identified the histones H1 and H3 as PKA targets (4, 5). Additionally, we have recently shown that FSH signals via PKA to increase extracellular regulated kinase (ERK) signaling in response to FSH by turning off an inhibitory ERK phosphatase (6). Furthermore, we and others have shown that FSH activates AKT downstream of phosphatidylinositol 3 (PI 3)-kinase (6–11).

FSH signaling results in increased transcription of a number of characteristic follicular differentiation markers, including the steroidogenic enzymes side-chain cleavage cytochrome P-450 and aromatase cytochrome P-450 (12), the luteinizing hormone receptor (LHR) (3), inhibin-α (13), the signaling intermediates microtubule-associated protein 2D (MAP2D) (14), and the PKA type IIβ regulatory subunit (RIIβ) (15). FSH also stimulates antrum formation and angiogenesis in the peripheral follicle thecal cells mediated in part by the vascular endothelial growth factor (VEGF) (16, 17). Our investigation into the mechanisms by which two of these FSH targets, MAP2D and RIIβ, are up-regulated in response to FSH revealed that their up-regulation at the protein level is blocked by the PI 3-kinase inhibitors LY294002 and wortmannin. FSH-mediated PI 3-kinase activation is also necessary for the expression of the cartilage link protein (8) in GCs and for optimal transferrin secretion and lactate production by Sertoli cells (9). In support of a role for PI 3-kinase/AKT activity in GC differentiation, insulin-like growth factor-1 (IGF-1), which activates the PI 3-kinase/AKT pathway via the IGF-1 receptor (18), synergizes with FSH in GCs to increase the expression of LHR (19), inhibin-α (20), and side-chain cleavage cytochrome P-450 and is sufficient for the expression of 3β-hydroxysteroid dehydrogenase/isomerase (21). The importance of AKT activation by PI growth factor-1; LHR, luteinizing hormone receptor; Luc, luciferase; MAP2D, microtubule-associated protein 2D; mTOR, mammalian target of rapamycin; p70S6K, p70 S6 kinase; PI 3-kinase, phosphatidylinositol-3-kinase; PKA, protein kinase A; PMSG, pregnant mare serum gonadotropin; Rheb, ras homolog enriched in brain; RIIβ, PKA type IIβ regulatory subunit; SF-1, steroidogenic factor-1; TK, thymidine kinase; VEGF, vascular endothelial growth factor.
growth factor-2 (IGF-2) and p21 (29). Our results show that FSH enhances HIF-1 activity by the PI 3-kinase/AKT-dependent activation of mTOR and that HIF-1 activity is necessary for the up-regulation of such FSH target genes as VEGF, inhibin-α, and the LHR.

**EXPERIMENTAL PROCEDURES**

**Materials**—A luciferase reporter of a truncated 24-mer containing 18 bp from the phosphoglycerate kinase promoter with the HRE (5′-TGTCAGCTCTTGACCGACTTCAATTG3′; HRE underlined) and an 8-bp linker sequence followed by a 50-bp minimal thymidine kinase (TK) promoter in a pGL2-basic backbone vector (Promega), HRE (3′)-TK-Luc, was described previously (30). The pA5 reporter construct for the rat inhibin-α-subunit gene (−2021 to +68) was described previously (31). A 2.1-kb fragment of the 5′-flanking region of the LHR (−2082 to +1) gene was excised with XhoI and BamHI digestion of a described previously LHR-DAX transgene (32) and subcloned into pGL3 basic vector (Promega) digested with XhoI and BglII. A VEGF-luciferase reporter construct (−2274 to +379) and an HIF-1α dominant negative expression vector, pCEP4/HIF-1αDN (33), were kindly provided by Dr. Gregg L. Semenza. The following items were purchased: ovine FSH (oFSH-19) was from the National Hormone and Pituitary Agency of the NIDDK, National Institutes of Health; PDB0809, H89, rapamycin, farnesyltransferase inhibitor-277 (FTI-277), and LY294002 were from Calbiochem; ethanol chloride, MG115, and anti-MAP2 antibody were from Sigma; the anti-HIF-1α (H1a67) and the anti-HIF-1β antibodies were from Novus Biologicals (Littleton, CO); anti-tubulin phosphorylated on Thr-1462, anti-S6 ribosomal protein phosphorylated on Ser-235/236, anti-P70S6 phosphorylated on Thr-389, anti-AKT phosphorylated on Ser-473, anti-4EBP1 phosphorylated on Ser-65, and anti-mTOR, and anti-AKT was from Cell Signaling Technologies; anti-CREB antibody was from Upstate Biotechnology; and the anti-PKA RIβ subunit antibody was from BD Transduction Laboratories.

