IGF1R Signaling Is Necessary for FSH-Induced Activation of AKT and Differentiation of Human Cumulus Granulosa Cells

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Context: FSH is routinely administered to in vitro fertilization patients to induce follicle maturation. During this process, granulosa cells differentiate and acquire specific functional characteristics that are required to coordinate ovulation and oocyte maturation.

Objective: The objective of the study was to gain insight into the molecular mechanisms regulating human granulosa cell differentiation.

Design, Setting, Patients, and Interventions: Cumulus and mural granulosa cells were isolated from the follicular aspirates of in vitro fertilization patients and analyzed immediately or cultured in serum-free media in the presence of FSH, IGFs, or an inhibitor of type I IGF receptor (IGF1R) activity.

Main outcome: We quantified the mRNA and protein levels of steroidogenic enzymes, components of the IGF system, and gonadotropin receptors; measured 17β-estradiol levels; and examined the activation of intracellular signaling pathways to assess the granulosa cell differentiation as well as the FSH and IGF actions in both cumulus and mural cells.

Results: In freshly isolated cells, LH receptor (*Lhr*) and steroidogenic acute regulator (*Star*) were expressed at lower levels in cumulus than mural cells, whereas FSH receptor (*Fshr*) and anti-Müllerian hormone (*Amh*) were expressed at higher levels in cumulus than mural cells. In vitro, the expression of *Igf2*, the differentiation markers *Lhr*, *Star*, and *Cyp19a1* (aromatase) as well as 17β-estradiol production remained low in untreated cumulus cells but increased significantly after FSH treatment. Strikingly, this stimulatory effect of FSH was abolished by the inhibition of IGF1R activity. FSH-induced activation of v-*akt* murine thymoma viral oncogene homolog 3 (AKT) required IGF1R activity, and overexpression of constitutively active AKT rescued the induction of differentiation markers and 17β-estradiol production by FSH in the presence of the IGF1R inhibitor.

Conclusions: The cumulus cell response to FSH resembles the differentiation of preantral to preovulatory granulosa cells. This differentiation program requires IGF1R activity and subsequent AKT activation. **(*J Clin Endocrinol Metab* 99: 2995–3004, 2014)**

In the United States, infertility affects approximately 7% of couples (1), and in nearly 40% of these cases the cause of sterility is due to female factors, of which 15%–30% are related to ovulatory disorders (2). Treatments of ovulatory disorders include the direct stimulation of follicular development via the administration of FSH, which constitutes one of the most commonly used ovarian stimulation protocols for in vitro fertilization (IVF). Nearly

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Abbreviations: ACTB, β-actin; AEW, NVP-AEW451; ca, constitutively activated; hCG, human chorionic gonadotropin; IGF1R, type I IGF receptor; IVF, in vitro fertilization; OHSS, ovarian hyperstimulation syndrome; qPCR, quantitative PCR; StAR, steroidogenic acute regulator.

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100 000 women undergo IVF cycles each year in the United States, with a success rate of approximately 40% for patients younger than 35 years and less than 10% for patients older than 40 years (3). These treatments are expensive and impose significant physical, financial, and emotional burdens on infertile couples. Additionally, IVF treatments are associated with multiple births (4) and carry the risk of ovarian hyperstimulation syndrome (OHSS) (5). Major advances are therefore needed to improve the success rates, lower the costs, and eliminate the unfavorable risks of IVF.

During the final phases of follicle development, as preantral follicles mature into preovulatory follicles, the preantral granulosa cells differentiate into the mural granulosa cells that line the wall of the follicle. Differentiation of preantral granulosa into mural granulosa cells is driven mainly by FSH in close interaction with IGFs (6). In contrast, cumulus cells that surround the oocyte in the preovulatory follicle are spared from the differentiation effects of FSH and IGF by oocyte-secreted factors (7). The FSH-induced differentiation of mural granulosa cells plays a central and crucial role in fertility as mural cell-derived hormones coordinate oocyte maturation with ovulation and prepare the female reproductive tract to transport the sperm, facilitate fertilization, and support early embryo development (8). However, many aspects of follicular development and the process of mural granulosa cell differentiation are poorly understood, especially in humans.

Numerous reports have examined the effect of FSH on the function of human granulosa cells (9–12) recovered from follicular aspirates during IVF; however, these studies were conducted in mural granulosa cells, which have already initiated the process of luteinization and may not fully recapitulate the characteristic response of undifferentiated preantral granulosa cells to FSH. Granulosa cells isolated from preantral follicles are an ideal system to study the differentiation process, and in fact, this system has been used extensively in rodents. Although human preantral follicles can be isolated from ovarian cortical biopsies or oophorectomy (13), access to this tissue is extremely limited. Because of the paucity of a source of preantral undifferentiated granulosa cells for study, many aspects of the process of granulosa cell differentiation are not well understood in humans.

Previous studies have demonstrated that the oocyte plays a dominant role in establishing the heterogeneity of the granulosa cells found in preovulatory follicles by preventing the differentiation of the cumulus granulosa cells (14). Specifically, it has been shown that the oocyte suppresses FSH-induced LH receptor (*Lhr*), *Cyp19a1* (also known as aromatase), and *Cyp11a1* (also known as P450 side chain cleavage) mRNA expression (15, 16), which are well-known markers of differentiation. This influence of the oocyte on cumulus cells appears to be mediated primarily by oocyte-secreted factors, such as growth differentiation factor-9 and bone morphogenetic proteins, and is not dependent on continued close contact between them (17). Based on these findings, we hypothesized that culturing cumulus cells from preovulatory follicles is a suitable approach to study granulosa cell differentiation because they are no longer under the influence of oocyte-secreted factors. Here we tested this idea and demonstrated that cumulus cells obtained from IVF patients respond to FSH by expressing several markers of differentiation.

