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# Insulin-like growth factor 1 enhances follicle-stimulating hormone-induced phosphorylation of GATA4 in rat granulosa cells

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## Abstract

Preovulatory granulosa cell (GC) differentiation is essential for the maturation and release of oocytes from the ovary. We have previously demonstrated that follicle-stimulating hormone (FSH) and insulin-like growth factors (IGFs) closely interact to control GC function. Similarly, we showed that GATA4 mediates FSH actions and it is required for preovulatory follicle formation. This report aimed to determine *in vivo* the effect of FSH on GATA4 phosphorylation and to investigate whether FSH and IGF1 interact to regulate GATA4 activity. In rat ovaries, treatment with equine chorionic gonadotropin (eCG) increased the phosphorylation of GATA4, which was confined to the nucleus of GCs. Using primary rat GCs, we observed that GATA4 phosphorylation at serine 105 increases the transcriptional activity of this transcription factor. Like FSH, IGF1 stimulated GATA4 phosphorylation at serine 105. Interestingly, GATA4 phosphorylation was significantly higher in cells cotreated with FSH and IGF1 when compared to FSH or IGF1 alone, suggesting that IGF1 augments the effects of FSH on GATA4. It was also found that the enhancing effect of IGF1 requires AKT activity and is mimicked by the inhibition of glycogen synthase kinase-3 β (GSK3β), suggesting that AKT inhibition of GSK3β may play a role in the regulation of GATA4 phosphorylation. The data support an important role of the IGF1/AKT/GSK3β signaling pathway in the regulation of GATA4 transcriptional activity and provide new insights into the mechanisms by which FSH and IGF1 regulate GC differentiation. Our findings suggest that GATA4 transcriptional activation may, at least partially, mediate AKT actions in GCs.

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Disclosure summary

The authors have nothing to disclose.

CRediT authorship contribution statement

**Scott Convissar:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, preparation, Writing – review & editing. **Jill Bennett-Toomey:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, preparation, Writing – review & editing. **Carlos Stocco:** Conceptualization, Formal analysis, Investigation, Writing – original draft, preparation, Writing – review & editing, Project administration, Funding acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2022.111807.

**Keywords**

Ovary; Granulosa cells; FSH; IGF1; GATA4; Cyp19a1; AKT

## 1. Introduction

During the final phases of ovarian follicle development, as preantral follicles mature into preovulatory follicles, the granulosa cells (GCs) differentiate into the mural GCs that line the wall of the follicle. As GCs differentiate they activate the *Cyp19a1* gene, which is encoded for the enzyme CYP19A1, also known as aromatase. Preovulatory GC differentiation and the expression of *Cyp19a1* are primarily driven by follicle-stimulating hormone (FSH). The FSH-induced differentiation of mural GCs plays a central and crucial role in fertility as these cells produce hormones such as estradiol, inhibin, and progesterone needed to coordinate oocyte maturation and ovulation by preparing the female reproductive tract to transport sperm, facilitate fertilization, and support early embryo development (Stocco, 2012; Baumgarten and Stocco, 2018). However, many aspects of follicular development and the process of mural GC differentiation are poorly understood.

Members of the GATA family of zinc finger transcription factors regulate critical steps of cellular differentiation during vertebrate development (Molkentin, 2000). Among the six members of the GATA family, GATA4 and GATA6 are highly expressed in ovarian GCs (Anttonen et al., 2003, Bennett et al., 2012, Heikinheimo et al., 1997, Lavoie et al., 2004). GATA4 and GATA6 activate the transcription of several gonadal genes (Kwintkiewicz et al., 2007,Tremblay and Viger, 2001); thus, their loss in GCs impairs folliculogenesis, which reduces fertility in mice (Bennett et al., 2012). Moreover, deletion of GATA4 and GATA6 in GCs of mice blocks folliculogenesis at the preantral or early antral stage (Bennett et al., 2012). Mechanistically, we showed that in the absence of GATA4, the mRNA and protein expression of the FSH receptor decreases significantly (Bennett et al., 2012), suggesting that GATA4 plays an essential role in the regulation of the response of GCs to FSH. Reciprocally, we have also shown that FSH phosphorylates GATA4 at serine 105, a modification that increases its transcriptional activity (Kwintkiewicz et al., 2007; Liang et al., 2001). These findings reveal that a positive feedback loop between FSH and GATA4 may be essential for GC differentiation.

Insulin-like growth factor 1 (IGF1) is an important regulator of body and organ size during postnatal development (Riedemann and Macaulay, 2006). In the ovary, we have demonstrated that IGF1 promotes GC differentiation and is required for FSH-induced stimulation of follicle growth (Zhou et al., 2013). Thus, similarly to the knockdown of GATA4, we showed that IGF1 receptor (IGF1R) activity is necessary for FSH to stimulate the expression of differentiation markers, including *Cyp19a1*, acute steroidogenic regulatory protein (Stard1, also known as StAR), and cholesterol side chain cleavage (CYP11a1, also known as P450scc) (Bennett et al., 2013). Remarkably, each of these genes is regulated by GATA factors (Lavoie et al., 2004; Silverman et al., 1999; Martin et al., 2005; Sher et al., 2007; Lavoie and King, 2009). Collectively, the evidence suggested that GATA4 and the IGF1 pathway may be linked throughout the preovulatory differentiation of GCs, which

led us to hypothesize that FSH and IGFs interact to control GATA4 transcriptional activity. This study aimed to further evaluate the regulation of GATA4 activity in ovarian GCs, specifically, to determine the effects and molecular signaling involved in the regulation of GATA4 by FSH and IGFs.

## 2. Material and methods

### GCs cultures –

GCs were isolated from 23 to 25 days old estradiol-treated immature rats and cultured as described previously (Bennett et al., 2012, 2013; Wu et al., 2013). The use of GCs from estradiol-treated immature rats is a well-established and valuable approach that provides an *in vitro* model for examining GC differentiation and the mechanisms involved in the regulation of GCs by FSH (Sanders and Midgley, 1982). Cells were treated with purified ovine FSH, IGF1, forskolin (FSK), dbcAMP, NVP-AEW541 (AEW), MK-2206, U0126, or SB216763 as described in the figure legends. All inhibitors and hormones were obtained from Tocris (Bristol, United Kingdom). The Institutional Animal Care and Use Committee at the University of Illinois at Chicago approved all animal experiments (ACC number: 20–173).