**Animals**—Sprague-Dawley rats (Charles River Laboratories, Inc.) were housed at Northwestern University animal care facilities and maintained in accordance with guidelines for the care and use of laboratory animals by protocols approved by the Northwestern University Animal Care and Use Committee.

**Pregnant Mare Serum Gonadotropin (PMSG) Treatment and Tissue Extract Preparation**—Immature female rats (26–27 days old) were injected subcutaneously with 25 IU of PMSG. Ovaries were harvested at the indicated times post PMSG injection and subjected to tissue extract preparations. Ovarian extracts were prepared as described previously (34). Protein concentrations were measured by the method of Lowry et al. (35) using crystalline bovine serum albumin as a standard. The samples were prepared for SDS-PAGE by suspension in SDS-containing sample buffer followed by heat denaturation (100 °C, 5 min). Western blotting was performed as described below.

**Granulosa Cell Culture and Western Blotting**—GCs were isolated from ovaries of 26- to 27-day-old Sprague-Dawley rats primed with subcutaneous injections of 1.5 mg of estradiol-17β on days 23–25 to promote the growth of pre-antral follicles. Cells were plated on fibronectin-coated (BD Biosciences) 33-mm plastic dishes at a density of 3 × 10^4 cells/dish in Dulbecco’s modified Eagle’s/F12 serum-free medium supplemented with 1 %, 10 ng/ml IGF-1, and 100 µg/ml streptomycin and treated with the indicated additions 20 h after plating. Treatments were terminated by aspirating medium and rinsing cells once with phosphate-buffered saline. Total cell extracts were collected by scraping cells in SDS sample buffer (36) followed by heat denaturation (100 °C, 5 min). Western blotting was performed as described below.

**Transfection and Luciferase Assays**—GCs were plated in 12-well plates at 10^5 cells/well in Dulbecco’s modified Eagle’s/F12 medium, 1 %, 10 ng/ml IGF-1, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were washed with PBS and transfected with various promoter-luciferase constructs (0.5 µg DNA/well) and the indicated expression constructs (0.05 µg of DNA per well) using LipofectAMINE 2000 (Invitrogen) as a transfection reagent.
described previously (37). Briefly, cells were incubated in the transfection mixture at 37 °C for 4–5 h, after which Dulbecco’s modified Eagle’s F12 serum-free medium supplemented with 1 nM estradiol-17β, 100 units/ml penicillin, and 100 μg/ml streptomycin was added to the cells. Approximately 20 h after transfection, cells were treated as indicated. Cells were lysed and analyzed for luciferase activity using a luminometer as described previously (37). Luciferase activity for each measurement was normalized to protein content using 280-nm absorbance measurements (38). Data are presented as the mean ± S.E. of triplicate samples. All transfection experiments were repeated three times with similar results unless otherwise indicated. Results were analyzed using Student’s t test (p < 0.05 denoted by **) (39). The percentage of inhibition in the presence of various inhibitors was calculated as (1 – (fold induction \( \text{FSH} + \text{inhibitor} / \text{fold induction FSH} \)) × 100.

RESULTS

FSH Stimulates Activation of AKT, Inactivation of Tuberin, and Activation of p70\text{S6k} and S6 Ribosomal Protein—AKT kinase activation requires the sequential phosphorylation of Thr-308 by phosphoinositide-dependent kinase 1 and Ser-473 by an unidentified kinase (40). It has been previously established that FSH stimulation of rat ovarian GCs leads to the activation of AKT phosphorylation (6, 7). We confirmed that AKT is activated in GCs under our culture conditions in response to FSH using an antibody specific for AKT phosphorylated at Ser-473. As shown in Fig. 2A, AKT phosphorylation was readily detected at 10 min post FSH treatment of GCs, had further increased by 1 h, and had subsided to undetectable levels by 4 h post FSH treatment. Total AKT is shown as a protein loading control.