Using this experimental approach, we examined the molecular mechanisms underlying the interaction between gonadotropins and the IGF system in the regulation of granulosa cell differentiation in humans. FSH and IGFs cooperate to up-regulate the production of estradiol and progesterone beyond that of either factor alone in undifferentiated granulosa cells of several species, including rodent (18), porcine (19), and bovine (20). This conservation across species speaks to the importance of the interactions between FSH and IGFs in granulosa cell differentiation. Moreover, using rat granulosa cells, we recently demonstrated that FSH fails to stimulate *Cyp19a1* and *Cyp11a1* expression and 17β-estradiol production when the activity of the type I IGF receptor (IGF1R) is inhibited (21). In humans, the molecular mechanisms that may foster the cooperation between FSH and IGFs have not been investigated. Herein we explore the relationship between FSH and IGF signaling during human granulosa cell differentiation using cultured cumulus cells. Our results suggest that the IGF1R signaling is necessary for FSH-induced activation of the v-akt murine thymoma viral oncogene homolog 1 (AKT), a kinase shown to be essential for rat granulosa cell differentiation (22), and the subsequent differentiation of human granulosa cells to the mural/preovulatory stage.

Materials and Methods

Human granulosa cell isolation and culture

Primary cumulus and mural granulosa cells were collected from the follicular aspirates of women undergoing IVF at the University of Illinois under an institutional review board-exempt protocol. No clinical information about patients can be provided under this protocol. Patients underwent controlled ovarian hyperstimulation, and transvaginal oocyte retrieval was performed 35 hours after human chorionic gonadotropin (hCG) administration, at which point cumulus cells are still in contact with the oocyte (23), and mural cells have initiated the luteinization process (24), as previously described (25). Cumulus oocyte com-

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plexes were removed from the follicular aspirates and placed in a separate dish, and cumulus granulosa cells were mechanically separated from the oocyte. Cumulus granulosa cells from all cumulus oocyte complexes of an individual patient were pooled, resuspended in phenol-red free DMEM/F12, and gently pipetted to break up clusters of cells to yield individual cells. At the same time, the remainder of the follicular aspirates containing mural granulosa cells were collected and applied to a 50% Percoll cushion (Sigma-Aldrich) and centrifuged to remove erythrocytes. An aliquot of cells was diluted in 0.4% Trypan and counted using a hemacytometer to estimate the cell number and the percentage of viability. An average of 80% viability was found for both cumulus and mural cells. Cumulus and mural cells were seeded on extracellular matrix-coated tissue culture plates at a density of approximately 1×10^5 cells/mL in serum-free, phenol-red free DMEM/F12 media supplemented with penicillin (50 IU/mL; Mediatech); streptomycin (50 μg/mL; Mediatech); gentamicin sulfate (0.5 mg/mL; Mediatech); and androstenedione (1 μM), transferrin (5 μg/mL), and selenium (2 μg/mL). Cumulus and mural granulosa cells were cultured for 24 hours before treatments with recombinant FSH (Serono), NVP-AEW451 (AEW) (Cayman Chemical Co), MK2206 (Selleck Chemicals), or their combination for 48 hours as described in the figure legends. It is known that the maximal stimulatory effect of FSH on aromatase occurs at 48 hours (26, 27). Cells from different patients were cultured separately; therefore, each independent experiment represents results from cumulus or mural cells from a single patient.

Polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen) as stated in the manufacturer's protocol. One microgram total RNA was reverse transcribed at 42°C for 1 hour using Moloney murine leukemia virus reverse transcriptase (Invitrogen). The resulting cDNA was diluted to 100 μL in H_2O and 5μL was used for each reaction. Quantitative PCR (qPCR) was carried out and quantified as described previously (21). The results are reported as the relative expression of the gene to ribosomal protein *Rpl19*, an internal control, unless otherwise noted. For one of the figures (see Figure 4), PCR was performed using the same cycling parameters as for qPCR, and 1% agarose gel electrophoresis was carried out to visualize the reaction product. All of the designed primer pairs are intron spanning, and sequences can be provided upon request.

17β-Estradiol measurement

Cell culture medium from the wells of cultured cumulus and mural granulosa cells was collected at the experimental end point. Medium samples were diluted 1:150, and 17β-estradiol levels were determined using the estradiol ELISA kit (DRG Instruments GmbH) following the manufacturer's protocol.

Western blotting

Cultured cumulus cells were harvested in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer being supplemented with protease inhibitor cocktail (Sigma) and Halt phosphatase inhibitor cocktail (Thermo Scientific). Protein quantification and Western blotting of whole-cell lysates was carried out as previously described (28). The primary antibodies used for Western blotting were β-actin (ACTB) (1:5000; Abcam), AKT (1:2000), phospho-AKT(Ser473) (1:2000), ERK1/2 (1:2000), phospho-ERK1/2(Thr202/Tyr204) (1:2000), IGF1R (1:1000), or phospho-IGF1R (1:1000; Cell Signaling Technology). Antibodies used for steroidogenic enzymes were CYP19A1 (1:1000; Epitomics), CYP11A1 (1:1000; Millipore), and steroidogenic acute regulator (StAR; 1:1000) kindly donated by Dr B. Hales (29). The band intensities were quantified using Image Lab software (Bio-Rad Laboratories) and adjusted relative to ACTB.

Lentivirus induction

Constitutively activated (ca)-AKT lentiviral construct, kindly provided by Michael Robinson (Children's Hospital of Philadelphia, Philadelphia, Pennsylvania), was subcloned into the pTY-CMV lentiviral transfer plasmid. The lentivirus containing this construct was generated in human embryonic kidney-293 cells and concentrated by ultracentrifugation. caAKT- or green fluorescent protein-expressing (control) lentivirus was added directly to cumulus granulosa cells 2 hours after plating. Cells were treated 36 hours after viral transduction, as specified in the figure legend.

Statistical analyses

Each experiment was run at least in triplicate, and data for continuous variables are presented as mean values ± SEM. Statistical comparisons of mean values between groups were performed with *t* tests, and multiple comparisons were performed with one-way ANOVA. Differences were considered to be statistically significant if the value was $P < .05$.