### RNA isolation and quantification –

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and reverse-transcribed using anchored oligo-dT primers (IDT, Coralville, IA) and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). Intron-spanning primers were used to amplify the gene of interest (GOI). The purified GOI cDNA was used for the generation of standard curves for each gene. Real-time PCR amplifications were performed with Brilliant II qPCR SYBR master mix (Agilent, Santa Clara, CA) using an AriaMx instrument (Agilent). For each sample, the number of cDNA copies corresponding to 10 ng of total RNA was computed for each GOI and ribosomal protein L19 (*Rpl19*) as an internal control. The expression of each GOI is reported as the ratio between the number of copies of the GOI and *Rpl19*.

### Promoter reporter assays –

The promoter region for the *cyp19* gene was cloned from genomic DNA using the following primers: forward — GCT CGA GCC ACA GAG ATC CTG ACA ACC; reverse — GAA GCT TTG TGG TAT TTT GCC TCA GAA GG. These primers amplify the region between −1100 and +63 of the C*yp19a1* gene, where +1 is the transcription initiation site (Fitzpatrick and Richards, 1993). Primers were designed based on a published sequence of the rat aromatase *Cyp19a1* promoter (Young and McPhaul, 1998). PCR products were cloned into pCS-LUC lentivirus using XhoI and HindIII restriction sites (CYP19-Luc) (Zhou et al., 2013). The GATA binding site found on the *Cyp19a1* promoter was mutated (GATA to tgTA) using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Lentivirus stocks were generated in 293FT cells (Invitrogen) cotransfected with CYP19a1-Luc or mGATA-CYP19a1-Luc lentivirus plasmid along with psPAX2 (packaging) and envelope (pMD2.G) plasmids (Addgene, Watertown, MA). Lentiviruses in 293FT supernatants were concentrated by ultracentrifugation. Viral stocks were titrated in 293FT cells aided by a green fluorescent protein marker present in the lentivirus plasmids. GCs

were infected with lentiviruses and after overnight incubation treated as indicated in the figure legends. Empty plasmids were used as transfection controls. Luciferase activity was determined in 50 µl of lysates and expressed relative to renilla luciferase, as previously described (Zhou et al., 2013).

### Overexpression experiments –

GATA4 wildtype or S105E mutant cDNAs were subcloned into the lentivirus pCS-GP vector, which was derived from the pCDH vector (System Biosciences, Mountain View, CA). Lentivirus stocks were generated in 293FT cells as described above but using pCS-GP (empty), GATA4, or S105-GATA4 lentiviral vectors. Viral stocks carrying empty plasmid (pCS-GP), GATA4, or S105-GATA4 were added directly to the GCs 2 h after plating at a multiplicity of infection of 10, followed by 24 h incubation to allow infection and expression of transfected cDNAs. Then, GCs were treated as described in each figure legend.

### Immunohistochemistry and Immunocytochemistry –

Thirty days old rats were treated with equine chorionic gonadotropin (5 IU, eCG, Sigma) and sacrificed 1 h later. eCG is known to activate the FSH receptor in non-equine species (Combarnous et al., 1984,Moudgal and Papkoff, 1982; Byambaragchaa et al., 2021). Ovaries were fixed in Bouin's solution before paraffin embedding. Five-micron sections were dewaxed and rehydrated. This was followed by antigen retrieval using citrate buffer solution (10 mM of citric acid and sodium citrate, pH 6) microwaved on high for 30 mins until boiling and then at low for 8 more minutes. After cooling at room temperature, slides were placed in 1% $H_2O_2$. Sections were then blocked utilizing the Avidin/Biotin Blocking kit (Vector Laboratories, Burlingame, CA) followed by 30 min of blocking in SuperBlock blocking buffer (Pierce Chemicals, Rockford, IL) before the addition of the primary antibody diluted in phosphate-buffered saline (PBS). The antibodies and dilution used are as follows: total GATA4 (Santa Cruz, Dallas, Texas: cat. # sc1237: 1/1000) and $pS^{105}$-GATA4 (Invitrogen, CA, USA, cat. # 44–948: 1/200). Following washes with PBS, slides were incubated with a secondary antibody for 30 min at room temperature followed by washing. Tissues were stained using the Vectastain DAB Elite ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer's recommendations. Slides were counterstained with hematoxylin before mounting. For immunocytochemistry, GCs were isolated as described above and cultured in Lab Tek II Chamber Slides (Thermo Fisher Scientific, Waltham, MA) for 24 h before treatment with FSH (50 ng/mL) for 1 h. Then, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 1% Triton X-100 and stained using ImmPACT SG Substrate, Peroxidase (Vector Laboratories) following manufacturer's instructions.

### RNA interference -

Short hairpin RNAs (shRNAs) under the control of the U6 promoter were used to specifically knock down the expression of GATA4. shRNA target recognition sequences used were shGATA4: GGA TTT AAT TCG TAT ATA T; and shLUC (control): GCC TGA AGT CTC TGA TTA AGT ACA A. Oligonucleotides and their corresponding antisense sequence separated by a short spacer sequence were chemically synthesized (Integrated DNA Technologies, Inc., Coralville, IA). These oligonucleotides were inserted into the

lentivirus shRNA vector pCS-U6-shRNA. Lentivirus stocks were produced and concentrated as described above. Viral stocks carrying shRNA were added directly to the GCs 2 h after plating at a multiplicity of infection of 20, followed by incubation for 24 h to allow shRNA synthesis and gene knockdown. At this time, cells were treated as described in each figure.

**Western blot analysis -**

Cytosolic and nuclear extracts were isolated from primary rat GCs as described previously (Wu et al., 2011). Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, Illinois). The protein samples were subjected to gel electrophoresis, transferred to nitrocellulose membranes, and processed by routine procedures. The primary antibodies and the dilutions used were total GATA4 (Santa Cruz, cat. # sc1237: 1/1000) and $pS^{105}$-GATA4 (Invitrogen, cat. # 44–948: 1/2000), Phospho-GSK3β (Ser9) (Cell Signaling, Danvers, MA; cat. # 9336: 1/2000), Lamin B1 (Cell Signaling, cat. # 17416: 1/500), GAPDH (Cell Signaling, cat. # 5174: 1/500). The secondary antibodies used were goat anti-rabbit IgG-HRP (Abcam, Cambridge, UK: cat. # 205718 1/10000) or goat anti-mouse IgG-HRP (Jackson ImmunoResearch Laboratory Inc., West Grove, PA; cat. # 115-035-003: 1/10000). Detection was performed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL). Protein expression quantification was performed with ImageJ software (National Institutes of Health, Bethesda, Maryland).