Tuberin, a component of the tuberous sclerosis complex, has been identified in various cellular models as a target of AKT kinase (41, 42). In its active, unphosphorylated state, tuberin prevents signaling of the downstream kinase mammalian target of rapamycin, mTOR (43). AKT phosphorylation of tuberin at Thr-1462 inhibits its activity, thus preventing it from impeding mTOR signaling (44). We therefore determined whether FSH stimulated the phosphorylation of tuberin in GCs. Using a Thr(P)-1462-specific anti-tuberin antibody, our results demonstrate that FSH treatment of GCs promoted phosphorylation of tuberin by 10 min, which was further increased at 1 h post FSH treatment and waned moderately by 4 and 8 h, but not, however, to the extent observed with AKT phosphorylation (Fig. 2A).

Because FSH promotes the phosphorylation of tuberin and thus the expected activation of mTOR, we explored whether previously identified targets of mTOR kinase activity, namely p70\text{S6k} (45) and 4E-binding protein-1 (4EBP-1) (46, 47) were phosphorylated in GCs in response to FSH. Phosphorylation of p70\text{S6k} at Thr-389 downstream of mTOR activity is indicative of p70\text{S6k} activation (48, 49). When active, p70\text{S6k} phosphorylates the S6 ribosomal protein. S6 is a component of the 40 S ribosome that promotes the translation of a subset of mRNAs containing a 5′-oligopyrimidine tract (50). Furthermore, inhibitory phosphorylation of 4EBP-1 at multiple sites downstream of mTOR, including Ser-65 (51), prevents 4EBP-1 from sequestering the eukaryotic translation initiation factor 4E, which promotes cap-dependent translation (52). Thus, phosphorylation of both p70\text{S6k} and 4EBP-1 leads to the activation of the translational machinery.

Western blotting with an antibody recognizing phosphorylated p70\text{S6k} (Thr(P)-389) demonstrated that FSH treatment of GCs resulted in p70\text{S6k} phosphorylation that was maximal at 1 h and remained elevated 4 h post FSH treatment (Fig. 2A). The phosphorylation of S6 by p70\text{S6k} in GCs treated with FSH was monitored with a Ser(P)-235/Ser(P)-236-specific anti-S6 antibody. S6 phosphorylation was observed at 10 min, elevated by 1 h, and remained detectable at 4 h after FSH treatment (Fig. 2A). Additionally, the inhibitory phosphorylation of 4EBP-1 at Ser-65 was detected by 1 h and increased by 2 h post FSH treatment (Fig. 2B). Taken together, these data demonstrate that FSH stimulation of GCs leads to the inactivating phosphorylation of tuberin, an inhibitor of mTOR, as well as to the phosphorylation of p70\text{S6k}, S6, and 4EBP-1, previously characterized targets downstream of mTOR kinase activity. These results suggest that FSH stimulates the translational machinery in GCs by activating S6 ribosomal protein and eukaryotic translation initiation factor 4E.

FSHR-dependent Phosphorylation of AKT, Tuberin, p70\text{S6k}, and S6 Occurs via cAMP-mediated Stimulation of PI 3-Kinase Activity—FSH signals to activate G\(_{\alpha}\)s and, consequently, adenylyl cyclase, promoting the synthesis of cAMP (3). In the following experiments, we investigated whether FSH-dependent signaling to AKT, tuberin, p70\text{S6k}, and S6 could be mimicked by the direct pharmacological adenylyl cyclase activator forskolin or the cell permeable cAMP analogue, 8-Clomephylthio (8-CPT)-cAMP. The results demonstrated that the treatment of GCs for 1 h with forskolin or 8-CPT-cAMP stimulated the phosphorylation of AKT, tuberin, p70\text{S6k}, and S6 (Fig. 3A, lanes 1–4). Therefore, cAMP is sufficient to bring about the phosphorylation of AKT, tuberin, p70\text{S6k}, and S6.
FSH-stimulated AKT activation in GCs has previously been demonstrated to require PI 3-kinase activity (7). We therefore used the PI 3-kinase inhibitor, wortmannin, to determine whether the phosphorylation of tuberin, p70\textsuperscript{S6k}, and S6, also required activation of PI 3-kinase. Wortmannin treatment of GCs prevented FSH from eliciting maximal phosphorylation of AKT, tuberin, p70\textsuperscript{S6k}, and S6 (Fig. 3A, compare lane 2 versus lane 6). Additionally, the phosphorylation of these proteins in response to forskolin (Fig. 3A, compare lane 3 versus lane 7) and 8-CPT-cAMP (Fig. 3A, compare lane 4 versus lane 8) was prevented.

