Role of the Phosphatidylinositol-3-Kinase and Extracellular Regulated Kinase Pathways in the Induction of Hypoxia-Inducible Factor (HIF)-1 Activity and the HIF-1 Target Vascular Endothelial Growth Factor in Ovarian Granulosa Cells in Response to Follicle-Stimulating Hormone

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FSH stimulation of granulosa cells (GCs) results in increased hypoxia-inducible factor (HIF)-1α protein levels and HIF-1 activity that is necessary for up-regulation of certain FSH target genes including vascular endothelial growth factor. We report that the role of the phosphatidylinositol (PI)-3-kinase/AKT pathway in increasing HIF-1α protein in FSH-stimulated GCs extends beyond an increase in mammalian target of rapamycin-stimulated translation. FSH increases phosphorylation of the AKT target mouse double-minute 2 (MDM2); a phosphomimetic mutation of MDM2 is sufficient to induce HIF-1 activity. The PI3-kinase/AKT target forkhead box-containing protein O subfamily 1 (FOXO1) also effects the accumulation of HIF-1α as evidenced by the ability of a constitutively active FOXO1 mutant to inhibit the induction by FSH of HIF-1α protein and HIF-1 activity. Activation of the PI3-kinase/AKT pathway in GCs by IGF-I is sufficient to induce HIF-1α protein but surprisingly not HIF-1 activity. HIF-1 activity also appears to require a PD98059-sensitive protein (kinase) activity stimulated by FSH that is both distinct from mitogen-activated ERK kinase1/2 or 5 and independent of the PI3-kinase/AKT pathway. These results indicate that FSH-stimulated HIF-1 activation leading to up-regulation of targets such as vascular endothelial growth factor requires not only PI3-kinase/AKT-mediated activation of mammalian target of rapamycin as well as phosphorylation of FOXO1 and possibly MDM2 but also a protein (kinase) activity that is inhibited by the classic ERK kinase inhibitor PD98059 but not ERK1/2 or 5. Thus, regulation of HIF-1 activity in GCs by FSH under normoxic conditions is complex and requires input from multiple signaling pathways. (*Endocrinology* 150: 915–928, 2009)

FSH acting via the G protein-coupled FSH receptor provides the primary stimulus leading to maturation of the ovarian follicle from a preantral to preovulatory state (1–3). FSH binding to its receptor results in increases in cAMP levels and the sub- sequent activation of protein kinase A leading to activation of the cAMP response element binding protein, chromatin remodeling via histone H3 modifications, and activation of the ERK1/2 via inactivation of an ERK protein-tyrosine phosphatase (4–8). The

Abbreviations: ChIP, Chromatin immunoprecipitation; C(t), cycle threshold; DMSO, dimethylsulf- oxide; E, estradiol-17β; EGF, epidermal growth factor; E/PS, E/penicillin and streptomycin; FOXO1, forkhead box-containing protein O, subfamily 1; GC, granulosa cell; GSK, glycogen synthase kinase; HIF, hypoxia-inducible factor; HRE, hypoxia response element; MDM2, mouse double-minute 2; MEK, mitogen-activated ERK kinase; mTOR, mammalian target of rapamycin; PI3-kinase, phos- phatidylinositol-3-kinase; PMSG, pregnant mare serum gonadotropin; p70 S6-kinase, 70-kDa ri- bosomal S6 protein kinase; raptor, regulatory associated protein of mTOR; RHEB, ras homolog enriched in brain; SDS, sodium dodecyl sulfate; TK, thymidine kinase; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

FSH-mediated increase in cAMP also results in up-regulation of the phosphatidylinositol-3 (PI3)-kinase/AKT pathway (9, 10).

Recent reports have demonstrated the centrality of signaling downstream of PI3-kinase/AKT for the induction of multiple follicular differentiation markers (9, 11, 12). We have described two FSH-regulated pathways downstream of PI3-kinase/AKT and begun to elucidate their contributions to the follicular maturation process. First, we have shown that in a proliferating model of granulosa cell (GC) development downstream of FSH and activin (13), the PI3-kinase/AKT target forkhead box O, subfamily 1 (FOXO1) inhibits GC proliferation by serving as a trans-acting repressor of *cyclin D2* (officially *Ccnd2*) transcription and inhibits GC differentiation by preventing the up-regulation of *steroidogenic factor-1* (*Nr5a1*), *inhibin-α* (*Inha*), *aromatase cytochrome P-450* (*Cyp19a1*), and *epiregulin* (*Ereg*). Therefore, the PI3-kinase/AKT-mediated phosphorylation/inhibition of FOXO1 is required to relieve FOXO1's inhibitory influence on GC proliferation and differentiation (13).

Second, we reported that the AKT target tuberin (protein product of the *Tsc2* gene) is phosphorylated by FSH in a PI3-kinase-dependent manner (9) at a site shown to correlate with inhibition of its GTPase activating protein activity on the small G protein ras homolog enriched in brain (RHEB), resulting in increased GTP bound/active RHEB (14, 15). A downstream effector of RHEB, mammalian target of rapamycin (mTOR; officially FRAP1) (16), is then activated in GCs. Activation of mTOR was detected by monitoring the phosphorylation of two of its downstream targets, the 70-kDa ribosomal S6 protein (p70 S6-) kinase and eukaryotic translation initiation factor-4E binding protein (9), events that have been shown to promote cap-dependent translation (17). We identified hypoxia inducible-factor (HIF)-1α as one target of FSH-mediated translational up-regulation downstream of the PI3-kinase/AKT/mTOR pathway (9), a pathway that has been shown to up-regulate HIF-1α in other cell types (18–20).

HIF-1α together with the constitutively expressed HIF-1β comprise the heterodimeric transcription factor HIF-1 (21). HIF-1α is best known for its up-regulation in response to hypoxic conditions that prevent its proteosomal degradation by an E3 ubiquitin ligase complex that contains the von Hippel-Lindau (VHL) tumor suppressor protein (22). HIF-1α can also be up-regulated under normoxic conditions in response to growth factors that enhance protein synthesis via the PI3-kinase pathway (23). Upon recruitment of the cAMP response element binding protein (CREB) binding protein/p300 to its C terminus (24), HIF-1 binds to consensus hypoxia-response elements (HRE; core -ACGTG-) in target genes characteristically activated under reduced oxygen concentrations, such as erythropoietin, glucose transporters, and various glycolytic enzymes, as well as in target genes that promote increased vascularity including the vascular endothelial growth factor (VEGF) receptor *FLT-1* and *VEGF* (22). We found that FSH treatment induced HIF-1 activity in GCs using a minimal HRE reporter. Using a dominant-negative HIF-1α construct, we identified *inhibin-α*, *LH receptor* (*Lhr*, officially *Lhcgr*), and *VEGF* as HIF-1 targets in FSH-stimulated GCs under normoxic conditions (9).

VEGF has been characterized as an FSH target (25) and shown to increase in expression with increasing follicle size (26, 27). Furthermore, inhibition of VEGF signaling is reported to prevent pregnant mare serum gonadotropin (PMSG)-stimulated antrum formation and steroidogenesis as well as thecal angiogenesis in mice (28). Despite the increased expression of VEGF by GCs during follicular maturation, the GC compartment remains avascular until after ovulation occurs. Emerging evidence suggests that VEGF may play a cytoprotective, autocrine role in GCs by preventing apoptosis (29). There are also reports that suggest that the misregulation of ovarian VEGF during ovulation induction can lead to ovarian hyperstimulation syndrome, a potentially fatal complication (30, 31).

In this report we sought to further elucidate the signaling pathways in GCs that are necessary for HIF-1 activity and the resulting induction of VEGF. HIF-1 activity was assessed using either a minimal HIF promoter-luciferase reporter or the VEGF-luciferase reporter, or chromatin immunoprecipitation (ChIP) assays with the VEGF promoter. Our results show that multiple targets of PI3-kinase signaling in addition to mTOR-stimulated translation contribute to the up-regulation of HIF-α protein and/or activity. These include the PI3-kinase/AKT target proteins FOXO1 and mouse double-minute 2 (MDM2). However, up-regulation of HIF-1α protein by IGF-I-mediated stimulation of the PI3-kinase/AKT pathway is not sufficient to induce HIF-1 activity in GCs. Additional signaling downstream of a protein whose activity is inhibited by the classic mitogen-activated ERK kinase (MEK)1/2 inhibitor PD98059 is necessary for HIF-1 activation, but this protein is neither MEK1/2 nor MEK5. Taken together, these results show that regulation of HIF-1 activity in GCs by FSH under normoxic conditions is complex and requires input from multiple signaling pathways.

Materials and Methods

Materials

A luciferase reporter of a trimerized 24-mer containing 18 bp from the phosphoglycerate kinase promoter with the HRE (5′-tgtcacgtcctg-cacgactctagt-3′, 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, Madison, WI), HRE-(3)-TK-Luc, was previously described (32). A VEGF-luciferase reporter construct (−2274 to +379) was kindly provided by Dr. Gregg L. Semenza (Johns Hopkins School of Medicine, Baltimore, MD) (33). Wild-type and S166D human (H)-MDM2 expression vectors were previously described (34). Constitutively active R4F mitogen-activated MEK-1 (also known as MKK1) was kindly provided by Dr. Natalie Ahn (University of Colorado at Boulder, Boulder, CO) (35). Recombinant human epidermal growth factor (EGF) was from Intergen (Purchase, NY). oFSH-19 was purchased from the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases (Torrance, CA); PD98059, rapamycin, and LY294002 were from Calbiochem (San Diego, CA); $CoCl_2$ and anti-ERK5 antibody (E1523) were from Sigma (St. Louis, MO); PD184352 was kindly provided by Dr. Simon Cook (Babraham Institute, Cambridge, UK); anti-HIF-1α (H1α67) antibody and anti-HIF-1β were from Novus Biologicals (Litteton, CO); anti-mTOR, anti-AKT, anti-phospho-MDM2 (Ser166), anti-phospho-GSK3α/β (Ser21/9), anti-phospho-ERK5 antibody (Thr218/Tyr220), and anti-phospho-ERK1/2 antibody (Thr202/Tyr204) were from Cell Signaling

Technologies (Beverly, MA); anti-FOXO1 antibody, anti-MDM2, and anti-VEGF were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

Sprague Dawley rats (Charles River Laboratories, Inc., Portage, MI) were housed either at Northwestern University or Washington State University animal care facilities and maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals by protocols approved by the Northwestern University or Washington State University Animal Care and Use Committees, respectively.