**Statistics –**

Data were analyzed using GraphPad Prism 6.0 (San Diego, CA). Differences between two groups were determined by Student's *t*-test. For multiple groups, one-way ANOVA was used, and differences between individual means were determined by the Tukey test. Data are represented as mean ± SEM. Significant differences were recognized at $p < 0.05$.

## 3. Results

### 3.1. FSH increases GATA4 phosphorylation at serine 105 in vivo

We have previously shown that FSH stimulates serine 105 phosphorylation of GATA4 ($pS^{105}$-GATA4) *in vitro* in rat GCs (Kwintkiewicz et al., 2007). Here, we aimed to validate this observation *in vivo*. To this end, we measured $pS^{105}$-GATA4 expression in ovarian sections of rats treated with eCG for 1 h. Staining for $pS^{105}$-GATA4 was more intense in the ovaries of eCG-treated rats compared to control animals (Fig. 1B). This effect of FSH can be seen more clearly in the absence of counter staining (Fig . 1C). No significant changes in the staining for total GATA4 were observed (Fig. 1A). Notably, total GATA4 was found in the cytoplasm and nucleus, whereas $pS^{105}$-GATA4 immunoreactivity was found exclusively in the nucleus (Fig .1). These results confirm *in vivo* that FSH increases GATA4 phosphorylation and suggest that phosphorylation of serine 105 increases GATA4 localization to the nucleus of GCs.

To further examine the effect of FSH on GATA4 subcellular localization, rat GCs were cultured in microscope slides, treated with FSH for 1 h, and then stained for total and $pS^{105}$-GATA4. $pS^{105}$-GATA4 immunostaining was again found in the nucleus and the signal

increased in cells treated with FSH (Supplemental Fig. 1). As expected, the total GATA4 signal was not altered by FSH treatment and was found in the cytoplasm and nucleus.

### 3.2. Phosphorylation of GATA4 at serine 105 increases Cyp19a1 expression and potentiates the effect of FSH and cAMP

To examine whether phosphorylation of serine 105 affects the capacity of GATA4 to stimulate ovarian gene expression, we used the promoter of aromatase (*Cyp19a1*), a gene that is targeted by GATA4 *in vivo* (Bennett et al., 2012) and *in vitro* (Kwintkiewicz et al., 2007; Tremblay and Viger, 2001). GCs were infected with lentivirus carrying the proximal promoter of *Cyp19a1* and cDNA for either wild-type GATA4 (WT-GATA4) or a mutant in which serine 105 was exchanged for glutamic acid (S105E-GATA4) to mimic phosphorylation. Overexpression of WT-GATA4 increased *Cyp19a1*-Luc activity (Fig 2A). Mutation of serine 105 to glutamic acid potentiated the stimulatory effect of GATA4 overexpression on *Cyp19a1*-Luc. In addition, mutation of the GATA response element found in the *Cyp19a1* promoter (Stocco, 2004) prevented the stimulatory effect of both GATA4 and the S105E-GATA4 mutant (Fig 2B). Lastly, FSH stimulation of *Cyp19a1* mRNA expression was amplified by the expression of WT-GATA4 (Fig. 2C). This amplification was significantly higher when S105E-GATA4 was overexpressed. These results demonstrate that serine 105 phosphorylation increases GATA4 transcriptional activity in ovarian GCs.

We have previously shown that GATA4 is needed for normal FSH receptor expression (Bennett et al., 2012). To determine whether the enhancing effects of GATA4 on FSH actions are not mediated by changes in FSH receptor expression, we next examined whether GATA4 enhances the effect of key components of the FSH receptor downstream signaling pathway. In GCs, the FSH activates Gα (a G-protein subunit) protein which stimulates adenylate cyclase (AC) activity and the production of cyclic adenosine 3′,5′-monophosphate (cAMP). Therefore, we overexpressed GATA4 and treated the cells with FSH, forskolin, a specific AC activator, or dibutyryl-cAMP (dbcAMP), a cell-permeable analog of cAMP. Treatment with FSH combined with overexpression of GATA4 led to a synergistic stimulation of *Cyp19a1* promoter activity. The overexpression of GATA4 alone also significantly stimulated *Cyp19a1* promoter activity. Treatment with increasing concentrations of forskolin or dbcAMP stimulated the activity of the *Cyp19a1* promoter. As with FSH treatment, GATA4 overexpression also enhanced the stimulatory effect of forskolin and dbcAMP on *Cyp19a1* activity at all concentrations (Fig. 3A and B), suggesting that GATA4 acts downstream of the FSH receptor.

### 3.3. IGF1 potentiates FSH-induced GATA4 phosphorylation at serine 105

We have previously reported that FSH actions require the input of the IGF system (Zhou et al., 2013; Hobeika et al., 2020; Baumgarten et al., 2017; Baumgarten et al., 2015; Baumgarten et al., 2014; Baumgarten et al., 2014). This evidence led us to examine whether the FSH-induced stimulation of GATA4 phosphorylation is also affected by the activation of the IGF1R. To test this possibility, serine 105 phosphorylation of GATA4 was quantified in GCs treated with FSH, IGF1, or their combination. As expected, FSH increased $pS^{105}$-GATA4. In addition, a significant increase in $pS^{105}$-GATA4 was found in cells treated with IGF1 alone (Fig. 4A). Treatment with FSH and IGF1 significantly increased the

pS$^{105}$-GATA4 levels above the levels observed in the presence of FSH or IGF1 alone. The results suggest that FSH and IGF1 signaling interact to synergistically activate GATA4.

As described in Fig. 1, pS$^{105}$-GATA4 appears to be present exclusively in the nucleus of GCs after FSH receptor activation. Therefore, the role of FSH, IGF1, or their combination on GATA4 subcellular localization was examined using cellular fractionation. Confirming IHC and ICC results, pS$^{105}$-GATA4 expression in the nuclear fraction increased in the presence of FSH and after treatment with FSH and IGF1 (Fig. 4B). No significant changes in the loading controls lamin B and β-actin were observed in either the nuclear or the cytosolic fractions, respectively. The results confirm the presence of pS$^{105}$-GATA4 mostly in the nuclear fraction and that FSH and IGF1 interact to regulate GATA4 activation.

### 3.4. Knockdown of GATA4 blunts FSH or FSH plus IGF1 stimulation of Cyp19a1 expression

Next, we examined whether FSH, IGF1, and their combination affect the steady-state levels of GATA4 mRNA after 48 h of treatment. As shown in Fig. 5A, GATA4 mRNA levels remain unchanged, suggesting that FSH and IGF1 only affect the phosphorylation of GATA4.