**Fig. 3.** FSH-stimulated inactivation of tuberin and activation of p70\textsuperscript{S6k} and the S6 ribosomal protein in GCs occurs downstream of PI 3-kinase, Rheb, and mTOR activation. **A.** GCs were pretreated with or without 100 nM wortmannin or 10 \( \mu \)M H89 for 1 h and then left untreated (CON) or treated with 50 ng/ml FSH, 10 \( \mu \)M forskolin, or 1 mM 8-CPT-cAMP for 1 h. Results are representative of two similar experiments. **B.** GCs were pretreated with and without 100 nM rapamycin for 15 min and then left untreated (CON) or treated with 50 ng/ml FSH for 1 h. Results are representative of three separate experiments. **C.** GCs were pretreated with and without 10 \( \mu \)M FTI-277 for 18 h then left untreated (CON) or treated with 50 ng/ml FSH for 1 h. Results are representative of three separate experiments. **D.** GCs were pretreated with or without 10 \( \mu \)M H89 for 1 h and then left untreated (CON) or treated with 50 ng/ml IGF-1 for 1 h. Western blots of total cell extracts were probed with the indicated antibodies. Phospho-specific (ph) antibodies are described under “Experimental Procedures.” AKT is used as a loading control for all experiments.
abrogated by wortmannin. These results thus show that in GCs, PI 3-kinase activity downstream of cAMP is required for the phosphorylation of AKT, tuberin, p70^65k, and S6.

**Rheb Activation of mTOR Downstream of AKT Leads to p70^65k and S6 Activation**—In the following experiments, we used the mTOR inhibitor rapamycin, a macrolide that inhibits mTOR in a selective manner (53), to verify that phosphorylation of p70^65k at Thr-389 and the subsequent activation of S6 occur downstream of mTOR activation in FSH-stimulated GCs. Treatment of GCs with rapamycin did not affect FSH-mediated phosphorylation of either AKT or tuberin but abrogated the phosphorylation of p70^65k and its target S6 (Fig 3B, compare lanes 1 and 2 versus lanes 3 and 4). These data place mTOR activation downstream of AKT phosphorylation/activation and tuberin phosphorylation/inactivation, but upstream of p70^65k and S6 phosphorylation/activation.

Recent studies have identified the mechanism by which the AKT substrate tuberin regulates mTOR activity. The small G-protein Rheb has been identified as an activator of mTOR, while tuberin has been identified as a negative regulator of Rheb by acting as its GTP-ase activating protein (54–56). In the following experiments, we sought to determine whether Rheb activity was necessary for mTOR signaling downstream of FSH in GCs. To ascertain the effects of inhibiting Rheb activity, we treated GCs with the farnesyltransferase inhibitor, FTI-277, because optimal Rheb function requires C-terminal farnesylation (56, 57). Treating GCs with the Rheb inhibitor FTI-277 did not affect the FSH-mediated phosphorylation of AKT or tuberin; however, FTI-277 reduced phosphorylation of p70^65k and S6 by 54 and 63%, respectively (Fig. 3C, compare lanes 1 and 2 versus lanes 3 and 4). This places Rheb downstream of AKT phosphorylation/activation and tuberin phosphorylation/inactivation, but upstream of mTOR signaling to p70^65k and S6.

The protein kinase inhibitor H89 is reported to inhibit p70^65k with an IC_{50} of 80 nM, and other kinases including PKA at higher concentrations (53). We therefore used H89 to confirm that the signaling pathway initiated in GCs by FSH and resulting in S6 phosphorylation requires p70^65k activity. As shown in Fig. 2A, 10 μM H89 inhibited S6 phosphorylation but did not block the phosphorylation of AKT, tuberin, or p70^65k (Fig. 3A, compare lane 1–4 versus lanes 9–12). The inability of H89 to inhibit AKT phosphorylation in FSH-stimulated GCs

![Fig. 4. PMSG treatment of rats results in activation of AKT, inactivation of tuberin, and activation of p70^65k and the S6 ribosomal protein.](image)

**Fig. 5. Follicular differentiation markers require signaling via PI 3-kinase/AKT/mTOR pathway.** In panel A, GCs were pretreated with and without 12.5 μM LY294002 for 1 h and then left untreated (CON) or treated with 50 ng/ml FSH for 1 h. In panel B, GCs were pretreated with and without 100 nM rapamycin for 15 min and then treated with 50 ng/ml FSH for 1 h. Western blots of total cell extracts were probed with the indicated antibodies. Phospho-specific (ph) antibodies are described under “Experimental Procedures.” AKT is used as a loading control. Results are representative of two separate experiments.