PMSG treatment and tissue extract preparation

Immature female rats (26–27 d old) were injected sc with 25 IU of PMSG. Ovaries were harvested at the indicated times after PMSG injection and subjected to tissue extract preparations, as described previously (9). Protein concentrations were measured by the method of Lowry *et al.* (36) using crystalline BSA as a standard. The samples were prepared for SDS-PAGE by suspension in sodium dodecyl sulfate (SDS)-containing sample buffer followed by heat denaturation (100 C, 5 min). Western blotting was performed as described below.

GC culture and Western blotting

GCs were isolated from ovaries of 26-d-old Sprague Dawley rats primed with sc injections of 1.5 mg of estradiol-17β (E) in 0.1 ml propylene glycol on d 23–25 to promote growth of preantral follicles. Cells were plated on fibronectin (BD Biosciences, San Jose, CA)-coated 60 mm plastic dishes at a density of about 3×10^6 cells/dish in DMEM/F12 serum-free medium supplemented with 1 nM E, 100 U/ml penicillin, and 100 μg/ml streptomycin (E/PS), and treated with indicated additions about 20 h after plating (7). Treatments were terminated by aspirating medium and rinsing cells once with PBS. Total cell extracts were collected by scraping cells in SDS sample buffer (37) followed by heat denaturation. Protein concentrations were controlled by plating identical cell numbers per plate in each experiment then loading equal volumes of total cell extract per gel lane; GCs do not proliferate under these culture conditions. Equal protein loading was confirmed by total AKT or mTOR Western blots, as indicated. GC proteins were separated by SDS-PAGE and transferred to Hybond C-extra nitrocellulose (Amersham Biosciences; Piscataway, NJ) (7). Blots were incubated with primary antibody overnight at 4 C, and antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham Biosciences). Western signals were quantitated with Molecular Analyst/PC Image Analysis software program (Bio-Rad Laboratories, Hercules, CA), divided by the densitometric signal for control protein load, and expressed relative to the maximal signal. Results were analyzed using Student's *t* test (38).

Transfection and luciferase assays

GCs were plated in 12-well plates at 3×10^5 cells/well in DMEM/F12, E/PS. Cells were washed with PBS and transfected with various promoter-luciferase constructs (0.5 μg DNA/well) and indicated expression constructs (0.05 μg DNA/well) using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) as described previously (39). Briefly, cells were incubated with the transfection mixture at 37 C for 4–5 h after which DMEM/F12 serum-free medium supplemented with E/PS was added to the cells. Approximately 18–20 h after transfection, cells were treated as indicated. Cells were lysed and analyzed for luciferase activity using a Veritas microplate luminometer (Turner Biosystems; Sunnyvale, CA). Data are presented as the mean ± SEM of triplicate samples. All transfection experiments were repeated a minimum of three times with similar results. Results were analyzed using Student's *t* test (38). Percent inhibition in the presence of various inhibitors was calculated as $[1 - (\text{fold induction}_{\text{FSH + inhibitor}}/\text{fold induction}_{\text{FSH}})] \times 100$.

RT-PCR

GCs were plated in 60-mm plates at 5×10^6 cells/plate as described above. The next day cells were pretreated for 1 h with 50 μM PD98059 or dimethylsulfoxide (DMSO) vehicle or 0.05 μM PD184352 or ethanol vehicle and then left untreated or treated with 50 ng/ml FSH for the indicated times, rinsed with PBS, and RNA was isolated using the RNEasy kit (no. 74104; QIAGEN, Valencia, CA). One sixth of the total RNA isolated was reverse transcribed for 75 min at 42 C with 1 mM deoxynucleotide triphosphates, random hexamers (no. C118A; Promega), and avian myeloma virus-reverse transcriptase (no. M510F; Promega), and one sixth of each reverse transcriptase reaction was used for PCR using a DNA engine cycler (Bio-Rad Laboratories, Hercules, CA) with primers for rat VEGF or for the rat ribosomal protein L19 (VEGF forward, 5'-ctttctgctctcttgggtgcactg-3', reverse, 5'-aagctcatctctcctatgtgctgg-3'; L19 forward, 5'-ctgaaggtcaaagggaatgtg-3', reverse, 5'-ggacagagtcttgatgatctc-3'). PCRs were resolved on agarose gels containing ethidium bromide and quantified using the Bio-Rad Quantity One program. Results were analyzed using Student's *t* test (38).

ChIP assay

ChIP assays were performed as described previously (13). Briefly, GCs were plated in 100-mm plates at 10×10^6 cells/ plate in DMEM/F12, E/PS. After various treatments, formaldehyde was added directly to the media for 10 min at room temperature at a final concentration of 1% to cross-link protein-DNA complexes. The cells were then washed once with cold PBS and harvested by scraping in cold PBS with a Complete EDTA free protease inhibitor tablet (Roche; Basel, Switzerland). The samples were then centrifuged at 6000 rpm for 30 min. Pellets were resuspended in 200 μl lysis buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8), and incubated on ice for 10 min before being sonicated for 1 min. Supernatants were then recovered after samples had been centrifuged for 10 min at 14,000 rpm. A fraction of this sample was retained as the input. The rest was diluted 10-fold in immunoprecipitation buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8), 167 mM NaCl, and protease inhibitors. The samples were precleared with protein A Sepharose/salmon sperm DNA (Upstate Biotechnology, Lake Placid, NY) for 45 min. The precleared sample was incubated with 10 μl HIF-1α 105 antibody (Novus-Biologicals, Littleton, CO) overnight at 4 C. The immunoreactive complexes were captured by adding protein A Sepharose/salmon sperm DNA; collected by centrifugation; washed three times with 2 mM EDTA, 20 mM Tris-HCl (pH 8), 150 mM NaCl and once with Tris/EDTA; and eluted at room temperature with 1% SDS and 0.1 M NaHCO$_3$. DNA was extracted by phenol/chloroform extraction followed by ethanol precipitation. The primers used for PCR correspond to regions in the VEGF promoter spanning a characterized HRE: forward, 5'-ggctctgtctgccag ctgtc-3' and reverse, 5'-gtgacactgagaacgggaagc-3'.

Samples were analyzed by real-time PCR using the Chromo4 real-time detector (Bio-Rad). Cycle threshold [C(t)] values for each sample were normalized using $\Delta C(t)_{\text{average}}$ values obtained for the corresponding input sample to obtain $\Delta C(t)$. PCR for each sample was done in triplicate. The average $\Delta C(t)_{\text{average}}$ was then calibrated to the sample with the highest $\Delta C(t)_{\text{average}}$ (the lowest expression level) to obtain a $\Delta\Delta C(t)$ for each sample. The normalized fold difference relative to the sample with the lowest expression was then calculated using $2^{-\Delta\Delta C(t)}$. Data are presented as the mean ± SEM of triplicate samples. All experiments were repeated three times with similar results. Results were analyzed using Student's *t* test (38).

Adenoviral infection of GCs

Infection with the adenovirus Ad-A3-FOXO1 or an empty adenovirus (Ad-E) was performed as previously described (13). In short, GCs were plated in 60-mm plates at 3×10^6 cells/ plate in DMEM/F12, E/PS. After the cells were allowed to adhere to fibronectin for 3 h, they were treated with the indicated concentration of adenovirus for 4 h. The adenovirus was subsequently washed off with PBS, and DMEM/F12 and E/PS was added to the cells. The following morning the cells underwent indicated treatments.
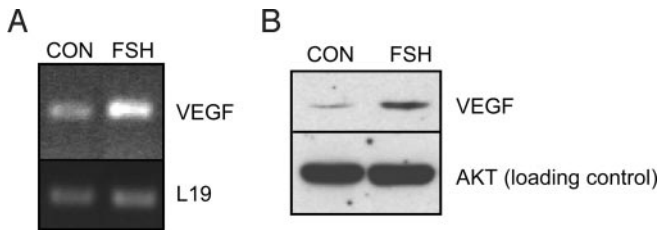
FIG. 1. FSH stimulates expression of VEGF mRNA and protein in GCs. A, GCs were untreated or treated with 50 ng/ml FSH for 24 h. RNA was isolated and subjected to RT-PCR as described in *Materials and Methods* using primers for VEGF and the rat ribosomal protein L19. Results of ethidium bromide-stained agarose gel shown are representative of three separate experiments. B, GCs were untreated or treated with 50 ng/ml FSH for 72 h. Total cell extracts were probed with indicated antibodies. Results are representative of three separate experiments. CON, Control.

Results

The contribution of the PI3-kinase pathway to FSH-mediated HIF-1 stimulation extends beyond increasing translation via mTOR

In this report we sought to elucidate the signaling pathways that contribute to the ability of FSH to induce HIF-1 activity in GCs. We previously reported that FSH induces HIF-1 activity in GCs measured both with a luciferase reporter containing 3 copies of the HRE from phosphoglycerate kinase [HRE-(3)-TK-Luc] and with a VEGF-luciferase reporter construct containing the HIF-1 binding site (9). That FSH indeed stimulates expression of VEGF mRNA and protein in GCs is shown in Fig. 1. We also previously reported that both HRE-luciferase and VEGF-luciferase activities are significantly inhibited by the PI3-kinase inhibitor LY294002, which broadly inhibits members of the PI3-kinase family including mTOR (40), and by the mTOR/regulatory associated protein of mTOR (raptor) selective inhibitor (41), rapamycin. Furthermore, we demonstrated that FSH-stimulation of GCs leads to activation of translational enhancers such as p70 S6-kinase downstream of mTOR and that the mechanism of FSH-mediated up-regulation of HIF-1α is primarily translational and not transcriptional (9). However, these studies suggested that effects of the PI3-kinase pathway were not exclusively mediated via mTOR because the PI3-kinase/mTOR inhibitor LY294002 more potently (72%) inhibited FSH-stimulated HIF-1 activity compared with the

mTOR selective inhibitor rapamycin (45%) at a concentration of rapamycin in which mTOR activity, as seen by phosphorylation of its downstream effectors, was completely abolished (9).