Since GATA4 mRNA expression does not change with FSH and/or IGF1 treatment, we examined whether GATA4 knockdown impacts the stimulatory effects of FSH or the synergistic effect of FSH and IGF1 on *Cyp19a1* expression that we have previously described (Zhou et al., 2013; Baumgarten et al., 2013). For this purpose, we infected GCs with lentivirus carrying a control shRNA (shSCR) or an anti-GATA4 shRNA (shGATA4). Expression of shGATA4 was highly effective at knocking down GATA4 (Fig. 5B, insert). The knockdown of GATA4 significantly reduced the synergetic stimulation of *Cyp19a1* by the combinatory treatment of FSH and IGF1 (Fig 5B).

### 3.5. IGF1R and AKT activities are required for the synergistic effect of FSH and IGF1 on pS$^{105}$-GATA4

Next, we examined the role of IGF1R activity on GATA4 phosphorylation by FSH and IGF1. For this purpose, we treated GCs with NVP-AEW541 (AEW), a specific inhibitor of IGF1R activity (Riedemann and Macaulay, 2006; Garcia-Echeverria et al., 2004), for 1 h before adding FSH and/or IGF1 to the media. Treatment with AEW did not affect the increase of pS$^{105}$-GATA4 induced by FSH alone but prevented the synergism between FSH and IGF1 (Fig 6A). We have demonstrated that AKT plays a key role in the regulation of GCs by FSH and IGF1 (Zhou et al., 2013; Baumgarten et al., 2014, 2015; Baumgarten et al., 2014a,b). To test whether AKT is involved in GATA4 serine 105 phosphorylation, we treated GCs with MK-2206, a specific inhibitor of AKT, in the presence of FSH or FSH plus IGF1. Treatment with MK-2206 prevented the synergism between FSH and IGF1 on serine 105 phosphorylation but did not affect FSH actions (Fig. 6B).

Finally, since ERK1/2 plays a key role in FSH-induced phosphorylation (Kwintkiewicz et al., 2007), we examined the role of ERK1/2 on GATA4 activation in the presence of FSH and IGF1. As shown in Fig. 6B, treatment with ERK1/2 inhibitor, U0126, blocked GATA4 phosphorylation by FSH alone and FSH plus IGF1. These findings demonstrate that IGF1

has a permissive effect on GATA4 activation by allowing FSH to fully activate GATA4. The permissive effect of IGF1 seems to be mediated at least in part by the IGF1R and its downstream target AKT.

### 3.6. Inhibition of GSK3β activity increases FSH and IGF1-induced GATA4 phosphorylation

Next, we aimed to elucidate the AKT-activated downstream signaling involved in GATA4 phosphorylation. Since GSK3β is a well-known downstream target of AKT and GSK3β and GATA4 interact to control cardiomyocyte hypertrophic (Condorelli et al., 2002; Morisco et al., 2000), we tested if GATA4 is regulated by the AKT/GSK3β pathway in GCs. First, we examined GSK3β phosphorylation in GCs treated with FSH and/or IGF1. As shown in Fig. 7A, FSH and IGF1 alone stimulated GSK3β phosphorylation at serine 9. Additionally, synergism between FSH and IGF1 on GSK3β phosphorylation was observed. Furthermore, inhibition of IGF1R activity with AEW prevented FSH phosphorylation of GSK3β (Fig. 7B, top) while inhibition of AKT activity with MK-2206 prevented its phosphorylation by FSH, IGF1, or their combination (Fig. 7B, bottom).

Phosphorylation on serine 9 is known to inhibit GSK3β (Dajani et al., 2001); therefore, we tested if blocking GSK3β activity affects FSH and IGF1 phosphorylation of GATA4. For this purpose, we used SB216763, an inhibitor of GSK3β. GSK3β inhibition alone stimulated $pS^{105}$-GATA4 and synergized with FSH to further increase $pS^{105}$-GATA4 levels (Fig. 7C). GSK3β inhibition also enhanced the effect of IGF1 and FSH plus IGF1 on the stimulation of $pS^{105}$-GATA4.

Finally, we examined if inhibition of GSK3β translates into changes in *Cyp19a1* expression. We observed that inhibition of GSK3β activity potentiated the stimulatory effect of FSH on *Cyp19a1* (Fig. 7D). However, inhibition of GSK3β did not augment the already strong stimulation of *Cyp19a1* expression by the combination of FSH and IGF1. To further test the capacity of GSK3β to inhibit FSH actions in GCs, cells were transduced with a lentivirus encoding a constitutively active form of GSK3β, which carries a mutation in serine 9 to alanine (GSK3β-CA) (Park et al., 2003) or with a virus expressing Luciferase (LUC) as a control. Twenty-four hours after infection, cells were treated with FSH and/or IGF1. The results show that overexpression of GSK3β-CA significantly reduced the induction of *Cyp19a1* by FSH and IGF1 (Fig. 8). These results further support the notion that GSK3β inhibits GATA4 activity and *Cyp19a1* expression in ovarian GCs.

## 4. Discussion

Our previous reports demonstrated that IGFs and GATA4 are key players in the regulation of GC differentiation and preovulatory follicle growth (Bennett et al., 2012, 2013; Zhou et al., 2013; Baumgarten et al., 2015, 2017; Convissar et al., 2015). Yet, the relationship between IGF1 and GATA4 has not previously been elucidated in the ovary. Our data indicate that GATA4 activation occurs downstream of IGF1 and FSH in GCs. Moreover, the findings suggest that GATA4 is essential for the synergistic effects of FSH and IGFs on GC differentiation.

*Activation of GATA4 by FSH in vivo* - evidence from transgenic mice revealed that GATA4 is crucial for ovarian development, GC differentiation, postnatal follicle growth, and luteinization (Bennett et al., 2012, 2013; Kwintkiewicz et al., 2007; Convissar et al., 2015). Thus, conditional knockdown of GATA4 in GCs at any stage of development leads to female subfertility. GATA factors impact female reproduction by regulating genes involved in steroidogenesis, hormone signaling, ovarian hormone expression, extracellular matrix organization, apoptosis, and cell division (Bennett et al., 2013). Moreover, women carrying an inactivating mutation in the FSH receptor show little or negligible GATA4 expression in the ovary (Vaskivuo et al., 2002), providing further evidence for a major role of GATA4 in the regulation of ovarian function. We have previously demonstrated that FSH induces GATA4 phosphorylation at serine 105 (Kwintkiewicz et al., 2007). Phosphorylation of serine 105 enhances the transcriptional potency of GATA4 (Liang et al., 2001). Accordingly, GATA4 overexpression, together with FSH stimulation, synergistically activates the expression and promoter activity of *Cyp19a1*, whereas the mutation of serine 105 prevents these effects. In this report, we demonstrate that the stimulatory effect of FSH on GATA4 serine 105 phosphorylation also occurs *in vivo* and show that in follicles of rats treated with FSH, $pS^{105}$-GATA4 is located exclusively in the nucleus, whereas total GATA4 can be detected in the cytoplasm and the nucleus. Our current findings also demonstrate that serine 105 phosphorylation increases GATA4 transcriptional activity on the *Cyp19a1* promoter in ovarian GCs. Moreover, GATA4 overexpression potentiates the effect of FSH, forskolin, and dbcAMP on the stimulation of the *Cyp19a1* promoter, suggesting that GATA4 acts downstream of cAMP and most probably PKA, the main target of cAMP.