![Fig. 6. LHR-Luc reporter assay.](image)

**Fig. 6. LHR-Luc reporter assay.** The FSH-treated GCs were transfected with LHR-α-Luc (CON) or treated with 50 ng/ml FSH for 6 h. Results are representative of two separate experiments.
has been reported previously (7). These results confirm that the S6 protein is phosphorylated downstream of p70 S6k in GCs treated with FSH, forskolin, or cAMP.

Because IGF-1 signaling via the IGF receptor has been shown in other cellular models to activate the PI 3-kinase/AKT/mTOR pathway and not PKA (58), we confirmed the site of H89 inhibition at p70S6k in IGF-1-stimulated GCs. As seen in Fig. 2D, IGF-1 treatment of GCs for 1 h led to the phosphorylation of AKT, tuberin, p70S6k, and S6. Consistent with the results seen with FSH-treated GCs, pretreatment of GCs with H89 did not affect IGF-stimulated phosphorylation of AKT, tuberin, or p70S6k, but did abrogate S6 phosphorylation (Fig. 3D, compare lanes 1 and 2 versus lanes 3 and 4). Thus, at 10 μM, H89 is an effective inhibitor of p70S6k activity in GCs.

Taken together, these data define a novel signaling pathway in GCs whereby AKT-stimulated inactivation of tuberin results in increased Rheb and, consequently, mTOR activities leading to p70S6k and S6 activation.

Tuberin Inactivation and p70S6k and S6 Activation Occur in Response to PMSG in Vivo—We also tested whether this pathway was activated in vivo in response to PMSG (an FSH source). Rats were injected subcutaneously with PMSG, and the phosphorylation of key intermediates was evaluated at indicated times post PMSG treatment by Western blotting of detergent-soluble ovarian extracts. Results demonstrated that AKT, tuberin, p70S6k, and S6 were phosphorylated at 8 h post PMSG treatment and, with the exception of p70 S6k, remained phosphorylated at 48 h after PMSG treatment (Fig. 4). Thus the AKT/mTOR pathway leading to S6 phosphorylation is also activated in vivo in ovarian cells by gonadotropin stimulation.

PI 3-Kinase/AKT/Rheb/mTOR Pathway Is Necessary for the Up-regulation of Genes Transcriptionally Up-regulated in Response to FSH—RlIβ (15), MAP2D (14), LHR (3), and inhibin-α (13) are transcriptionally up-regulated in response to FSH and comprise markers of follicular differentiation. The dependence of LHR and inhibin-α up-regulation on AKT signaling has recently been demonstrated using a dominant negative AKT adenovirus (22). To test whether the activation of the Rheb/mTOR pathway downstream of PI 3-kinase/AKT is involved in the up-regulation of these genes, we used either reporter as-

FIG. 6. FSH stimulation of GCs results in increased HIF-1α protein levels occurring by increased translation. In panel A, GCs were either untreated (CON) or treated with 50 ng/ml FSH for 4 h in the presence of either 150 μM CoCl2 or 30 μM MG115, as indicated, to prevent degradation of HIF-1α. Western blots of total cell extracts were probed with anti-HIF-1α antibody. CREB is used as a loading control. Results are representative of three separate experiments. In panel B, GCs were treated with 50 ng/ml FSH for the indicated times. Western blots of total cell extracts were probed with anti-HIF-1β antibody. Results are representative of two separate experiments. AKT is used as a loading control. In panel C, GCs were pretreated for 1 h with 8 μM actinomycin D (ACT D) or 30 μM cyclohexamide (CHX) where indicated. GCs were then left untreated (CON) or treated with 50 ng/ml FSH for 4 h in the presence or absence of 150 μM CoCl2 as indicated. CREB is used as a loading control. Results are representative of three separate experiments.