To identify the contribution of the PI3-kinase pathway beyond the contribution made by mTOR, we initially examined the relative effect of these inhibitors on FSH-mediated accumulation of HIF-1α protein levels (Fig. 2). GCs were untreated (CON) or treated with FSH for 4 h in conjunction with various inhibitors. All samples used the hypoxia mimetic CoCl$_2$ to inhibit the degradation of HIF-1α, as described previously (9). Whereas the mTOR inhibitor rapamycin inhibited FSH-mediated accumulation of HIF-1α protein by 30% (Fig. 2, lane 2 *vs.* lane 8) at a dose at which the phosphorylation of mTOR effectors was abolished (9), LY294002 inhibited the accumulation of HIF-1α by 90% (Fig. 2, lane 2 *vs.* lane 4). These results suggest that the greater inhibition in HIF-1 activity caused by LY294002 relative to rapamycin that we previously reported (9) is at least partially a result of the ability of LY294002 to cause a greater reduction of HIF-1α protein, and that the PI3-kinase pathway might regulate HIF-1α protein levels not only via mTOR but also via additional pathways. We thus investigated whether there were additional PI3-kinase targets that may play a role in FSH-mediated HIF-1α protein accumulation in GCs.

FSH phosphorylates MDM2 at Ser166, and phosphorylated MDM2 enhances HIF-1 activity

A PI3-kinase target reported to promote the accumulation of HIF-1α is the ubiquitin-ligase MDM2. MDM2 is phosphorylated at Ser166 and Ser188 by AKT (34, 42–44). AKT-mediated phosphorylation of MDM2 has been shown to enhance the stability of MDM2 by protecting it from proteosome-dependent degradation (34, 44). Moreover, the transient expression of MDM2 in various cancer cell models is reported to increase HIF-1 activity (45) by increasing HIF-1α protein translation (42). Studies using MDM2 in which S166 is mutated to Asp or Ala demonstrate that AKT-stimulated phosphorylation of S166 further enhances the accumulation of HIF-1α (42). We thus investigated whether FSH promoted the expression of MDM2 in GCs using an MDM2 antibody that detects total levels of MDM2 and/or the phosphorylation of MDM2 at Ser166 using a phospho-specific MDM2 antibody. Total MDM2 protein levels were relatively stable over the 8-h time course of this experiment (Fig. 3A, *lower panel*). However, MDM2 phosphorylation *in vitro* in GCs was detected by 30 min post FSH, reached a maximal response at 1 h, and subsided by 4 h (Fig. 3A, *top panel*, lanes 3–5). FSH-stimulated phosphorylation of AKT at Ser473 followed a similar pattern (Fig. 3A). We confirmed that phosphorylation of MDM2 occurred *in vivo* in animals treated with a sc injection of PMSG. Maximal MDM2 phosphorylation was observed 8 h after PMSG and was decreased but still detectable at later time points (Fig. 3B). The retarded response seen *in vivo* likely reflects slower delivery of PMSG to the ovary compared with direct addition of FSH to GCs. We further tested the effect of overexpressing wild-type H-MDM2 (human ortholog of MDM2) or a phospho-mimetic S166D mutant of H-MDM2 on
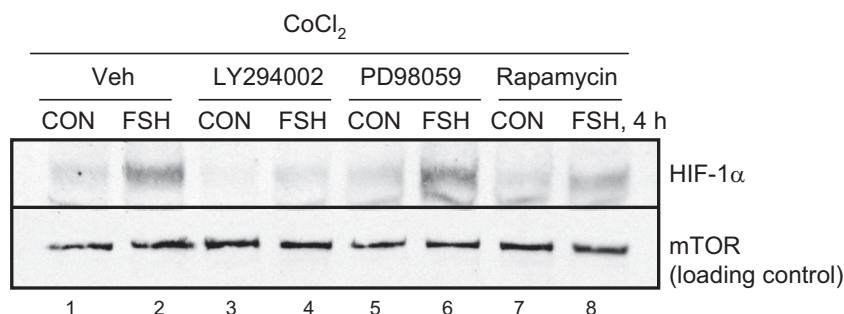

FIG. 2. The PI3-kinase/mTOR inhibitor LY294002 inhibits FSH-stimulated HIF-1α accumulation more than the mTOR inhibitor rapamycin. GCs were pretreated with DMSO vehicle (Veh) or 12.5 μM LY294002 for 1 h, 50 μM PD98059 for 1 h, or 100 nM rapamycin for 15 min and then left untreated (CON) or treated with 50 ng/ml FSH for 4 h in the presence of 150 μM CoCl$_2$ to retard degradation of HIF-1α, as indicated. Results are representative of three similar experiments. Western blots of total cell extracts were probed with the indicated antibodies. mTOR is used as a loading control. Western signals were quantitated with Molecular Analyst/PC Image Analysis software program, divided by the densitometric signal for mTOR, and expressed in text relative to the untreated (CON) sample.
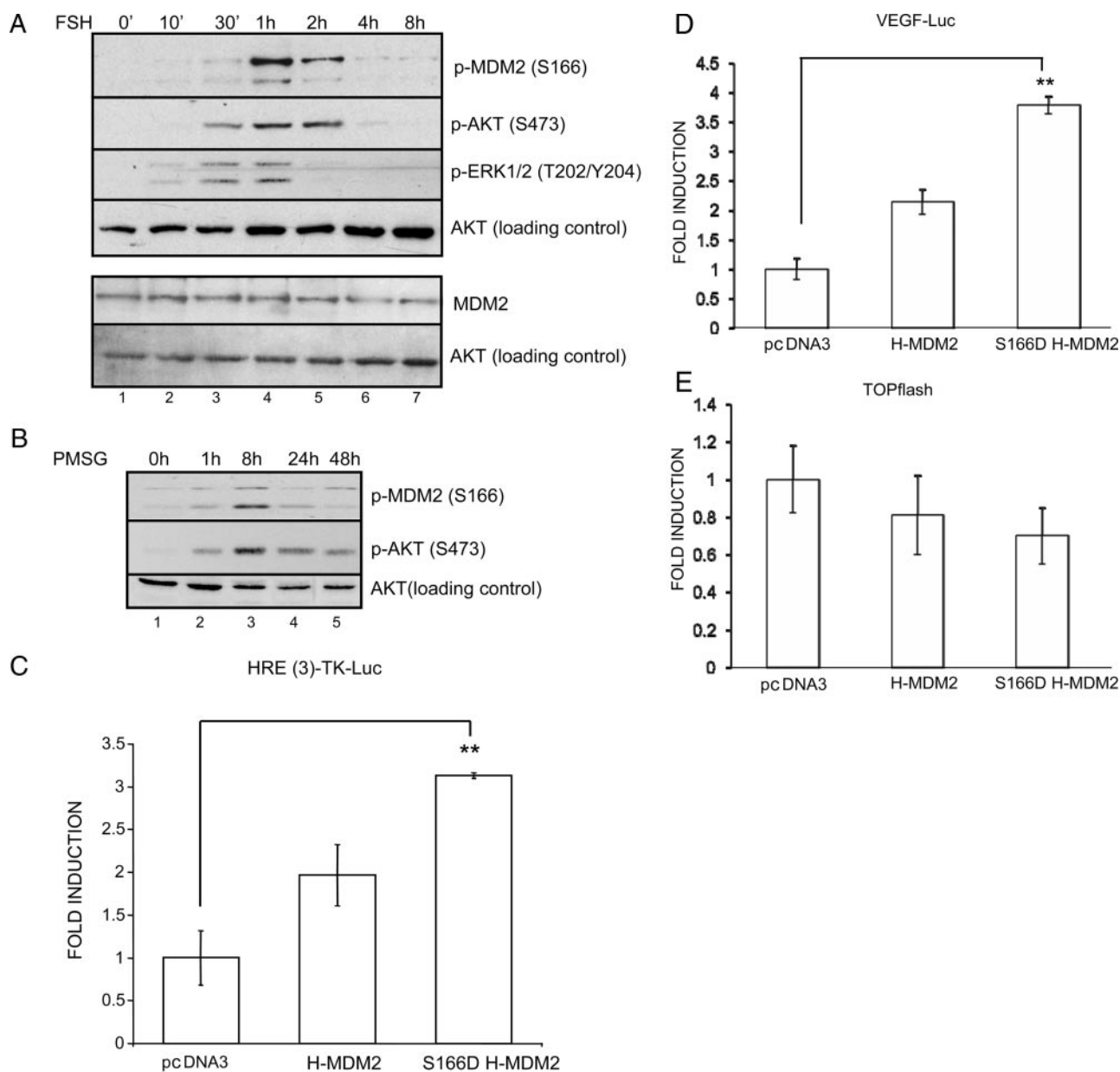
FIG. 3. FSH stimulates the phosphorylation of MDM2 at Ser166; S166D H-MDM2 is sufficient to induce HIF-1 activity. A, GCs were treated with 50 ng/ml FSH for the indicated times. Western blots of total cell extracts were probed with the indicated antibodies. Phospho (p)-specific antibodies are described in *Materials and Methods*. AKT is used as a loading control. Lower two blots for total MDM2 and AKT reflect separate samples from an identical experiment. Signal for p-MDM2 was detected about 130 and 96 kDa; signal for total MDM2 was detected about 96 kDa. Results are representative of three similar experiments. B, Rats were injected sc with 25 IU PMSG, and ovarian extracts were prepared for Western blotting at the indicated times after PMSG injection, as described in *Materials and Methods*. C–E, GCs were transfected with promoter-Luc constructs as described in *Materials and Methods* and with 50 ng of pcDNA3, WT-H-MDM2, or S166D-H-MDM2, as indicated. GCs transfected with HRE-(3)-TK-Luc, VEGF-Luc, or TOPflash and the indicated expression vector were harvested 24 h after transfection. Values are expressed as a mean ± SEM of triplicates and representative of three separate experiments. Student *t* test for compared values: **, Significant difference with $P \leq 0.05$.