### FSH and IGF1 interact to control GATA4 Activity -

IGF1 is required for GC differentiation to the preovulatory stage (Zhou et al., 2013). IGF1 stimulates the PI3K-AKT pathway in GCs (Baumgarten et al., 2014, 2017; Mack et al., 2012; Mani et al., 2010). Activation of AKT has been linked to increased GATA4 activity in rabbit cardiomyocytes (Yoshida et al., 2014). Hence, we hypothesized that GATA4 might be activated downstream of the IGF1R in GCs and play an important role in the synergistic effect between FSH and IGF1 on the induction of *Cyp19a1* expression. To test this hypothesis, we compared the effects of IGF1 to FSH on the phosphorylation of GATA. We found that IGF1 stimulates GATA4 phosphorylation at serine 105 but at lower levels than those induced by FSH. More interestingly, the data demonstrated that IGF1 enhances FSH induction of GATA4 phosphorylation. The role of GATA4 in mediating IGF1 effects in the ovary is further supported by the observation that the knockdown of GATA4 drastically reduces the stimulation of *Cyp19a1* by FSH and IGF1 cotreatment. The findings suggest that the coordinated activation of GATA4 by FSH and IGF1 may play a major role in the synergistic effect these two hormones have on the induction of *Cyp19a1*. Interestingly, we previously showed that knockdown of GATA4 in GCs leads to a decrease in IGF1R activation due to a reduction of IGF1 expression and an increase in IGF1 binding protein (Bennett et al., 2013). Taken together, these findings suggest a positive feedback loop between GATA4 and the IGFs system that could significantly contribute to follicle development.

The participation of IGF1 in regulating GATA4 was further confirmed by the observation that inhibition of the IGF1R activity eliminates the synergistic actions of FSH and IGF1 on GATA4 phosphorylation. Similar results were obtained when AKT activity was blocked. Our results also confirm our previous report demonstrating that FSH induction of GATA4 phosphorylation is mainly controlled by the MAPKs as inhibiting ERK1/2 activity (U0126 treatment) abolished FSH-induced GATA4 phosphorylation. Thus, we conclude that FSH induces GATA4 phosphorylation in an ERK1/2-dependent manner and that IGF1 enhances the effect of FSH by increasing AKT activity.

### GSK3β blocks GATA4 activity -

AKT phosphorylates numerous proteins, among which is GSK3β. GSK3β is a serine/threonine kinase that is inhibited via phosphorylation at serine 9 (Dajani et al., 2001). In rat and porcine GCs, GSK3β phosphorylation at serine 9 is increased by FSH via a PI3K-dependent mechanism (Alliston et al., 2000,Gillio-Meina et al., 2005). GSK3β has been shown to catalyze the phosphorylation and inhibition of initiation factor 2B, β-catenin, and cAMP response element-binding protein (Kaytor and Orr, 2002; Fiol et al., 1994; Morisco et al., 2001). However, the role that GSK3β plays in the ovary remains unknown. It has also been shown that GSK3β stimulates the exportation of GATA4 from the nucleus (Ku et al., 2011), while inhibition of GSK3β by lithium chloride causes nuclear accumulation of GATA4, suggesting that GSK3β negatively regulates the nuclear expression of GATA4 (Morisco et al., 2001). Our finding suggests that, in GCs, IGF1 and FSH work in synchrony to inhibit GSK3β activity. Moreover, our findings suggest that AKT may be a key mediator of the combined effect of FSH and IGF1 on GSK3β inhibition as blocking AKT activity prevents GSK3β phosphorylation by FSH, IGF1, or their combination. Thus, we propose that inhibition of GSK3β activity plays an important role in the activation of GATA4. Supporting this idea, inhibition of GSK3β activity with SB216763 enhances the stimulatory effect of FSH and IGF1 on GATA4 phosphorylation, which correlates with the potentiation of *Cyp19a1* expression. Further support is provided by data showing that overexpression of constitutively active GSK3β blunts the stimulation of *Cyp19a1* expression by FSH and IGF1.

The molecular mechanism by which GSK3β regulates GATA4 phosphorylation and activity remains to be determined. A deletion analysis suggested that GSK3β phosphorylates the amino terminus of GATA4 (Morisco et al., 2001); however, it is not known which specific residues are targeted. Examination of the amino acid sequence of the amino terminus of GATA4 reveals the presence of several potential GSK3β sites around serine 105; therefore, GSK3β may phosphorylate GATA4 directly. Further research is needed to determine the effects of GSK3β on GATA4 activity in ovarian GCs.

In conclusion, our data provide new insights into the mechanisms by which FSH and IGF1 regulate GCs differentiation. The data support an important role of the IGF1R/AKT/GSK3β signaling pathway in the regulation of GATA4 transcriptional activity in ovarian GCs. AKT has been shown to play a crucial role in the regulation of GCs by FSH and IGFs, but the downstream targets of AKT have not been elucidated. Our findings suggest that GATA4 transcriptional activation may, at least partially, mediate AKT actions in GCs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data availability

Data will be made available on request.