Results

Expression of differentiation markers in human cumulus and mural granulosa cells

To determine whether cumulus cells are protected from the differentiating effect of FSH during follicle maturation in humans, we evaluated the expression of differentiation and luteinization markers in human cumulus and mural cells obtained from preovulatory follicles. The results demonstrated that, despite the down-regulation of *Lhr* expression that occurs in mural cells during luteinization (30), cumulus cells expressed significantly lower levels of *Lhr* when compared with mural cells (Figure 1A). In contrast, the expression of *Cyp19a1* and *Fshr* remain highly expressed in cumulus cells with respect to mural cells. In addition, the expression of *Cyp11a1* and *Star*, which are required for progesterone production in luteal cells, was higher in mural than in cumulus cells. Finally, the expression of anti-Müllerian hormone (*Amh*), whose expression decreases toward the preovulatory stage, was 4-fold higher in cumulus than in mural cells (Figure 1A). The fact that cumulus cells express low levels of *Lhr* but retain *Cyp19a1*, *Fshr*, and *Amh* expression suggests that they do not undergo luteinization, even after hCG administration; in contrast, their gene expression pattern resembles that of granulosa cells undergoing preantral to preovulatory differentiation.

Next, we examined whether cumulus cells maintain an undifferentiated phenotype in culture. As shown in Figure

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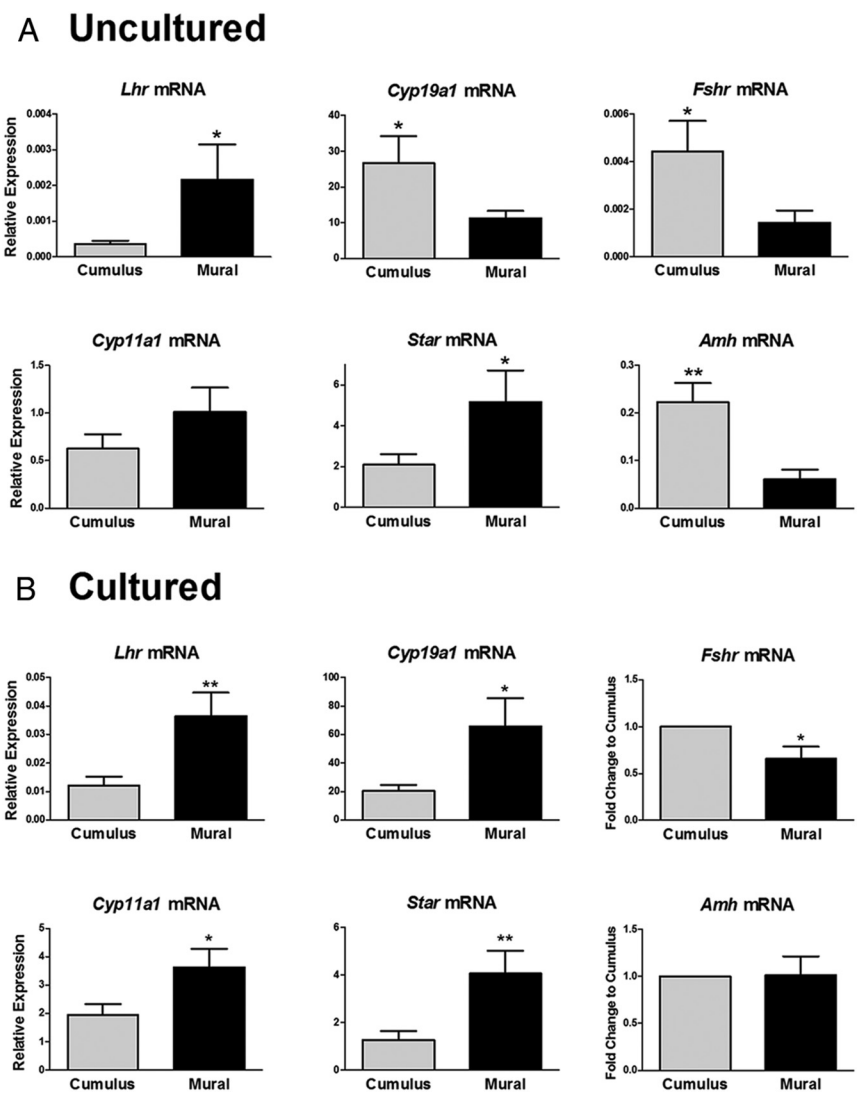

Figure 1. Cumulus and mural cells have distinct gene expression profiles. A, RNA was isolated from freshly purified, uncultured cumulus and mural cells collected from patients undergoing IVF. The expression of key gene markers of granulosa cell differentiation and luteinization, including *Lhr*, *Cyp19a1*, *Fshr*, *Cyp11a1*, *Star*, and *Amh*, were measured by qPCR. Columns represent the mean ± SEM for six independent experiments. B, RNA was isolated from cumulus and mural granulosa cells after culture in serum-free media for 72 hours, and the expression of key differentiation and luteinization genes was measured by qPCR. Columns represent the mean ± SEM for 14 independent experiments. *, $P < .05$; **, $P < .01$, *t* test.

We next assessed whether FSH is able to stimulate the expression of differentiation markers in cultured cumulus cells. Treatment of cumulus cells with FSH for 48 hours significantly increased the expression of *Cyp19a1*, *Cyp11a1*, and *Star* mRNA (Figure 2B). In contrast, FSH had no effect on the expression of these genes in mural cells. Treatment of cumulus cells with FSH for 48 hours also led to an increase in the protein levels of CYP19A1, CYP11A1, and StAR (Figure 2C). In conjunction with the significant increase in CYP19A1 expression, 17β-estradiol levels in the culture medium of cumulus cells treated with FSH was significantly higher than in the culture medium of control cells (Figure 2D).