HIF-1 activity via reporter assays. Although H-MDM2 did not induce a significant increase in the activity of HRE-(3)-TK-Luc, S166D H-MDM2 promoted a 3.1-fold ($P < 0.05$) increase in HRE-(3)-TK-Luc activity over pcDNA3-transfected GCs (Fig. 3C). Similarly, expression of S166D H-MDM2 significantly ($P < 0.05$) increased reporter activity of the HIF-1 target VEGF, measured using a VEGF-promoter-luciferase construct (Fig. 3D), whereas the activity of the synthetic T-cell factor reporter TOPflash (61) was not affected (Fig. 3E). These data demonstrate that MDM2 is constitutively expressed in immature GCs and is phosphorylated on Ser166 in response to FSH. These results also show that HIF-1 activity can be positively influenced by S166D H-

MDM2 levels in GCs. Taken together, these results suggest that the PI3-kinase/AKT target MDM2 may contribute to FSH-stimulated HIF-1 activity in GCs.

FOXO1 inactivation by PI3-kinase/AKT phosphorylation is necessary for FSH-mediated increase in HIF-1α protein and HIF-1 activity

The PI3-kinase/AKT target FOXO4, a member of the forkhead box O containing transcription factor family, in its active conformation has been shown to negatively affect HIF-1α stability (46). We hypothesized that the PI3-kinase/AKT tar-
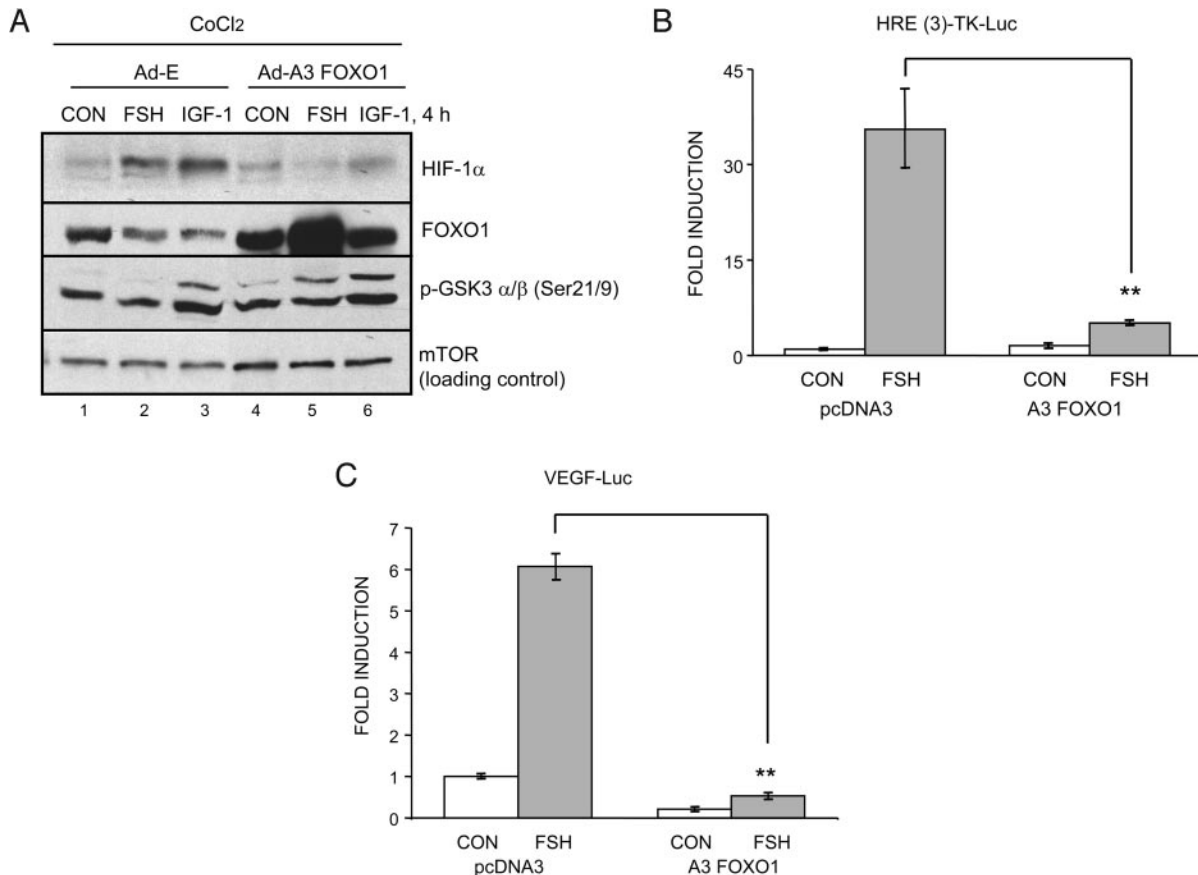
FIG. 4. Constitutively active mutant of FOXO1 inhibits FSH-mediated up-regulation of HIF-1α and thus affects HIF-1 activity. A, GCs were allowed 3 h after isolation to adhere to fibronectin-coated dishes and then were treated for 4 h with 5 PFU/cell of either an empty adenoviral construct, Ad-E, or Ad-A3-FOXO1, as indicated. The following morning GCs were either untreated (CON) or treated with 50 ng/ml FSH or 50 ng/ml IGF-I for 4 h in the presence of 150 μM CoCl₂ as indicated. Western blots of total cell extracts were probed with the indicated antibodies. Phospho (p)-specific antibodies are described in *Materials and Methods*. mTOR is used as a loading control. B and C, GCs were transfected with promoter-Luc constructs as described in *Materials and Methods* and with 50 ng of pcDNA3 or A3-FOXO1, as indicated. GCs transfected with HRE-(3)-TK-Luc (B) or VEGF-Luc (C) were left untreated (CON) or treated with 50 ng/ml FSH for 6 h. Values are expressed as a mean ± SEM of triplicates and representative of three separate experiments. Student's *t* test for compared values: **, significant difference with $P \leq 0.05$.

get in GCs FOXO1 might similarly regulate the stability of HIF-1α. To ascertain whether active FOXO1 affects HIF-1α accumulation in GCs, we transduced GCs either with an adenovirus expressing a constitutively active mutant of FOXO1 in which the three AKT phosphorylation sites were mutated from Thr/Ser to Ala (Ad-A3-FOXO1) or with an empty adenovirus (Ad-E) (13, 47). We then evaluated the accumulation of HIF-1α in response to both FSH and, as a positive control, the ubiquitous activator of the PI3-kinase/AKT pathway, IGF-I. In the presence of empty adenovirus, FSH and IGF-I enhanced the accumulation of HIF-1α by 2.3 ± 0.3 (n = 3) and 4.6 ± 0.7 (n = 3)-fold, respectively. Expression of constitutively active FOXO1 mutant in GCs diminished both the FSH- and IGF-I-mediated up-regulation of HIF-1α protein (Fig. 4A, compare lanes 2 and 3 *vs.* lanes 5 and 6). Using a FOXO1 antibody, we confirmed that transduction with Ad-A3-FOXO1 resulted in increased expression of FOXO1[1] (Fig. 4A, compare lanes 1–3 and 4–6).

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[1] We previously reported increased expression of FOXO1 in GCs infected with Ad-A3-FOXO1 and treated with FSH compared with controls (13).

To ensure that the diminished accumulation of HIF-1α in the presence of overexpressing A3-FOXO1 was not caused by a non-specific inhibition of AKT activity, we assessed the phosphorylation of glycogen synthase kinase (GSK)-3α/β, a recognized AKT target (48), using a phospho-specific GSK3α/β antibody. Results show that overexpression of A3-FOXO1 did not prevent AKT-mediated phosphorylation of GSK3α/β in response to FSH or IGF-I (Fig. 4A, compare lanes 1–3 and 4–6). The elevated phosphorylation of GSK3 in the presence of Ad-A3 FOXO1 likely reflects the elevated protein load in lane *5 vs.* lane 2. Additionally, an inverse relationship between endogenous FOXO1 and the expression of HIF-1α is apparent in the Ad-E-treated samples (Fig. 4A, compare HIF-1α and FOXO1 levels in lanes 1–3). These results suggest that growth factor-mediated down-regulation of FOXO1 in GCs (in response to FSH or IGF-I) contributes to HIF-1α stabilization. Next we tested the effect of the A3-FOXO1 mutant on HIF-1 activity via reporter assays in GCs transfected with HRE-(3)-Luc. Cotransfection of the A3-FOXO1 expression vector significantly ($P < 0.05$) inhibited the FSH-stimulated activity of HRE-(3)-TK-Luc by 86% (Fig. 4B). Similarly, expression of A3-FOXO1 also significantly ($P < 0.05$) inhibited FSH-mediated up-regulation of reporter activity of the HIF-1 target VEGF, measured using a VEGF promoter-luciferase

construct, by 92% (Fig. 4C). These results indicate that the active conformation of FOXO1, in which the three AKT phosphorylation sites are not phosphorylated, inhibits up-regulation of FSH target genes such as VEGF by destabilizing HIF-1α and thus preventing HIF-1 transcriptional activity.