FIG. 7. FSH stimulation of GCs leads to the induction of HRE (3)-TK-Luc and VEGF-Luc activity that is inhibited by the PI 3-kinase inhibitor LY294002. GCs were transfected with promoter-Luc constructs as described under “Experimental Procedures.” In panels A and B, GCs transfected with HRE (3)-TK-Luc (A) or VEGF-Luc (B) were left untreated (CON) or treated with 50 ng/ml FSH for 6 h. For LY294002 treatments, cells were pretreated with 12.5 μM LY294002 for 1 h prior to control (CON) or FSH treatment. The percentage of inhibition in the presence of inhibitor is calculated as stated under “Experimental Procedures” and indicated at the right of each figure. Values are expressed as the mean ± S.E. of triplicates and are representative of three separate experiments. Student’s t test was used for compared values; ** indicates significant difference with p ≤ 0.05.
**HIF-1α Protein Levels and HIF-1 Activity are Up-regulated by FSH in GCs**—HIF-1 is a heterodimeric transcription factor whose activity is up-regulated in other cellular models downstream of the PI 3-kinase/AKT/mTOR pathway (24, 25, 59–62). HIF-1 consists of a typically regulated subunit, HIF-1α, and an unregulated subunit, HIF-1β (63). In the following experiments, we examined whether FSH stimulation of GCs affected HIF-1α and HIF-1β protein levels. Because the half-life of HIF-1α is ~5 min under normoxic conditions (64), we used either the proteasome inhibitor MG115 (65) or CoCl₂ (66), both of which inhibit degradation of HIF-1α, to investigate whether FSH stimulation of GCs promoted the accumulation of HIF-1α relative to untreated cells. GCs were treated with FSH for 4 h in the presence of either one of these inhibitors. In Fig. 6A, Western blotting of total cell extracts demonstrated that FSH induced the accumulation of an ~120-kDa protein reactive with an anti-HIF-1α antibody, relative to untreated cells. The levels of HIF-1β did not change relative to control levels in response to 2 or 4 h of FSH treatment (Fig. 6B) even in the presence of reagents that prevent HIF-1α degradation (data not shown).

In contrast to hypoxia, which stabilizes HIF-1α, growth factor/hormone stimulation may increase HIF-1α translation (23–25). We therefore ascertained in GCs whether the FSH-stimulated increase in the HIF-1α protein, detected when HIF-1α degradation is blocked, is sensitive to transcriptional or translation inhibitors. Results show that the accumulation of HIF-1α induced by 4 h of FSH treatment of GCs was unaffected when the cells were pretreated for 1 h with the transcriptional inhibitor actinomycin D (Fig. 6C). However, when the cells were pretreated for 1 h with the translational inhibitor cycloheximide we were unable to detect any protein in either untreated or FSH-treated cells (Fig. 6C). These data suggest that the FSH-stimulated increase in the HIF-1α protein is largely dependent upon de novo translation and not transcription. For the increase in HIF-1α protein in FSH-stimulated GCs to be functionally relevant, FSH should also increase HIF-1 activity. Therefore, in the following experiments, we tested whether HIF-1 activity was up-regulated in GCs in response to FSH treatment. To ascertain whether FSH stimulated HIF-1 transcriptional activity, GCs were transfected with a luciferase reporter containing three copies of the HRE from the phosphoglycerate kinase promoter (HRE (3)-TK-Luc). FSH stimulation of GCs for 6 h resulted in a ~12-fold increase in the activity of the HRE (3)-TK-Luc reporter relative to the untreated control cells (Fig. 7A). Accumulating evidence suggests that VEGF, which is an established HIF-1 target in other systems (33), is up-regulated in GCs in response to FSH (16, 17). Using a VEGF-luciferase construct (VEGF-Luc) (33), we found that stimulation of GCs for 6 h resulted in a ~6-fold stimulation of VEGF-Luc activity relative to untreated cells (Fig. 7B). These results demonstrate that FSH enhances HIF-1 activity in GCs. Because the PI 3-kinase pathway has been implicated in the up-regulation of HIF-1 activity, we investigated whether HIF-1 activation in GCs is dependent on the PI 3-kinase/AKT/mTOR pathway. Addition of the PI 3-kinase inhibitor LY294002 significantly inhibited the FSH-dependent activation of both HRE (3)-TK-Luc and VEGF-Luc reporter activities by 72 and 85%, respectively (Fig. 7, A and B). These data are in accord with
reports that growth factor or hormone stimulation of HIF-1 activity requires the activation of the PI 3-kinase pathway (24, 25, 59–62).