These findings indicate that freshly isolated cumulus granulosa cells express lower levels of differentiation genes compared with mural granulosa cells and that incubation of these cells in serum-free media leads to further dedifferentiation. Dedifferentiated cumulus cells respond to FSH by up-regulating the expression of several mural granulosa cell markers, supporting the idea that cultured cumulus granulosa cells behave as granulosa cells from preantral or early antral follicles. Thus, we propose that cultured cumulus cells can be used to explore the mechanisms by which FSH stimulates human granulosa cell differentiation. In particular, we wanted to determine the relationship between FSH and the IGF system in the process of granulosa cell differentiation in humans.

1B, cumulus cells cultured for 72 hours in serum-free media expressed significantly lower levels of *Lhr*, *Cyp19a1*, *Cyp11a1*, and *Star* than mural cells. Moreover, the relative expression of *Cyp19a1*, a marker of preovulatory differentiation, was decreased in cultured cumulus cells when compared with freshly isolated cells. *Amh* mRNA expression was similar in cultured cumulus and mural cells. Furthermore, the steady-state levels of the *Fshr* mRNA in cumulus cells cultured in serum-free increased at 48 hours and remained highly expressed 96 hours later (Figure 2A). Together these findings suggest that cultured cumulus cells resemble undifferentiated granulosa cells from early antral follicles.

Cultured cumulus cells express *Igf2* but not *Igf1*

Prior to exploring the relationship between FSH and the IGFs, we assessed the expression of key components of the IGF system in cumulus granulosa cells. Previous findings have demonstrated that human granulosa cells express exclusively *Igf2* (31) and that FSH stimulates *Igf2* expression in small antral (class 2) human follicles (32). We found that cultured cumulus granulosa cells expressed

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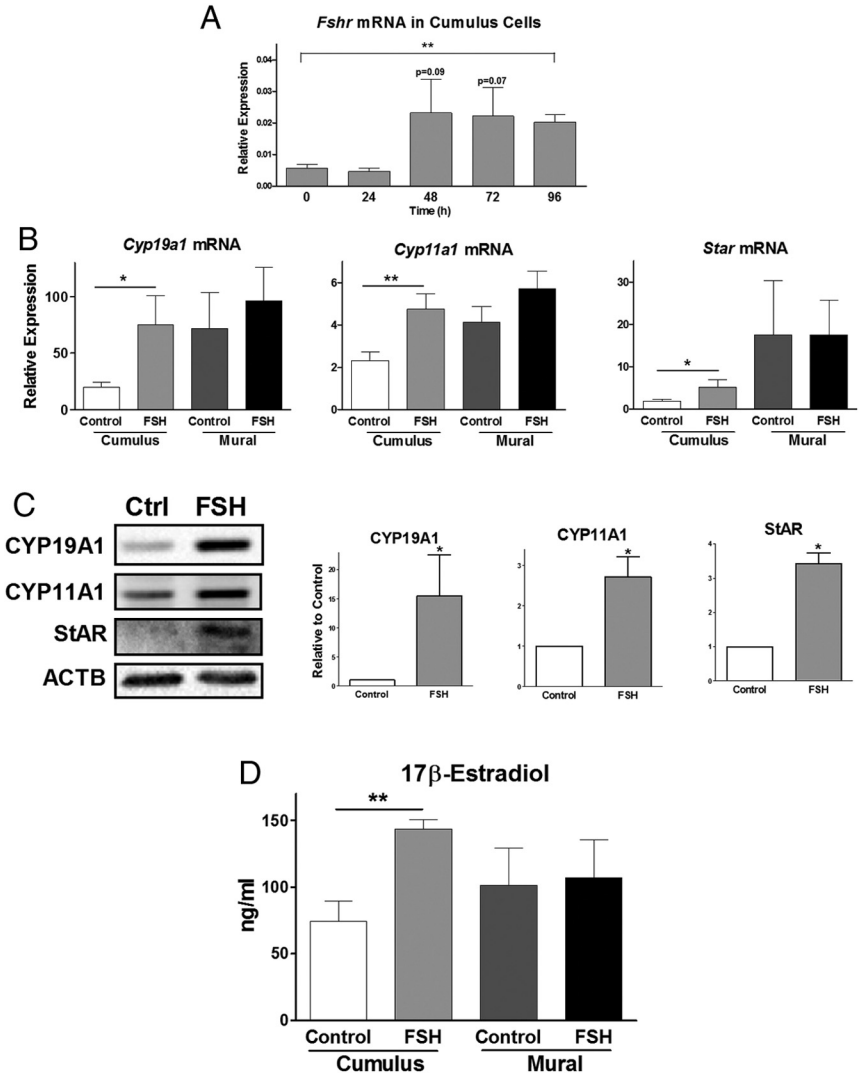

Figure 2. Cumulus cells differentiate in response to FSH. A, *Fshr* expression was measured in cumulus cells cultured for increasing periods of time (0–96 h) by qPCR. Columns represent the mean ± SEM for three independent experiments. B, Cumulus and mural cells were cultured in serum-free media for 24 hours and then treated with FSH (50 ng/mL) or left untreated (control) for 48 hours. RNA was isolated from the cells, and the expression of *Cyp19a1*, *Cyp11a1*, and *Star* was measured by qPCR. Columns represent the mean ± SEM for 10 independent experiments. C, Cumulus cells were cultured in serum-free media for 24 hours and then treated with FSH (50 ng/mL) or left untreated (Ctrl) for 48 hours. Total protein was isolated from these cells and CYP19A1, CYP11A1, StAR, and ACTB protein levels were assessed by Western blot. Columns represent the mean ± SEM for three independent experiments. D, 17β-Estradiol levels were measured in media collected from cultures of cumulus and mural cells that received FSH treatment (50 ng/mL) or no treatment (control) for 48 hours. Columns represent the mean ± SEM for three independent experiments. *, $P < .05$; **, $P < .01$, *t* test.