IGF-I induces HIF-α protein but not HIF-1 activity in GCs

Taken together our results show that multiple AKT targets, including tuberin/mTOR (9) and FOXO1 and possibly MDM2 contribute to FSH-stimulated HIF-1 activity in GCs. Because IGF-I is sufficient to activate the PI3-kinase/AKT/ mTOR pathway in GCs (9) and IGF-I stimulates the accumulation of HIF-1α protein in the presence of $CoCl_2$ (see Fig. 4A), we asked whether IGF-I was sufficient to induce HIF-1 activity, assessed using both the minimal HIF-1 promoter-luciferase construct and the HIF-1 target VEGF luciferase-promoter. GCs treated for 4 h with IGF-I in the presence of $CoCl_2$ showed a 3.4-fold accumulation of HIF-1α in IGF-I-treated cells relative to untreated cells (Fig. 5A, lanes 3 and 4), consistent with results shown in Fig. 4A. Surprisingly, however, IGF-I was unable to stimulate HIF-1 activity relative to untreated (CON) GCs in reporter assays of GCs transfected with HRE-(3)-TK-Luc (Fig. 5B, compare lanes 1 and 5), whereas FSH consistently induced a significant activation ($P < 0.05$) of this reporter (Fig. 5B, compare lanes 1 and 3). This result was unexpected because IGF-I consistently promoted a greater accumulation of HIF-1α compared with FSH (see Fig. 4A). We also tested to see whether IGF-I synergized with FSH to increase HIF-1 activity but found no significant difference in HRE-(3)-TK-Luc activity in IGF-I plus FSH-treated GCs relative to FSH-treated GCs (Fig. 5B, compare lanes 3 and 7). Previously the sensitivity of the reporter assays allowed us to detect the induction of HIF-1 activity by FSH in the absence of $CoCl_2$ (9). However, we wanted to ensure that the discrepancy we detected between the ability of IGF-I to induce HIF-1α protein by Western blots $vs.$ the inability of IGF-I to activate HIF-1 activity by reporter assays was not due to insufficient HIF-1α protein levels. Even with the addition of the hypoxia mimetic $CoCl_2$ to stabilize HIF-1α levels (Fig. 5B, $hatched\ bars$), IGF-I did not stimulate HRE-(3)-TK-Luc activity in GCs. Similarly, reporter activity of the HIF-1 target VEGF, measured using a VEGF promoter-luciferase construct, was induced 7-fold by FSH treatment of GCs but was unaffected by IGF-I treatment, and there was no significant synergism between FSH and IGF-I (Fig. 5C). These data indicate that the PI3-kinase pathway may be sufficient only to promote the accumulation of HIF-1α protein in GCs but not sufficient to stimulate HIF-1 activity. These results further suggest that signaling events independent of the PI3-kinase pathway that occur downstream of FSH appear to be necessary to stimulate HIF-1α activity.

ERK1/2 activity does not appear to be necessary for HIF-1 activity

There is abundant evidence from a number of cell models that HIF-1 activity is positively regulated by ERK1/2, based largely on effects of the MEK 1/2 inhibitor PD98059 or constitutively
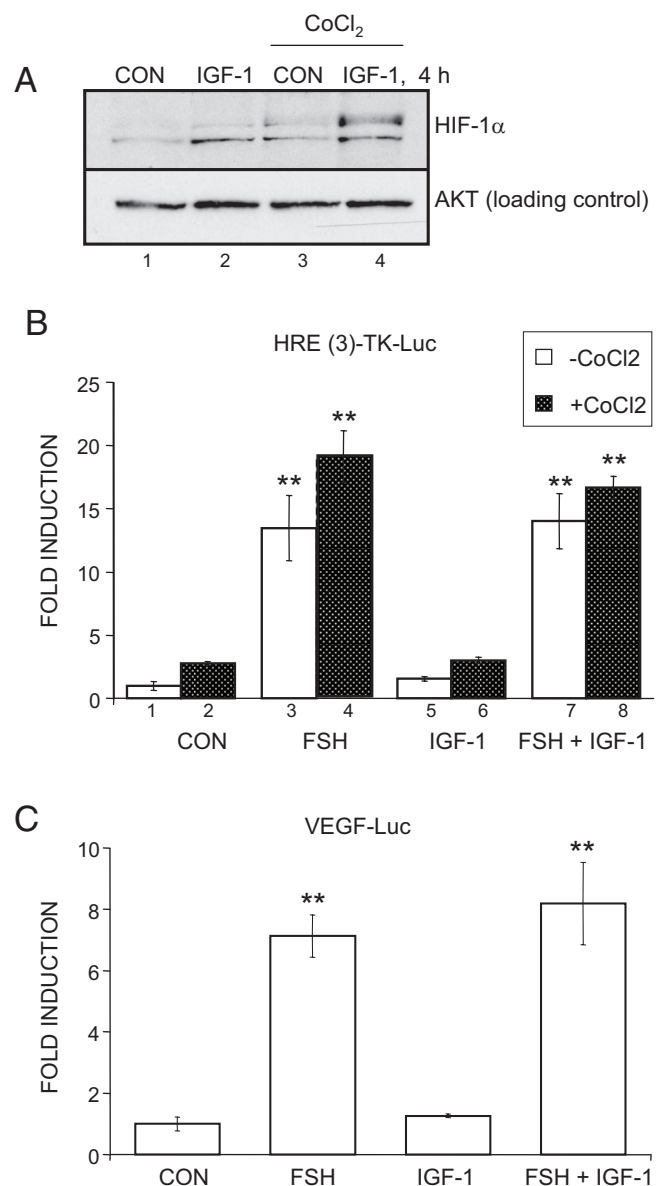

FIG. 5. IGF-I is sufficient to induce HIF-α protein but not HIF-1 activity. A, GCs were either untreated (CON) or treated with 50 ng/ml IGF-I for 4 h in the presence or absence of 150 μM $CoCl_2$ to retard degradation of HIF-1α. Western blots of total cell extracts were probed with the indicated antibodies. AKT is used as a loading control. B and C, GCs were transfected with promoter-Luc constructs as described in *Materials and Methods.* GCs transfected with HRE-(3)-TK-Luc (B) or VEGF-Luc (C) were left untreated (CON) or treated with 50 ng/ml FSH, 50 ng/ml IGF-I, or 50 ng/ml FSH + 50 ng/ml IGF-I for 6 h in the presence or absence of 150 μM $CoCl_2$ as indicated. Values are expressed as a mean $\pm$ SEM of triplicates and representative of three separate experiments. Student t test for compared values: **, significant difference with $P \leq 0.05$ compared with equivalent control.

active MEK1/2, although the site of ERK1/2 phosphorylation on HIF-1α remains controversial (49–55). Because FSH is recognized to activate the ERK1/2 pathway in GCs (4), as shown in Fig. 3A, we first asked whether IGF-I similarly activated ERK1/2 in GCs. IGF-I promotes ERK activation in many cellular models (53, 56, 57) but fails to do so in other cellular models as a result of the dephosphorylation of insulin receptor substrate-1 at the Grb2/SOS binding site by the tyrosine phosphatase Src homology 2-containing protein phosphatase (SHP_2) (58). To ascertain
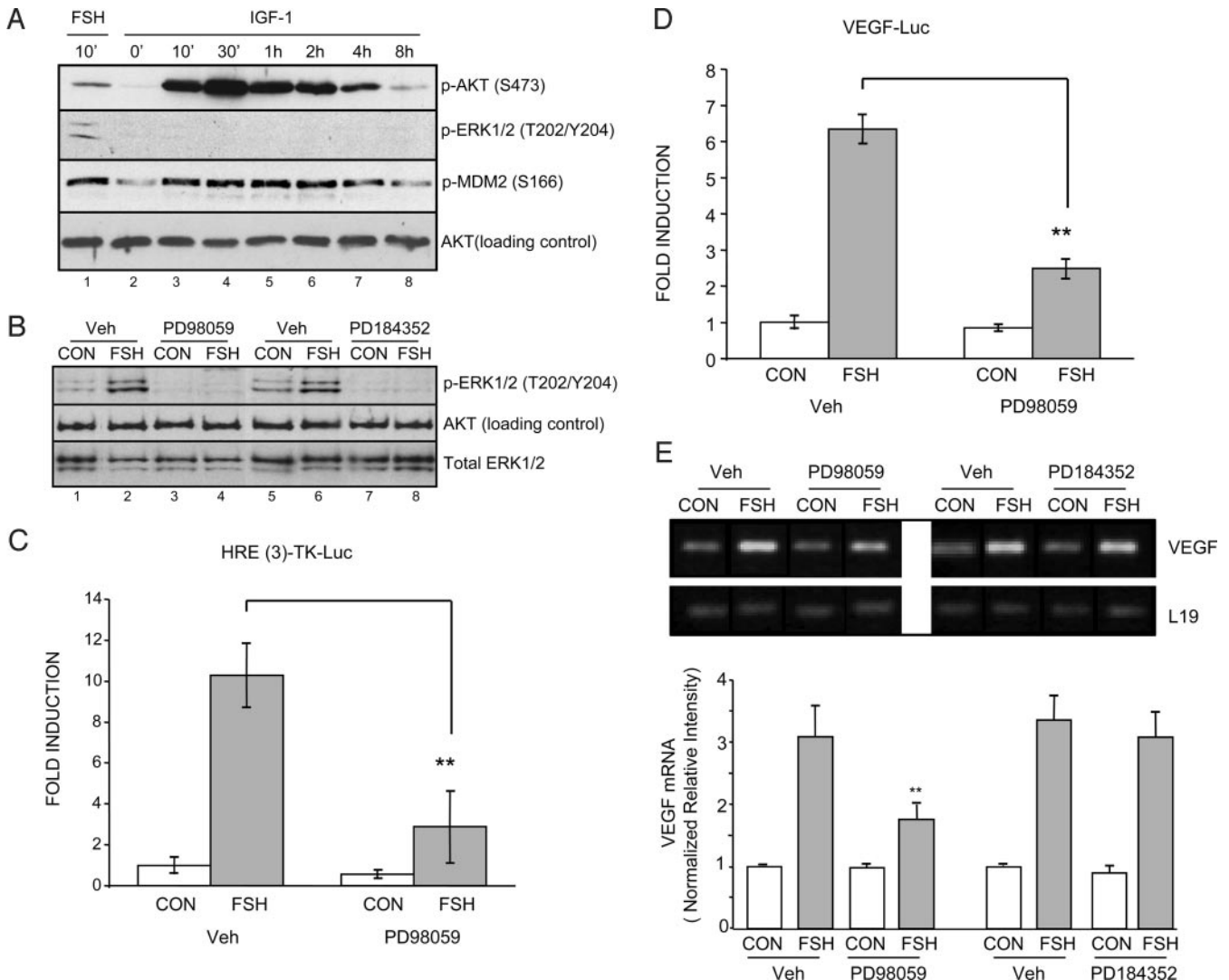
FIG. 6. The selective MEK1/2 inhibitor PD98059 inhibits FSH-stimulated HIF-1 and VEGF activity, whereas the specific MEK1/2 inhibitor PD184352 does not inhibit FSH-stimulated HIF-1 activity. A, GCs were treated with 50 ng/ml IGF-I or FSH for the indicated times. Results are representative of two similar experiments. Western blots of total cell extracts were probed with the indicated antibodies. AKT is used as a loading control. B, GCs were pretreated with DMSO vehicle or 50 μM PD98059 or with ethanol vehicle or 0.05 μM PD184352, 1 h before treatment without or with 50 ng/ml FSH for 10 min. Results are representative of three separate experiments. C and D, GCs transfected with HRE-(3)-TK-Luc (C) or VEGF-Luc (D) were left untreated (CON) or treated with 50 ng/ml FSH for 6 h. Cells were pretreated with DMSO vehicle or 50 μM PD98059 for 1 h before control (CON) or FSH treatment. Values are expressed as a mean ± SEM of triplicates and representative of three separate experiments. Student *t* test for compared values: **, significant difference with $P \leq 0.05$. E, GCs were pretreated for 1 h with DMSO vehicle or 50 μM PD98059 or with ethanol vehicle or 0.05 μM PD184352 and then treated without or with 50 ng/ml FSH for 1 h. RNA was isolated and subjected to RT-PCR as described in *Materials and Methods* using primers for VEGF and the rat ribosomal protein L19. *Top panel* is a representative ethidium bromide agarose gel. *Lower panel* shows results as mean ± SEM of five separate experiments. **, significant difference with $P \leq 0.02$.