## References

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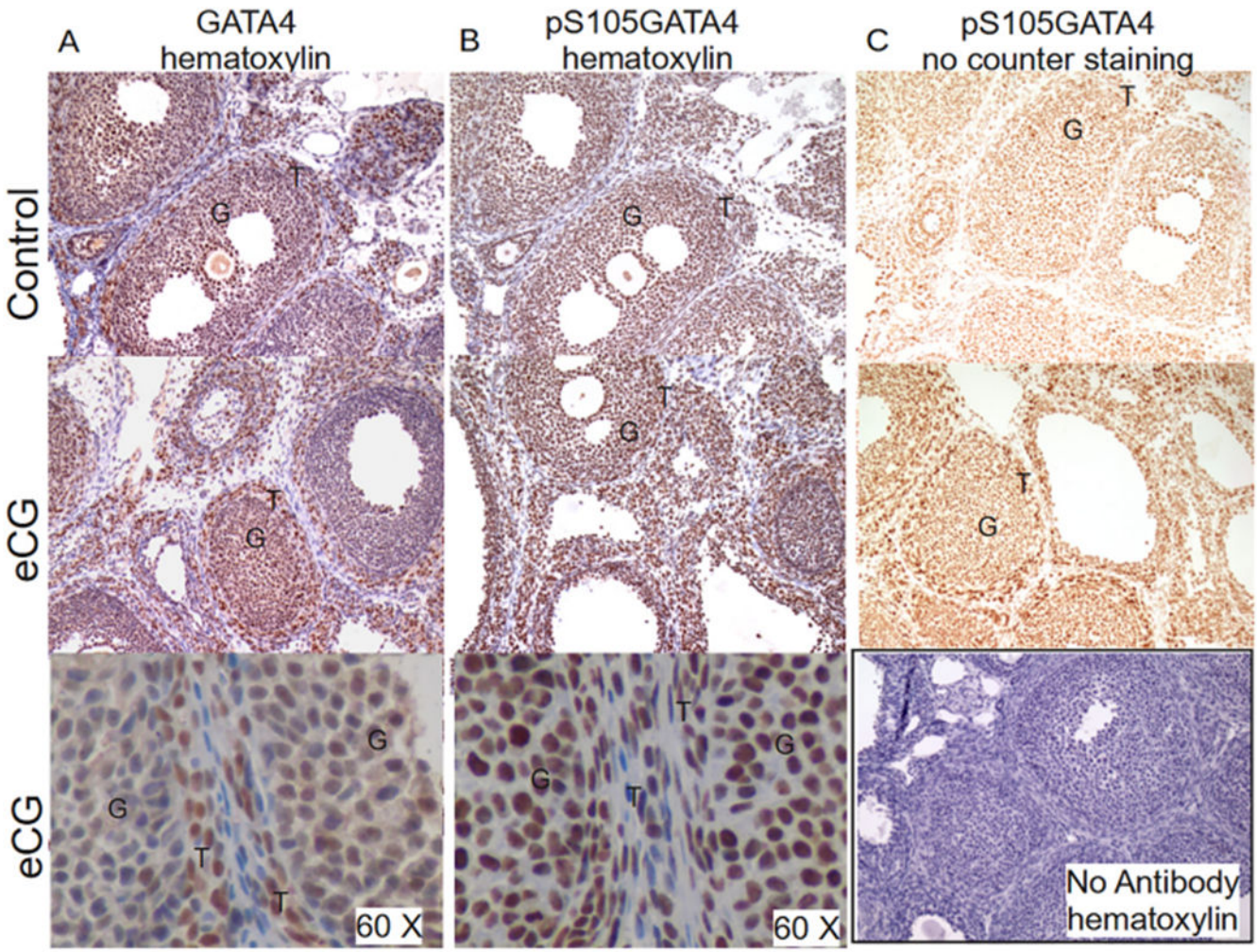


**Fig. 1. Immunohistochemical localization of pS$^{105}$-GATA4 and total GATA4 in the ovaries of immature rats treated with eCG.**

Positive staining is shown in brown. Sections were probed with an anti-GATA4 (A) or an anti-pS$^{105}$ -GATA4 (B and C) antibody. In the negative (C bottom panel), the first antibody was omitted. Sections were counterstained with hematoxylin in A and B columns. In the C column, top and middle rows were left without counterstaining to better show GATA4 levels. The third row shows high magnification to illustrate the nuclear localization of pS$^{105}$-GATA4. Stains were repeated in three different animals for each treatment. Representative data are shown. G: Granulosa cells; T: Theca cells.

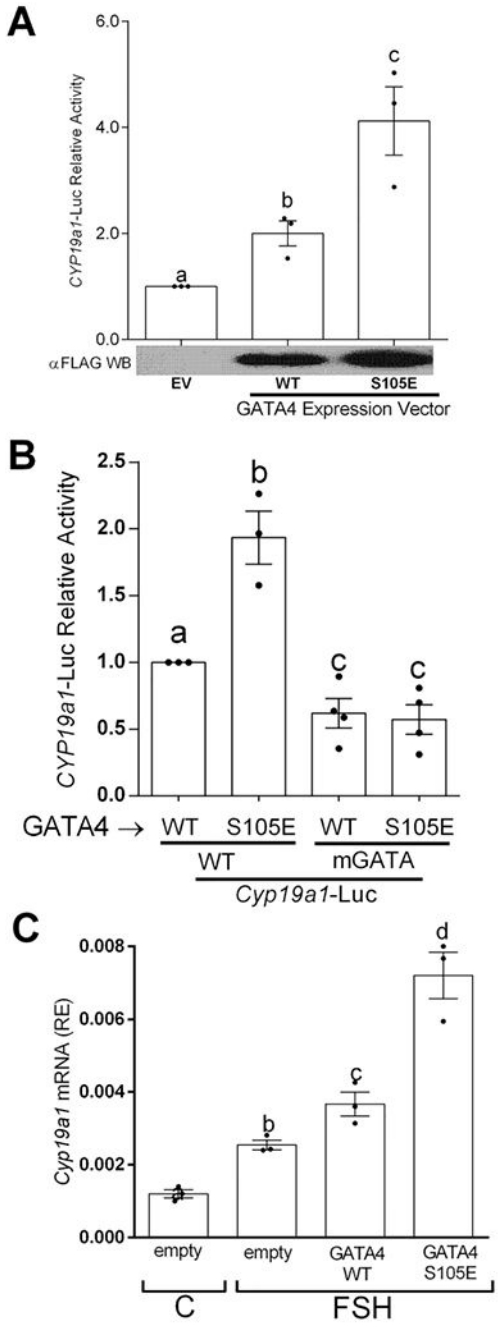


**Fig. 2. - Phosphorylation of serine 105 increases GATA4 capacity to stimulate gene expression.**
**A-** GCs were transfected with empty vector (EV), GATA4 wildtype (WT), or S105E-GATA4 along with *Cyp19a1*-Luc reporter. Luciferase activity was quantified 48 h after transfection. Columns with different letters differ significantly $p < 0.01$, $n = 3$. **B-** GCs were transfected with GATA4 wildtype (WT) or S105E-GATA4 along with wildtype (WT) or mutant GATA (mGATA) *Cyp19a1*-Luc reporter. Luciferase activity was quantified 48 h after transfection. Columns with different letters differ significantly $p < 0.01$, $n = 3$. **C-** GCs were transfected with empty plasmid (empty), GATA4 wildtype (WT), or S105E-GATA4. 24 h

after transfection, cells were treated with vehicle (C) or FSH (50 ng/mL). *Cyp19a1* mRNA levels were quantified 48 h later. Columns with different letters differ significantly a-b, b-d, c-d $p < 0.01$, b-c $p < 0.05$, $n = 3$ different experiments, each perfomed using technical duplicates.