Next, the Rheb inhibitor FTI-277 and the mTOR inhibitor rapamycin were used to determine whether Rheb and mTOR activities are necessary for FSH activation of HIF-1 transcriptional activity. Reporter assays using the HRE (3)-TK-Luc as well as the VEGF-Luc were performed in the presence and absence of the inhibitors. Treatment with FTI-277 inhibited FSH-mediated up-regulation of HRE (3)-TK-Luc and VEGF-Luc activities 45 and 48%, respectively (Fig. 8, A and B). Similarly, rapamycin inhibited the FSH-mediated up-regulation of HRE (3)-TK-Luc and VEGF-Luc activities by 44 and 60%, respectively (Fig. 8, C and D). These data demonstrate that both Rheb and mTOR activation downstream of PI 3-kinase/AKT are necessary to up-regulate HIF-1 activity.

**Expression of a Dominant Negative HIF-1α Interferes with FSH-mediated Up-regulation of Follicular Differentiation Markers**—Finally, we sought to determine whether HIF-1 transcriptional activity was necessary for FSH-stimulated induction of VEGF in GCs. A dominant negative construct of HIF-1α lacking the DNA binding domain, as well as the transactivation domain of HIF-1α (33), was transfected in GCs along with VEGF-Luc. Dominant negative HIF-1α inhibited the FSH-mediated up-regulation of VEGF by 54% (Fig. 9A). We then sought to determine whether inhibiting HIF-1 activity prevented the up-regulation of the follicular differentiation markers inhibin-α and LHR. Co-transfection of the HIF-1α dominant negative construct inhibited the FSH-mediated up-regulation of the inhibin-α and LHR reporter activities 55 and 69%, respectively (Fig. 9, B and C). These data demonstrate that FSH-stimulated HIF-1 activity is necessary for the up-regulation of VEGF, LHR, and inhibin-α.

**DISCUSSION**

FSH-stimulated differentiation of GCs in ovarian follicles is obligatory for ovulation and the resumption of oocyte meiosis (1, 2). The differentiation response consists, in part, of the induction of a number of proteins including the LHR (3), inhibin-α (13), MAP2D (14), and RIIβ (15), as well as the proteins necessary for steroidogenesis, gap junction, and antrum formation (67, 68). In addition to the important role for PKA in chromatin remodeling (5) and CREB phosphorylation (69) as well as ERK activation (6), recent studies from a number of laboratories have implicated the PI 3-kinase/AKT pathway in FSH-stimulated GC differentiation (7, 22).

In this report, we demonstrate that FSH promotes the PI 3-kinase-dependent activation of p70S6k by mTOR. mTOR is emerging in many cellular models as a key regulator of protein synthesis, cell growth, and cell survival (70). mTOR integrates signals from growth factor receptors and G-protein coupled receptors to stimulate mRNA translation by activating p70S6k and, consequently, activating the 40 S ribosomal protein S6 and inhibiting the translational repressor 4EBP-1 (71, 72). mTOR is activated upon FSH treatment by a pathway that is mediated by cAMP and requires the activation of PI 3-kinase/AKT, the inactivation of tuberin, and the activation of Rheb, as summarized in Fig. 1. Our results in GCs show that mTOR activation in response to FSH is indeed necessary for the induction of LHR, inhibin-α, MAP2D, and RIIβ, proteins that characterize GCs of the preovulatory phenotype. Taken together, these results indicate that in GCs, FSH-stimulated mTOR activity is obligatory for GC differentiation.