whether IGF-I activates ERK1/2, GCs were treated with IGF-I for various times (leading up to, including and beyond the times we measured HIF-1α protein levels and HIF-1 activity). Despite robust IGF-I-stimulated phosphorylation of both AKT and MDM2, we were unable to detect IGF-I-stimulated ERK1/2 phosphorylation (Fig. 6A).[2] These results suggest that perhaps the inability of IGF-I to induce HIF-1 activity reflects the inability of IGF-I to activate ERK1/2.

We thus sought to test the hypothesis that ERK1/2 is neces-

sary for HIF-1 activity and that the inability of IGF-I to activate HIF-1 reflects its inability to activate ERK1/2. We first determined whether the ERK1/2 pathway was indeed necessary for FSH to stimulate either the accumulation of HIF-1α protein and/or induction of HIF-1 activity by treating cells without and with the MEK1/2 inhibitor PD98059 (59). Results show that whereas treatment of GCs with PD98059 blocked FSH-stimulated ERK1/2 phosphorylation (Fig. 6B, lanes 1–4), PD98059 did not inhibit the induction of HIF-1α protein in response to FSH (Fig. 2, compare lanes 2 and 6). However, PD98059 at the same concentration significantly ($P < 0.05$) inhibited the FSH-mediated induction of HRE-(3)-TK-Luc by 72% (Fig. 6C) and the FSH-mediated up-regulation of VEGF-Luc activity by 61% (Fig. 6D). PD98059 also significantly ($P < 0.02$) inhibited FSH-

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[2] We have observed the association of IRS1 and SHP2 in rat GCs, as assessed by immunoprecipitation (Maizels, E., and M. Hunzicker-Dunn, unpublished). This association may explain the unresponsiveness of the ERK pathway to IGF-I stimulation in GCs.

stimulated induction of VEGF mRNA by 45% (Fig. 6E). These results thus suggest that indeed the ERK1/2 pathway contributes to the ability of FSH to induce HIF-1 activity, but that this pathway is not necessary for FSH to promote the accumulation of HIF-1α protein.

Because the MEK1/2 inhibitor PD98059 disrupted only the transactivational activity of HIF-1, and not HIF-1α accumulation, we hypothesized that the ERK1/2 pathway may be involved in targeting HIF-1 to the promoters of its target genes. We therefore tested to see whether the ERK1/2 pathway was necessary for HIF-1 to interact with a characterized HRE in the VEGF promoter. ChIP assays with a HIF-1α antibody followed by real-time PCR using primers spanning an HRE in the VEGF promoter were performed. Results demonstrate that FSH treatment of GCs for 4 h enhanced the interaction of HIF-1α with an HRE in the VEGF promoter 2-fold relative to untreated GCs (Fig. 7). IGF-I did not promote this interaction relative to untreated cells. Furthermore, treatment of GCs with PD98059 prevented FSH-mediated HIF-1 interaction with the VEGF promoter (Fig. 7).

Based on these results, we next determined whether activating the ERK1/2 pathway was sufficient to induce HIF-1 activity in GCs. Overexpression of a constitutively active MEK1/2 mutant (R4F) induced HRE-(3)-TK-Luc activity 16-fold compared with empty vector in untreated GCs (Fig. 8A, compare lanes 1 and 2). However, no notable additional HRE-(3)-TK-Luc activity was observed with the addition of IGF-I (Fig. 8A, compare lanes 2 and 6). R4F MEK synergized with FSH treatment to further induce HRE-(3)-TK-Luc activity beyond that observed with either treatment alone (Fig. 8A, lanes 3 and 4). The addition of IGF-I plus FSH did not further enhance HRE-(3)-TK-Luc activity over that of FSH alone (Fig. 8A, lanes 3 and 4 *vs.* 7 and 8). Similarly R4F MEK significantly ($P <$ 0.05) enhanced VEGF-Luc activity compared with empty vector in

untreated GCs, but did not significantly synergize with FSH or IGF-I treatments (Fig. 8B).

Taken together, these results indicate that the ERK1/2 pathway does not play a role in HIF-1α protein induction. Based on results with MEK1/2 inhibitor PD98059, the ERK1/2 pathway appears to be necessary for FSH-dependent HIF-1 transcriptional activity and to be a limiting factor in FSH-dependent HIF-1 activation. Yet ERK1/2 activity does not appear to be sufficient to rescue IGF-I-stimulated HIF-1 activity. Thus, although IGF-I enhances HIF-1α protein levels, IGF-I is unable to direct HIF-1 to the VEGF promoter in GCs, and ERK1/2 signaling cannot rescue this response.

FSH does not stimulate activation of ERK5

Based on our evidence that ERK1/2 activity was not sufficient to rescue IGF-I-stimulated HIF-1 activity and on recent evidence that PD98059 inhibits not only MEK1/2 but also MEK5 as well as cyclooxygenases 1 and 2 (60), we hypothesized that PD98059 might inhibit FSH-stimulated HIF-1 activity by inhibiting a kinase/enzyme other than MEK1/2. To test the hypothesis that a PD98059-sensitive enzyme/kinase distinct from ERK1/2 might facilitate activation of HIF-1, we evaluated the effect of a recently developed and more selective MEK1/2 inhibitor, PD184352 (60). Results show that whereas PD184352 indeed blocked FSH-dependent ERK1/2 phosphorylation (Fig. 6B, lanes 5–8), PD184352 did not inhibit FSH-stimulated HRE-(3)-TK-Luc activity (Fig. 9A) or induction of VEGF mRNA (Fig. 6E). Similar divergent effects of PD98059 and PD184352 on the ability of IGF-I to stimulate HIF-1 activity in various tumor cell models was recently reported (53). These results suggest that indeed FSH-stimulated HIF-1 activity is regulated by a PD98059-sensitive kinase/ enzyme that is distinct from ERK1/2.

Based on evidence that PD98059 also inhibits MEK5 (60), we hypothesized that perhaps FSH but not IGF-I activated MEK5 and that the MEK5 target ERK5 facilitated activation of HIF-1 activity. ERK5 activation has recently been linked to the regulation of a number of HIF-1 targets by microarray analysis, and ERK5 and ERK1/2 share some but not all substrates (63). We therefore tested the hypothesis that FSH but not IGF-I may selectively activate ERK5 to regulate HIF-1 activity and that PD98059 inhibition of FSH-stimulated HIF-1 activity was the consequence of its inhibition of ERK5 rather than ERK1/2. Results showed, however, that FSH did not activate ERK5, evidenced by the absence of an upshifted band detected by a total ERK5 antibody, whereas treatment with EGF, an established activator of ERK5 (64), promoted more than a 3-fold activation of ERK5 (Fig. 9B). Similar results were obtained using the antibody that detects phosphorylation of ERK5 at Thr218/Tyr220. Consistent with the absence of a role for ERK5 in regulating HIF-1 activity in GCs, EGF does not stimulate HRE-(3)-TK-Luc activity in GCs (data not shown). Taken together, these results suggest that whereas MEK1/2 can enhance HIF-1 activity, FSH-stimulated HIF-1 activity appears to require a protein whose activity is inhibited by PD98059 that is distinct from MEK1/2 and MEK5.
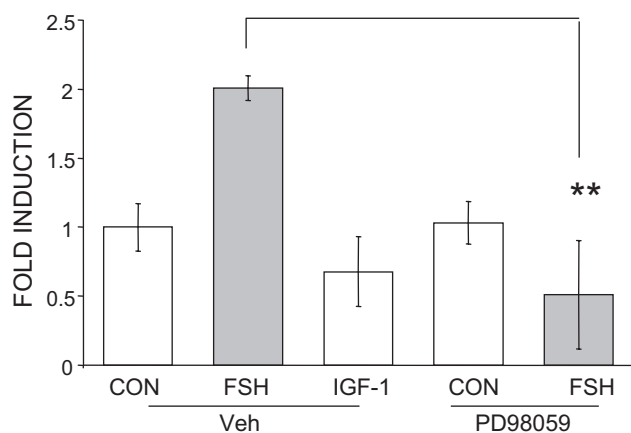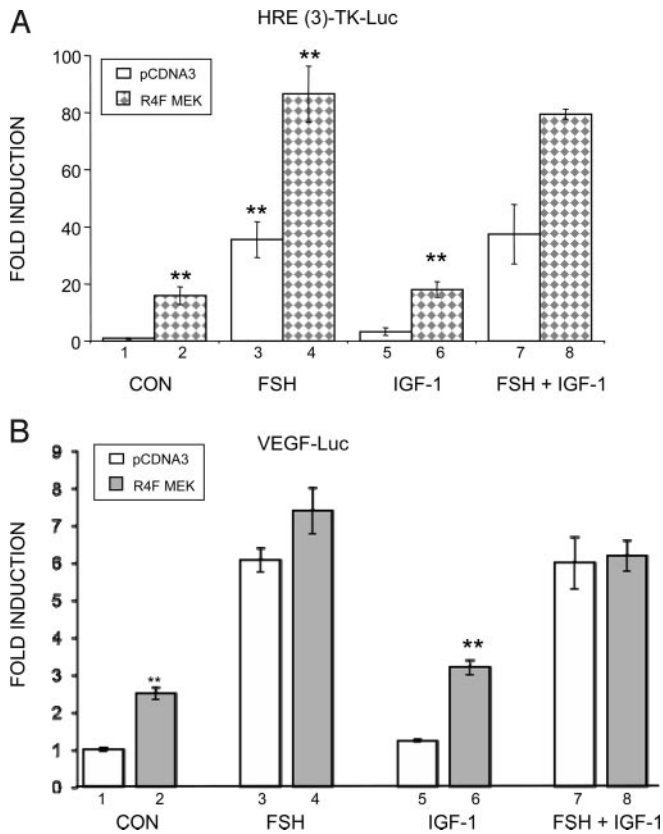