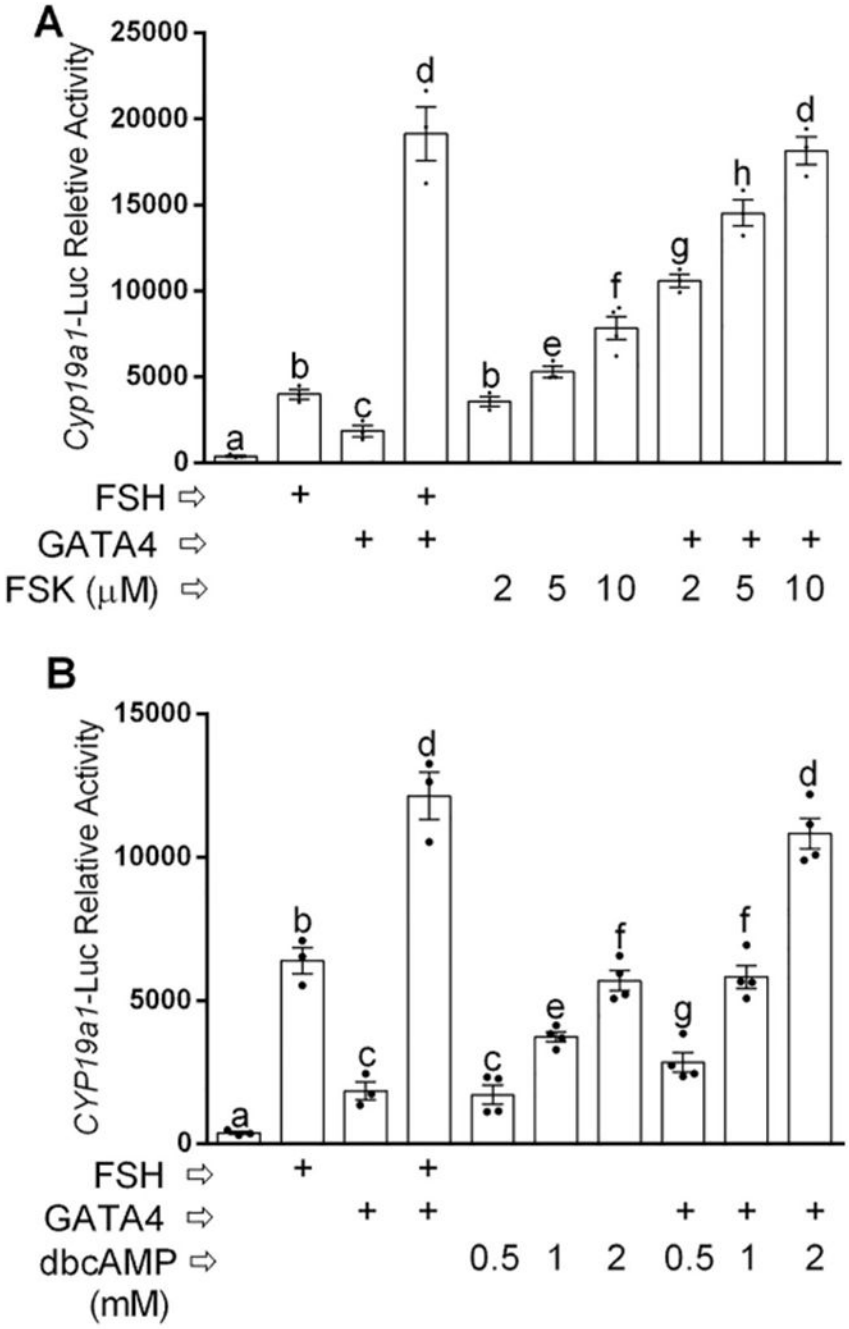


**Fig. 3. –. GATA4 potentiates forskolin and dbcAMP activation of *Cyp19a1* promoter activity.**
GCs were transfected with empty plasmid or GATA4 wildtype (+) along with *Cyp19a1*-Luc reporter. 24 h after transfection, cells were treated with vehicle, FSH (50 ng/mL) and/or increasing concentration of forskolin (FSK) (**A**) or dbcAMP (**B**). Luciferase activity was quantified 48 h after transfection. Columns with different letters differ significantly a-b, b-d, c-d $p < 0.01$, b-c $p < 0.05$, $n = 3$ different experiments, each perfomed using technical duplicates.