Igf2 as well as *Igf1r* and *Igf2r*, but not *Igf1* (Figure 3A). Additionally, we demonstrated that these cells expressed significantly higher levels of *Igf1r* than *Igf2r*. Furthermore, we observed that FSH induced a 3-fold increase in *Igf2* expression, but it had no effect on the expression of either receptor (Figure 3B), suggesting that gonadotropins could modulate the IGF pathway in granulosa cells by up-regulating *Igf2*. These results provide further support for the use of cultured cumulus cells as an approach to examine FSH and IGF action in human granulosa cells.

IGF1R activity is necessary for FSH-induced expression of steroidogenic genes in cumulus cells

Because FSH strongly stimulates *Igf2* expression in human cumulus cells, we investigated whether the activation of IGF1R by endogenous IGF-II plays a role in the regulation of differentiation genes by FSH. For this purpose, cells were stimulated with FSH in the presence or absence of AEW, a highly specific inhibitor of IGF1R activity (33). We found that the FSH-induced up-regulation of *Cyp19a1*, *Cyp11a1*, and *Star* mRNA levels was inhibited by cotreatment with AEW (Figure 4A). Further experiments demonstrated that AEW inhibits FSH-induced *Cyp19a1* expression in a dose-dependent (0.05–2 μM) manner (data not shown). In agreement with our findings at the level of mRNA expression, the stimulation of CYP19A1, CYP11A1, and StAR protein expression by FSH was significantly decreased by cotreatment with AEW (Figure 4B). Finally, the inhibition of the IGF1R prevented the increase of 17β-estradiol production induced by FSH (Figure 4C). These findings suggest that IGF1R activity is required for the induction of well-known markers of granulosa cell differentiation and for the stimulation of 17β-estradiol synthesis by FSH in human cumulus granulosa cells.

FSH-induced AKT phosphorylation requires IGF1R activity

To investigate the mechanisms by which IGF1R may regulate FSH-induced granulosa cell differentiation, we first examined whether FSH enhances the phosphorylation of the IGF1R itself. In good agreement with our previous report in rat granulosa cells (21), we found that FSH does not phosphorylate the IGF1R (Figure 5A), suggesting that the interaction between FSH and IGF1R signaling occurs downstream of this receptor. Therefore, we examined the effect of either FSH or AEW alone or combined on

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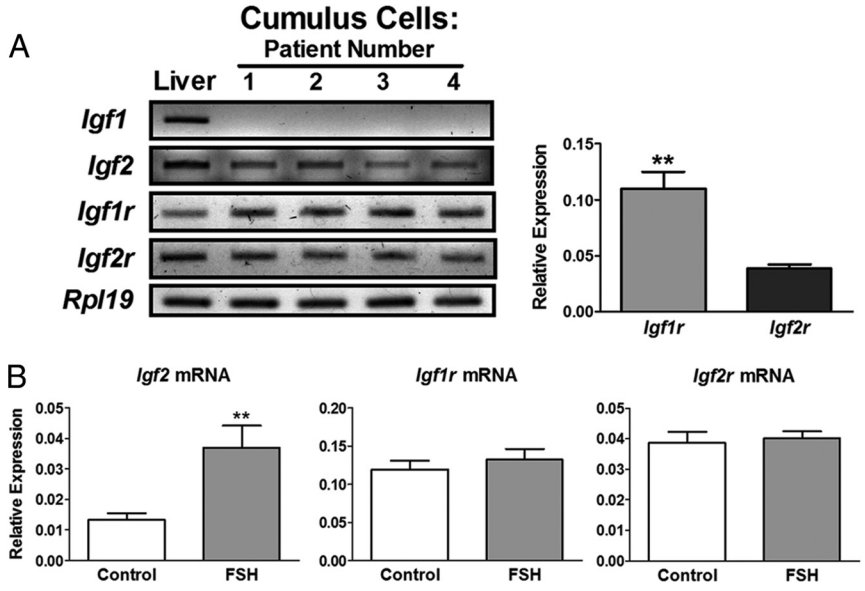

Figure 3. Cumulus granulosa cells express *Igf2* but not *Igf1*. Cumulus cells were cultured in serum-free media for 72 hours before RNA isolation. A, Cumulus cell RNA from four different patients as well as human liver RNA was reverse transcribed, and PCR was used to detect expression of *Igf1*, *Igf2*, *Igf1r*, *Igf2r*, and the housekeeping gene *Rpl19*. PCR products were visualized on agarose gels. *Igf1r* and *Igf2r* expression levels in cumulus cells were quantified by qPCR. B, Cumulus cells were cultured in serum-free media for 24 hours and then treated with FSH (50 ng/mL) or left untreated for 48 hours. RNA was isolated from these cells, and the expression of *Igf2*, *Igf1r*, and *Igf2r* was measured by qPCR. For qPCR results, columns represent the mean ± SEM of at least seven independent experiments. **, $P < .01$, *t* test.

the activation of AKT, a well-known downstream target of IGF1R and an essential component of FSH-mediated rat granulosa cell differentiation (21, 22). Phospho-S473AKT was low in untreated cumulus cells but increased significantly after treatment with FSH for 1 hour (Figure 5A, right panel). Remarkably, the inhibition of IGF1R activity by AEW not only prevented AKT activation by FSH but also reduced AKT phosphorylation to undetectable levels in cells treated with AEW alone. In marked contrast, lack of IGF1R activity had no effect on the phosphorylation of ERK1/2 by FSH (Figure 5A). This result represents the first demonstration of a stimulatory effect of FSH on AKT activity in human granulosa cells. These findings also suggest that basal IGF1R and AKT activation are likely maintained by locally produced IGF-II and that IGF1R activity is required for FSH-induced AKT phosphorylation.

Next, we compared the effect of AEW, the IGF1R inhibitor, with that of an AKT-specific inhibitor, MK2206. As shown in Figure 5B, inhibition of either IGF1R or AKT activity prevented the stimulatory effect of FSH on *Cyp19a1* mRNA accumulation as well as CYP19A1 activity, as indicated by the significantly suppressed production of 17β-estradiol in the presence of IGF1R and AKT inhibitors (Figure 5B, right panel). This finding suggests that AKT activation is required for the stimulation of granulosa cell differentiation by FSH in humans.