FIG. 7. Interaction of HIF-1 with the VEGF promoter is abrogated by PD98059. ChIP assays were performed as described in *Materials and Methods*. GCs were pretreated with DMSO vehicle or 50 μM PD98059 for 1 h and then left untreated (CON) and treated with 50 ng/ml FSH or 50 ng/ml IGF-I for 4 h, as indicated, in the presence of 150 μM CoCl$_2$ to retard degradation of HIF-1α. Samples were cross-linked with formaldehyde, collected, and sonicated to fragment DNA. A fraction was removed as input before immunoprecipitations were performed with a HIF-1α antibody. Real-time PCR was performed on both input DNA and immunoprecipitated DNA. C(t) values were normalized to input and then to the CON sample. Values are expressed as a mean $\pm$ SEM of triplicates and representative of three similar experiments. Student t test for compared values: **, significant difference with $P \leq 0.05$.

FIG. 8. Constitutively active MEK is sufficient to induce HIF-1 activity in GCs. GCs were transfected with HRE-(3)-TK-Luc construct (A) or VEGF-Luc (B), as described in *Materials and Methods* and with 50 ng of pCDNA3 or R4F-MEK, as indicated. GCs were left untreated (CON) or treated with 50 ng/ml IGF-I, 50 ng/ml FSH, or 50 ng/ml IGF-I + 50 ng/ml FSH, as indicated, for 6 h. Values are expressed as a mean ± SEM of triplicates and representative of three separate experiments. Student's *t* test for compared values: **, significant difference with $P \le 0.05$ between lanes 1 and 2, 1 and 3, 2 and 4, and 1 and 6 in A and between lanes 1 and 2 and 5 and 6 in B. Lanes 2 and 6 in B are not significantly different.

Discussion

FSH initiates a temporally defined differentiation program that leads to maturation of the ovarian follicle, resumption of oocyte meiosis, and subsequent ovulation of the oocyte (3). FSH induces a number of genes that define the preovulatory phenotype including *Lhr* (3), *microtubule-associated protein 2D* (65), *inhibin-α* (66), *protein kinase A type II regulatory subunit-β* (67), and *Cyp19a1* (68). In addition to initiating gene expression encompassing the up-regulation of both early (69, 70) and late genes in the context of the differentiation program, we have previously shown that FSH initiates an early translational event via the PI3-kinase/AKT/mTOR pathway (9). We have also shown how this early translational event is involved in the induction of the transcription factor HIF-1α and that HIF-1 plays a role in the up-regulation of FSH targets including LHR, inhibin-α, and VEGF (9).

In this report, we further examined the signaling events that are required for FSH mediated up-regulation of HIF-1α and activation of HIF-1 and its target gene VEGF. As modeled in Fig. 10A, we show that in addition to mTOR signaling, at least one additional target of the PI3-kinase/AKT pathway is involved in

HIF-1 activation, namely FOXO1. The transcriptional activator/repressor FOXO1 needs to be phosphorylated by AKT and thereby inactivated for maximal FSH-mediated HIF-1α up-regulation. We previously showed that HIF-1α accumulates in GCs in response to FSH via increased translation and not via increased transcription (9). Our observation that transduction of a constitutively active FOXO1 mutant into FSH-treated GCs prevents the accumulation of HIF-1α indicates that active FOXO1, present normally only in the absence of FSH, must negatively affect the stability of any HIF-1α that may be present in GCs.

Perhaps one way that HIF-1 activity is prevented in the avascular follicle until FSH stimulation, even if follicle cells are experiencing potential hypoxia-mediated HIF-1α up-regulation, is by destabilizing HIF-1α in a FOXO1-mediated manner. We show destabilization of HIF-1α occurs even in the presence of $CoCl_2$ (see Fig. 4A), which is primarily thought to stabilize HIF-1α by preventing E3 ubiquitin-ligase VHL-mediated degradation (71, 72). This result suggests that FOXO1 mediates HIF-1 degradation via a VHL-independent pathway in GCs, as was the case for FOXO4 in a HeLa cell model (46), perhaps by inducing a distinct ubiquitin-ligase that targets HIF-1α. Further studies will be required to determine the mechanism by which the transcriptional activator/repressor FOXO1 destabilizes HIF-1α and whether FOXO1 DNA binding or transcriptional activity is required for this activity. FOXO1 might also function in GCs to repress HIF-1 transcriptional activity by interfering with the coactivator activity of p300, as has been recently reported for FOXO3a (73). Further studies will also be required to determine whether the trans-acting repressor activity of FOXO1 extends beyond *cyclin D2*, *steroidogenic factor-1*, *inhibin-α*, *Cyp19a1*, and *epiregulin* (13) to include HIF-1 target genes in GCs such as *VEGF* and *Lhr* (9).

Taken together, these results suggest that in addition to promoting mTOR-stimulated translation, a further mechanism by which FSH promotes differentiation is by affecting protein stability. Thus, HIF-1α appears to be stabilized with the phosphorylation/inactivation of FOXO1 and FOXO1 protein levels are negatively regulated by FSH treatment (see Fig. 4A).

The second PI3-kinase/AKT target in GCs that may be involved in the regulation of HIF-1 activity is MDM2. MDM2 is best known for its ability to repress the transcriptional activity of p53 and to target p53 for ubiquitination via its E3 ligase activity (74). MDM2 also functions independently of p53: MDM2 is reported to promote degradation of the cell cycle inhibitor p21 independently of its ligase activity (75); to stabilize the transcriptional activator E2F1 (76); to express intrinsic chaperone activity (77); to interact with ERK-phosphorylated FOXO3a leading to FOXO3a degradation via its ubiquitin ligase activity (78); and to enhance expression of HIF-1α (42). Phosphorylation of MDM2 on S166 by AKT is reported to enhance p53 degradation (79), stabilize MDM2 (34, 42, 44), and increase expression of HIF-1α (45) by increasing its translation rather than increasing its half-life (42) in various cellular models. Our results constitute the first report of the phosphorylation of MDM2 at Ser166 downstream of FSH in GCs. Phosphorylation of MDM2 on S166 in GCs does not appear to enhance its stability; rather, MDM2 appears to be constitutively expressed in GCs of im-
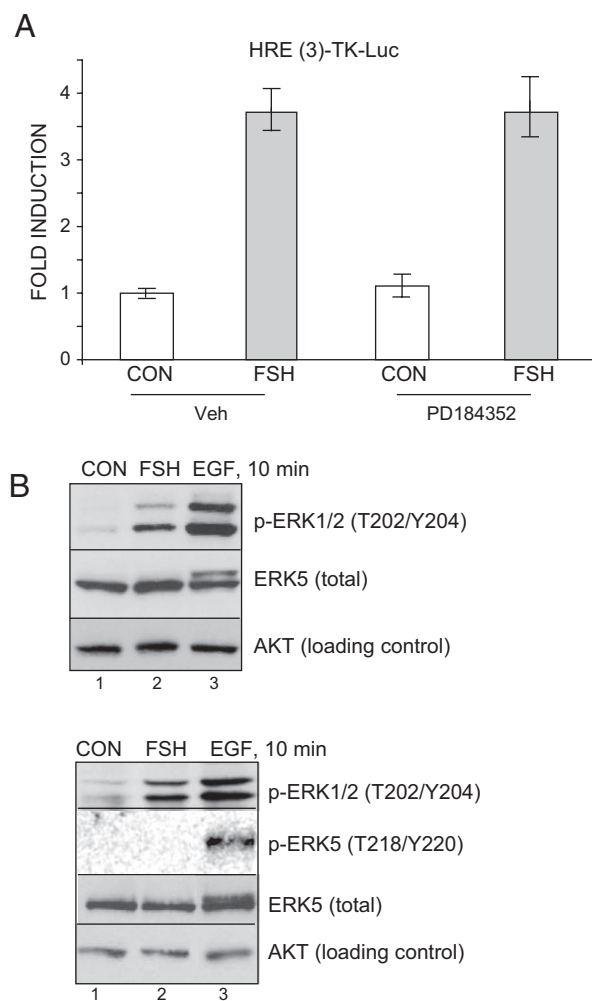
FIG. 9. The MEK1/2-specific inhibitor PD184352 does not inhibit FSH-stimulated HIF-1 activity. A, GCs transfected with HRE-(3)-TK-Luc were pretreated with ethanol vehicle or with 0.05 μM PD184352 for 1 h and then left untreated (CON) or treated with 50 ng/ml FSH for 6 h. Values are expressed as a mean ± SEM of three separate experiments. B, GCs were left untreated (CON) or treated for 10 min with 50 ng/ml FSH or 50 ng/ml EGF. Total cell lysates were probed with indicated antibodies. Results are representative of three separate experiments (two of which are shown) except for result using p-ERK5 (T218/Y220) antibody, which is from a single experiment.

mature follicles (see Fig. 3A). Moreover, overexpression of the S166D H-MDM2 mutant in GCs leads to significantly increased HIF-1 activity. Whereas increased HIF-1 activity likely results at least in part from an increase in HIF-1α protein, we cannot confirm an increase in HIF-1α protein because of the low transfection efficiency of primary GCs. It is unlikely, however, that an increase in HIF-1α protein is sufficient to increase HIF-1 activity, based on our results with IGF-I (that increases HIF-1α protein but not HIF-1 activity). Thus, the mechanism by which MDM2 increases HIF-1 activity is not known. FSH-stimulated phosphorylation of MDM2 at S166 may contribute to the ability of FSH to increase HIF-1 activity in GCs. However, proof that AKT-phosphorylated MDM2 participates in FSH-stimulated activation of HIF-1 activity requires the down-regulation of MDM2 and/or transduction of GCs with adenoviral-MDM2 S166A mutant.