We show that FSH stimulation of the PI 3-kinase/AKT/mTOR pathway results in the stimulation of HIF-1 activity. We also detect induction of HIF-1α protein in FSH-treated GCs when its degradation is inhibited, consistent with reports that HIF-1α is rapidly degraded under normoxic conditions (64). The binding partner for HIF-1α, HIF-1β, is constitutively expressed in GCs, and its levels are not affected by FSH. HIF-1α and HIF-1β dimerize to regulate the transcription of a number of genes (29). Our results show that FSH enhances HIF-1 activity, as detected by using a minimal HRE reporter (30) as well as a reporter for VEGF, a well known HIF-1 target (33). To our knowledge, these data are the first report that HIF-1 activity is up-regulated by FSH in GCs. To further assess the role of HIF-1 transcriptional activity in the FSH-mediated induction of follicular differentiation markers, we used a HIF-1α dominant negative that has been shown previously to inhibit, in a dose dependent manner, the HIF-1-mediated activity of reporters containing the VEGF (33) and erythropoietin (73) promoter sequences. The dominant negative HIF-1α blocked the FSH-dependent induction of VEGF, inhibin-α, and LHR in GCs, indicating that HIF-1 activity is critical for the up-regu-
luation of these genes. The importance of HIF-1 activity in the follicular maturation process is further supported by the observation that the conditional knockout of HIF-1α in mice results in a sub-fertile phenotype (74).

Although our results demonstrate that the expression of a dominant negative HIF-1α reduces the FSH-mediated increase of LHR and inhibin-α reporter activities, in this report we do not address the mechanism(s) of HIF-1 action in GCs. There are several possible mechanisms by which HIF-1α may promote transcription of LHR and inhibin-α. For example, HIF-1 could directly bind to HREs in LHR or inhibit-α promoters to increase transcription of these genes. Searching the promoter sequences of both LHR and inhibit-α revealed the presence of putative HREs in each of their promoters (75). Functional analyses of these sites are currently underway in our laboratory. HIF-1 may also be an important component of a multi-protein complex necessary for the transcriptional regulation of LHR and inhibit-α. Indeed, direct interactions of steroidogenic factor-1 (SF-1), CREB, and associated coactivators such as the CREB-binding protein induce transactivation of the inhibit-α gene (31). In Leydig cells, Sp1 and Sp3 have been shown to transactivate the LHR gene (76). In various cell types, HIF-1 has been shown to interact with other transcription factors such as Sp1 (77) and Smad (78) and to recruit the CREB-binding protein as part of a multi-protein complex to transactivate target genes (79). Studies are underway to elucidate possible interactions between HIF-1 and transcription factors known to regulate FSH-responsive genes in GCs. Alternatively, the role of HIF-1 in LHR and inhibit-α induction may be to promote the expression of transcriptional activators that, in turn, act at specific sites on the LHR and inhibit-α promoters.

In addition to these possible mechanisms by which HIF-1 could regulate the expression of inhibit-α and LHR, it is also well known that inhibit-α and LHR are not coincidentally regulated by FSH in GCs. An association between phosphorylated histone H3 and the inhibit-α promoter is detected by 1 h post FSH (5), and increased inhibit-α mRNA is detected by 10 h post FSH (80). Although we do not know whether FSH stimulates a similarly rapid association of phospho-histone H3 and the LHR promoter, increased LHR mRNA is not detected until 36 h post FSH (81). These results indicate, as discussed above, that distinct factors must be involved in producing the discrete chronological expression pattern observed for these two genes. The relatively rapid induction of inhibit-α most likely can be attributed to the rapid regulation of both CREB and SF-1 activities by FSH-stimulated phosphorylation events (5, 82), followed by their interactions with the CREB-binding protein and, in some manner, with HIF-1. However, the mechanism by which FSH regulates the activities of Sp1 and Sp3 and perhaps those of newly synthesized transcriptional activators, in association with or in response to HIF-1 and leading to the delayed regulation of LHR expression, is not understood. Recruitment of various cofactors to HIF-1 may also contribute to the different timing of inhibit-α versus LHR expression (83). Although the expression of IIβ and MAP2D requires mTOR activity, we do not yet know if they are regulated by HIF-1 in GCs. The IIβ promoter contains a putative HRE (84), but the MAP2D promoter region has not yet been identified. Studies are underway to determine whether HIF-1 regulates expression of these two FSH targets.

HIF-1 activity is also necessary for the up-regulation of VEGF reporter activity. VEGF, a recently identified FSH target (17), has two well characterized HREs in its promoter (33), and it is possible that the up-regulation of VEGF by FSH occurs through HIF-1 interaction directly with these elements. Recent reports emphasize the importance of VEGF to follicle develop-

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