To test this possibility, we overexpressed an active form of AKT to investigate whether it could overcome the inhibitory effect of AEW on FSH-induced *Cyp19a1* expression. For this purpose, cumulus cells were infected with increasing concentrations of lentivirus carrying a construct encoding human caAKT (21). As shown in Figure 5C, in the presence of AEW, caAKT rescued the stimulatory effect of FSH on *Cyp19a1*expression in a dose dependent manner. The response to caAKT varied among patients such that in some patients caAKT restored *Cyp19a1* expression to the levels observed after FSH treatment alone, whereas in others, FSH plus caAKT increased *Cyp19a1* expression to levels significantly higher than those stimulated by FSH alone. Despite these individual differences, caAKT was able to overcome the inhibitory effect of AEW in four experiments conducted using cells from different patients (Figure 5C and data not shown). Moreover, overexpression of caAKT also rescued 17β-estradiol production in the presence of AEW (Figure 5C, right panels). This effect of caAKT on 17β-estradiol production followed a similar pattern between patients to that observed for *Cyp19a1* expression.

Discussion

Herein we report findings that significantly advance our understanding of granulosa cell differentiation in humans. First, we showed that cumulus cells from preovulatory follicles do not luteinize after exposure to the ovulation trigger hCG in vivo and that, once separated from the oocyte, these cells respond in vitro to FSH with an increase in the expression of differentiation markers and 17β-estradiol production. Therefore, cumulus cells are a useful experimental approach to study human granulosa cell differentiation. Second, we demonstrated that the induction of human granulosa cell differentiation by FSH depends on the simultaneous activation of IGF1R and its main downstream target AKT.

Currently a robust approach to study human granulosa cell differentiation is lacking, largely due to the paucity of preantral undifferentiated granulosa cells available for

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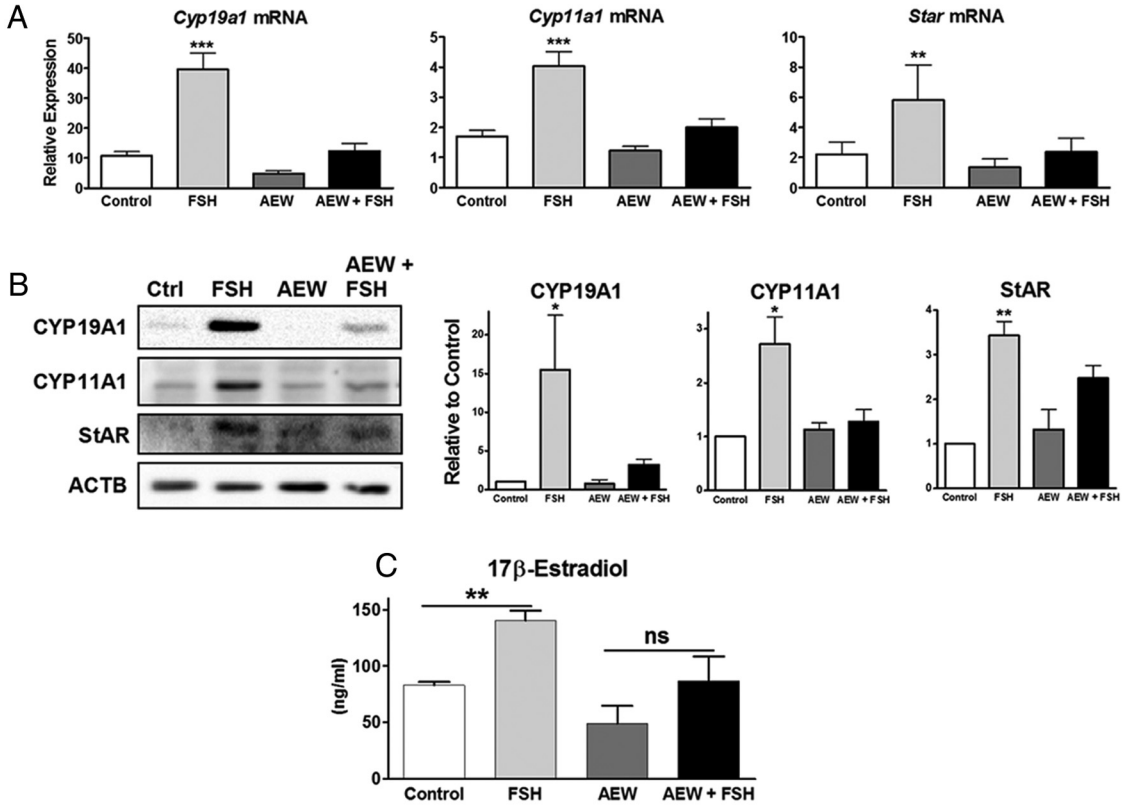

Figure 4. FSH action in cumulus granulosa cells requires IGF1R activity. Cumulus cells were cultured in serum-free media for 24 hours and then pretreated with AEW (0.5 μM) or vehicle (dimethyl sulfoxide, DMSO) for 1 hour before the addition of FSH (50 ng/mL) for 48 hours. RNA (A) and total protein (B) were isolated and the mRNA and protein levels of CYP19A1, CYP11A1, and StAR were measured by qPCR and Western blot. For mRNA quantification, columns represent the mean ± SEM for five independent experiments. For protein quantification, columns represent the mean ± SEM for the same three independent experiments presented in Figure 2. C, 17β-Estradiol levels were measured in media collected from cultures of cumulus cells by ELISA. Columns represent the mean ± SEM for three independent experiments. *, $P < .05$; **, $P < .01$; ***, $P < .001$, one-way ANOVA.

study. Although preantral follicles can be isolated from ovarian cortical biopsies (13), access to this tissue is rare. In contrast, cumulus granulosa cells are readily available from the follicular aspirates of IVF patients. Our current understanding of the actions of FSH and other factors in human granulosa cells has been deduced from studies in cultured mural granulosa cells that have undergone luteinization. Breckwoldt et al (12) demonstrate that these cells regain response to gonadotropins after long periods of hormone-free culture; however, luteinization is irreversible. Consequently, mural cells are not an ideal model to study preantral to preovulatory granulosa cell differentiation.