We further examined the role of the PI3-kinase pathway in follicular maturation using the growth factor IGF-I. IGF-I has been shown to play a vital role in fertility. IGF-I knockout mice are infertile in part because of an inability to induce FSH receptor and to promote follicular maturation beyond the early antral stage (80). IGF-I has also been shown to synergize with FSH in up-regulating a number of follicular differentiation markers (81–83). However, IGF-I was not sufficient to promote HIF-1 activity despite its ability to promote accumulation of HIF-1α, as depicted in Fig. 10B. Moreover, IGF-I did not synergize with FSH in the induction of either HIF-1 or VEGF reporter activity. Importantly, our studies begin to differentiate between the responses elicited downstream of FSH *vs.* IGF-I in GCs.

Both FSH and IGF-I activate the PI3-kinase/AKT pathway leading to phosphorylation of tuberin and resulting activation of mTOR and downstream p70 S6-kinase and eukaryotic translation initiation factor-4E (9) and accumulation of HIF-1α (9) (see Figs. 4 and 5), phosphorylation of GSK 3α/β, phosphorylation of MDM2 (see Fig. 6A), and phosphorylation of FOXO1 (13) (Hunziker-Dunn, M., unpublished data) in GCs. The unexpected inability of IGF-I to promote HIF-1 activity and direct HIF-1 to the VEGF promoter in GCs, despite the increase in HIF-1α and phosphorylated MDM2 in response to IGF-I, indicates that IGF-I likely regulates an additional signal that inhibits HIF-1 activity (based on phospho-MDM2 results) and/or that FSH regulates signals to activate HIF-1 that are not activated by IGF-I.

Regulation of HIF-1 activity under hypoxia or normoxia via growth factors is incompletely understood. Whereas mTOR, via its binding partner raptor, has recently been reported to bind HIF-1α and to increase its transcriptional activity (84), both IGF-I and FSH activate mTOR and downstream targets, so mTOR/raptor is unlikely to distinguish signaling between IGF-I and FSH regarding activation of HIF-1. Many previous reports in a variety of cellular models have demonstrated a role of the ERK1/2 pathway in up-regulation of both HIF-1α protein levels and/or HIF-1 activity, based almost exclusively on results using PD98059 or constitutively active MEK1/2. Proposed roles for ERK1/2 include a role in HIF-1α mRNA or protein up-regulation (53, 55) in cooperation with the PI3-kinase pathway as well as trans-activation by direct phosphorylation of HIF-1α or of the required coactivator p300 (49–52, 54, 85, 86). However, the phosphorylation of HIF-1α by ERK1/2 is controversial and does not appear to correlate with HIF-1 activity, and p300 phosphorylation by ERK1/2 has not been shown in an intact cell model (54).

We found that FSH but not IGF-I activates ERK1/2 in GCs and thus considered ERK1/2 as a potential candidate to promote HIF-1 activation in GCs. Based on results using the MEK1/2 inhibitor PD98059 and constitutively active MEK1/2, the ERK1/2 pathway appears to contribute to FSH stimulation of HIF-1 activity in GCs, including the association of HIF-1 with the VEGF promoter, and in turn contributes to the up-regulation of VEGF promoter-reporter activity and VEGF mRNA. The ERK1/2 pathway, however, is not involved in FSH-mediated induction of HIF-1α protein in GCs. However, to our surprise, results in GCs using the recently developed and more selective MEK1/2 inhibitor PD184352 indicate that MEK1/2 and hence
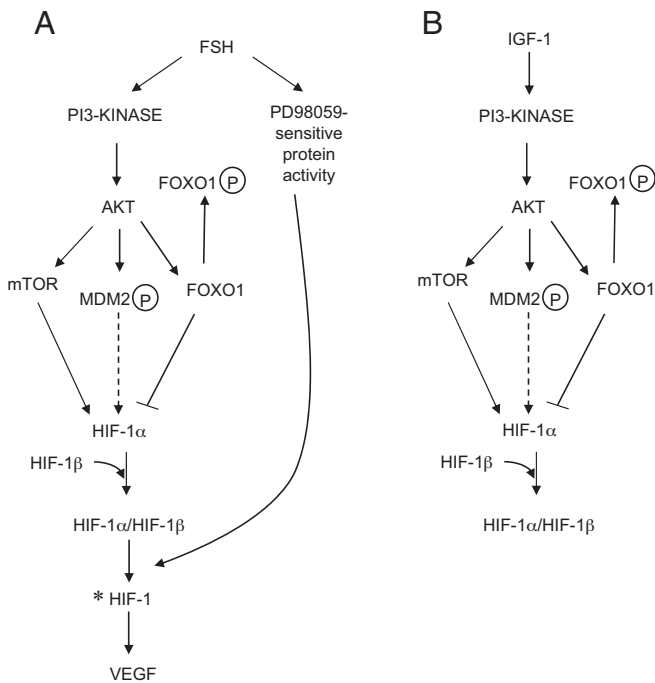
FIG. 10. Combinatorial effects of signaling pathways downstream of FSH lead to activation of the transcription factor HIF-1 leading to induction of HIF-1-responsive genes such as VEGF in GCs. Results support the schematic model in which FSH (A) via PI3-kinase/AKT leads to the accumulation of HIF-1α protein levels by inactivating FOXO1, by stimulating the translation of HIF-1α via mTOR, as previously described (9) and possibly by promoting the phosphorylation of MDM2. Activation of a PD98059-sensitive protein (kinase) activity is also required for HIF-1 activity (indicated by *asterisk*) to promote the interaction of HIF-1 with target promoters such as the VEGF promoter, leading to expression of VEGF. Whereas IGF-I promotes an accumulation of HIF-1α protein, IGF-I does not promote HIF-1 activity (B).

ERK1/2 is not necessary for FSH-stimulated HIF-1 activation and induction of VEGF mRNA. Our results thus suggest that an enzyme/kinase sensitive to PD98059 but distinct from MEK1/2 is necessary to activate HIF-1 in GCs. We tested whether this kinase could be MEK5 because this kinase is inhibited by PD98059 but not PD184352 (60), but our results show that FSH does not activate MEK5. Thus, the PD98059-sensitive protein (kinase) that is necessary for FSH-dependent HIF-1 activity remains elusive. It is likely that this protein is selectively regulated by FSH and not IGF-I in GCs. It is also expected that the PD98059-sensitive protein (kinase) activity that is necessary for HIF-1 activity in GCs is also required for HIF-1 activity in other cell models. Further work will be necessary to identify the PD98059-sensitive protein activity that contributes to FSH mediated HIF-1 activation.

We demonstrated previously (9) and in this report that $CoCl_2$ stabilized HIF-1α protein in GCs. We used this reagent to monitor the accumulation of HIF-1α because the half-life of HIF-1α under normoxic conditions is about 5 min (87). However, $CoCl_2$ like the proteosomal inhibitor MG115 (9) is not sufficient to induce HIF-1 activity to the degree that FSH does in GCs at 6 h nor does it synergize with FSH to activate HIF-1 (see Fig. 5B). $CoCl_2$ is sufficient to induce robust HIF-1 activity in other cellular models (62) and is commonly thought of as a hypoxia mimetic. Future studies will be required to determine whether physiological hypoxia seen by GCs in the avascular compartment of

the follicle synergizes with FSH to up-regulate specific HIF-1 targets or perhaps acts in another manner.

In summary, our results show that the PI3-kinase pathway has a complex role in HIF-1 induction. As summed in the model presented in Fig. 10, we can surmise from our results that the PI3-kinase pathway is necessary for HIF-1-mediated up-regulation of FSH target genes such as VEGF. Our results show that the PI3-kinase pathway is involved in HIF-1α protein up-regulation in GCs. In addition to the PI3-kinase/AKT target mTOR, our results show that the AKT target FOXO1 in its active conformation negatively affects the accumulation of HIF-1α in GCs. The AKT target MDM2 also increases HIF-1 activity; however, we do not know the mechanism(s) by which MDM2 achieves this function. We further demonstrate the necessity of a PD98059-sensitive protein (kinase) for HIF-1 activity independent of the PI3-kinase pathway and show that a PD98059-sensitive protein is required to induce binding of HIF-1 to the VEGF promoter in response to FSH. However, this PD98059-sensitive kinase is neither MEK1/2 nor MEK5. Identification of the protein activity that regulates HIF-1 activity in GCs and likely other cells awaits future studies. Further understanding of how FSH leads to the up-regulation of the transcription factor HIF-1 and target genes such as VEGF will help us to understand the role that these target genes play in the follicular maturation process as well as how dysregulation of this process can lead to disease.

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