Our findings suggest that human cumulus cells are protected from FSH-induced differentiation and subsequent hCG-induced luteinization. Gene expression studies in freshly isolated cumulus and mural cells revealed that cumulus cells expressed lower levels of *Lhr* as well as key genes necessary for progesterone synthesis, indicating that they had not begun the luteinization process. In fact, we speculate that cumulus cells are not able to luteinize because of the low expression of *Lhr*. Moreover, our findings suggest that cumulus cells have not undergone full differentiation to the preovulatory stage because *Amh* expression, which is strongest in preantral and small antral follicles but wanes in preovulatory follicles (34), is maintained in cumulus cells at significantly higher levels than in mural cells. This finding suggests that cumulus cells resemble granulosa cells from early antral follicles.

We also present evidence suggesting that when cultured free of the influence of the oocyte, cumulus cells further dedifferentiate, whereas mural cells maintain a luteinized phenotype. Thus, cumulus cells maintained in culture for 72 hours expressed low levels of the differentiation markers *Cyp19a1*, *Cyp11a1*, *Star*, and *Lhr*. Additionally, we demonstrated that the expression of these genes is significantly stimulated by FSH only in cumulus cells. Taken together, the fact that cumulus granulosa cells are protected from the influence of gonadotropins in vivo, further dedifferentiate in culture, and differentiate in response to FSH suggest that cultured cumulus cells from preovulatory follicles are an adequate and physiologically relevant approach to gain insight into the process of human granulosa cell differentiation.

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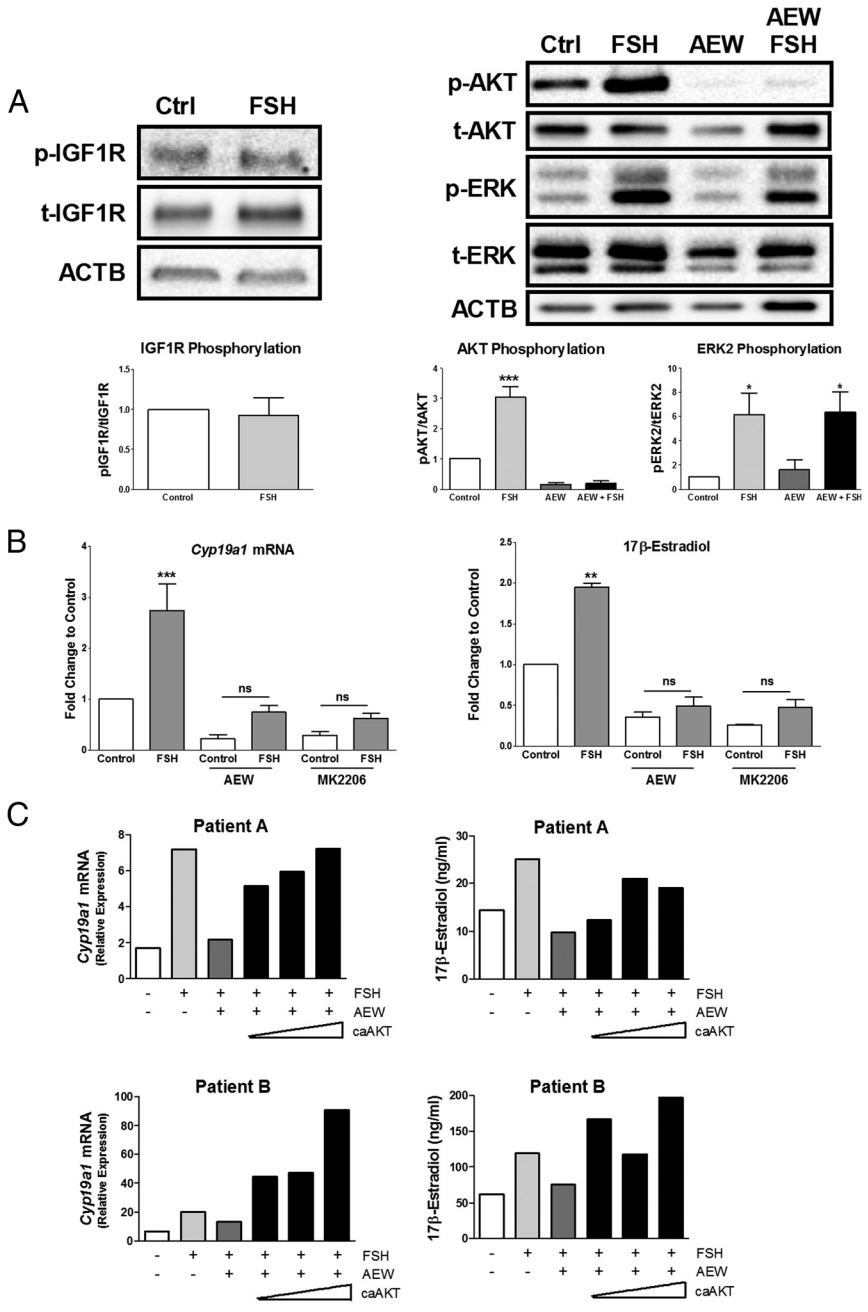

Figure 5. FSH-induced AKT phosphorylation requires IGF1R activity. A, left panel, Cumulus cells were cultured in serum-free media for 24 hours and then treated with FSH (50 ng/mL) for 1 hour. Total protein was isolated from these cells and phosphorylated and total IGF1R were detected by Western blot. Protein levels were normalized to ACTB, and the ratio of phosphorylated to total IGF1R is reported. Columns represent the mean ± SEM for three independent experiments. A, right panel, Cumulus cells were cultured in serum-free media for 24 hours and pretreated with AEW (0.5 μM) or vehicle (dimethylsulfoxide) for 1 hour before the addition of FSH (50 mg/mL) for 1 hour. Phosphorylated and total AKT and ERK were assessed by Western blot of whole-cell lysates. Protein levels were normalized to ACTB, and the ratio of phosphorylated to total AKT and ERK are reported. Columns represent the mean ± SEM for three independent experiments. B, Cumulus granulosa cells were cultured in serum-free media for 24 hours and then pretreated with AEW (0.5 μM), MK2206 (1 μM), or vehicle (dimethyl sulfoxide, DMSO) for 1 hour followed by treatment with FSH (50 ng/mL) for 48 hours. RNA was isolated from the cells, and the expression of *Cyp19a1* was measured by qPCR. Columns represent the mean ± SEM for five independent experiments. 17β-Estradiol was measured in media collected from treated cells, and columns represent the mean ± SEM for two patients. C, Cumulus granulosa cells were cultured in serum-free media for 24 hours and then pretreated with AEW (0.5 μM) for 1 hour before the addition of FSH (50 ng/mL) for 48 hours in the presence of lentivirus-expressing green fluorescent protein or caAKT. The expression of *Cyp19a1* as well as the production of 17β-estradiol is reported for two patients. *, $P < .05$; **, $P < .01$; ***, $P < .001$, one-way ANOVA. Ctrl, control; ns, not significant.

We used cumulus cells to gain an understanding of the relationship between FSH and IGF action in the process of granulosa cell differentiation. Previous studies demonstrated that IGFs can synergize with FSH to stimulate CYP19A1 activity (10), progesterone production (11), and inhibin B secretion (35) in humans; however, mechanisms underlying this synergistic relationship were not explored. Here we report that IGF1R activity is required for FSH-induced granulosa cell differentiation. In the presence of AEW, an inhibitor of IGF1R activity, FSH is unable to stimulate the expression of steroidogenic genes and 17β-estradiol production, which are essential for proper function of preovulatory mural granulosa cells. Our results provide a possible mechanism to explain the inhibitory effect of IGF binding proteins or anti-IGF1R antibodies in FSH-induced steroidogenesis in humans (31, 36); however, the molecular mechanisms involved in the integration of IGF1R and FSHR signaling are not known.

In this regard, our findings provide evidence suggesting that FSHR and IGF1R signaling synergistically activate AKT and that this cooperation is essential for human granulosa cell differentiation. The FSHR activates adenylyl cyclase activity resulting in an increase of intracellular cAMP and the subsequent activation of protein kinase A, which in turn activates cAMP response element-binding protein (CREB) and ERK1/2 (37). The IGF1R is a member of the tyrosine kinase receptor family that, once activated by either IGF-I or IGF-II, stimulates both the MAPK/ERK1/2 and phosphatidylinositol 3-kinase/AKT pathways. Previous reports in rodents demonstrated that FSH also stimulates AKT (37) and that the activity of this kinase is essential for the induction of 3β-hy-

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droxysteroid dehydrogenase, α-inhibin, and LHR by FSH (22, 38, 39). Here we demonstrate that AKT activity is necessary for FSH-induced *Cyp19a1* expression in humans, suggesting that AKT is a pivotal factor underlying the cooperation of FSH and the IGF system in human granulosa cell differentiation as well.

We demonstrate that FSH increases AKT phosphorylation in cultured cumulus granulosa cells, but the mechanisms by which FSH stimulates AKT are unclear. We observe AKT phosphorylation in untreated cumulus cells, which is eliminated by incubation with an IGF1R inhibitor, suggesting that endogenous IGF-II acts in an autocrine manner to stimulate the IGF1R and AKT. Strikingly, FSH is unable to stimulate AKT in the presence of the IGF1R inhibitor. This suggests that FSH does not phosphorylate AKT directly; rather, it enhances IGF1R-mediated AKT activation in human granulosa cells. Studies in rodents suggest that FSH could enhance AKT activation by down-regulating phosphatases to increase IGF1R phosphorylation and activity (40). Additionally, FSH could stimulate AKT phosphorylation by activating insulin receptor substrate-1 through a protein kinase A-dependent mechanism (41). Further studies are warranted to determine the molecular cross talk between FSH and IGF1R signaling that leads to AKT phosphorylation in both rodent and human granulosa cells.

These observations have critical implications for granulosa cell differentiation and represent a shift in the current understanding of how FSH promotes human follicle development. We have provided evidence demonstrating that the IGF system does not merely enhance FSH actions but that it is essential for FSH-induced granulosa cell differentiation. The activation of AKT by IGF-II through the IGF1R may prime granulosa cells to adequately respond to FSH resulting in granulosa cell differentiation and follicle development. We propose that FSH amplifies IGF1R signaling and/or removes inhibitory influences present in unstimulated cells. This hypothesis implies that only those follicles exposed to increasing levels of FSH can benefit from locally produced IGF-II, and only follicles with high endogenous IGF-II production are able to fully respond to FSH. Thus, the interaction between the endocrine effects of FSH and the autocrine actions of IGF-II might play an essential role in the establishment of selection and follicle dominance in humans.

Our findings could lead to the identification of new targets and therapies to improve the success of IVF protocols. Thus, it is possible to suggest that intrafollicular IGF-II levels and/or IGF1R expression/activity could be predictors of a patient's response to FSH stimulation during controlled ovarian stimulation. This is supported in the clinic by observations in patients with polycystic ovarian syndrome, who tend to have higher circulating IGF-II levels (42) and have an increased risk for developing OHSS after FSH administration (43). In the presence of increased IGF1R activation, a result of high levels of IGF-II, FSH could stimulate steroidogenesis beyond normal levels, rendering patients more susceptible to OHSS. On the other hand, patients who do not respond well to FSH stimulation may have an underlying insufficiency in the activation of the IGF system or in the local production of IGF-II. These examples point to the importance of gaining an understanding of FSH action, along with other critical factors, in granulosa cells that may impact IVF success while minimizing patient risk.

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