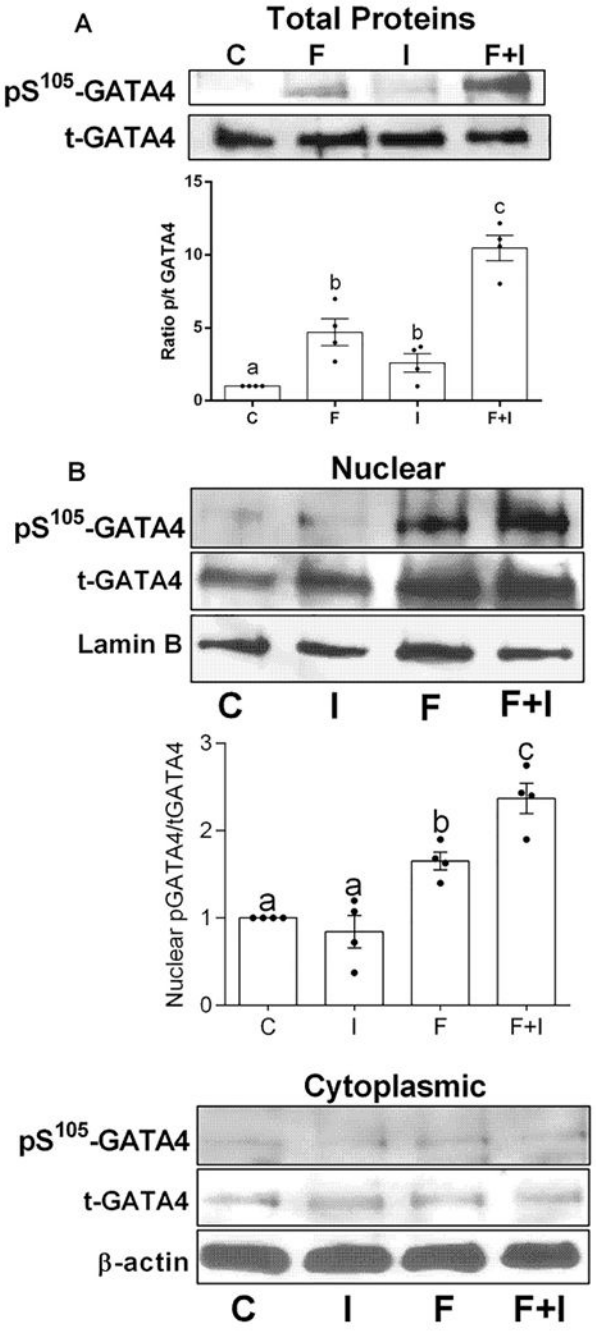


**Fig. 4. IGF1 enhances FSH-induced phosphorylation and nuclear localization of GATA4.**

A- GCs were treated with vehicle (C), FSH (F, 50 ng/mL), IGF1 (I, 50 ng/mL), or their combination (F + I). One hour later, total and pS$^{105}$-GATA4 were determined by Western blot in total cell lysates. The experiment was repeated 5 times, bands quantified, and the ratio phosphorylated to total GATA4 calculated and plotted. Columns with different letters differ significantly a-b, b-c $p < 0.01$, $n = 4$. B- GCs were treated as in Fig 4A and nuclear and cytosolic proteins were used of total and pS$^{105}$-GATA4 determination by Western

blot. Columns with different letters differ significantly a-b, b-c $p < 0.01$. A representative experiment is show from 4 biological replicates.

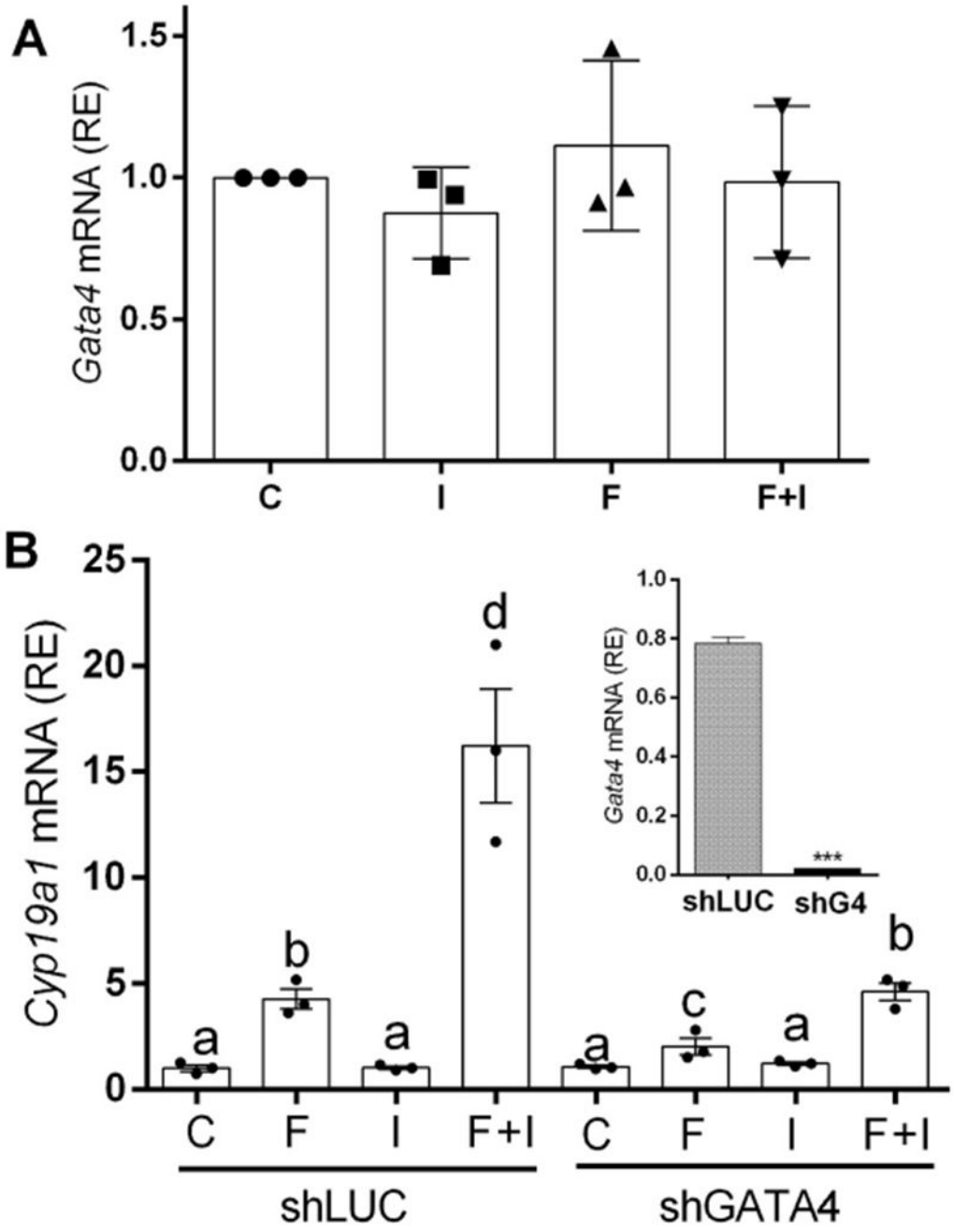


**Fig. 5. Knockdown of GATA4 decreases FSH and FSH plus IGF1 synergism.**

A- GCs were treated with vehicle (C), FSH (F, 50 ng/mL), IGF1 (I, 50 ng/mL), or their combination (F + I). *Gota4* mRNA levels were quantified 48 h later. The experiment was repeated 3 times. No changes in Gata4 mRNA levels were observed. B- GCs were infected with lentivirus carrying control shRNA (shLUC) or anti-GATA4 siRNA (shGATA4, shG4). 24 h after infection, cells were treated with vehicle (C), FSH (F, 50 ng/mL), IGF1 (I, 50 ng/mL) (I), or their combination (F + I). *Cyp19a1* mRNA levels were quantified 48 h after initiation of treatments. Insert graph shows Gata4 mRNA levels in cells infected with shLUC

of shG4. ***$p < 0.001$ vs shLUC. Columns with different letters differ significantly a-b, b-c, a-c $p < 0.05$; a-d $p < 0.01$, $n = 5$.

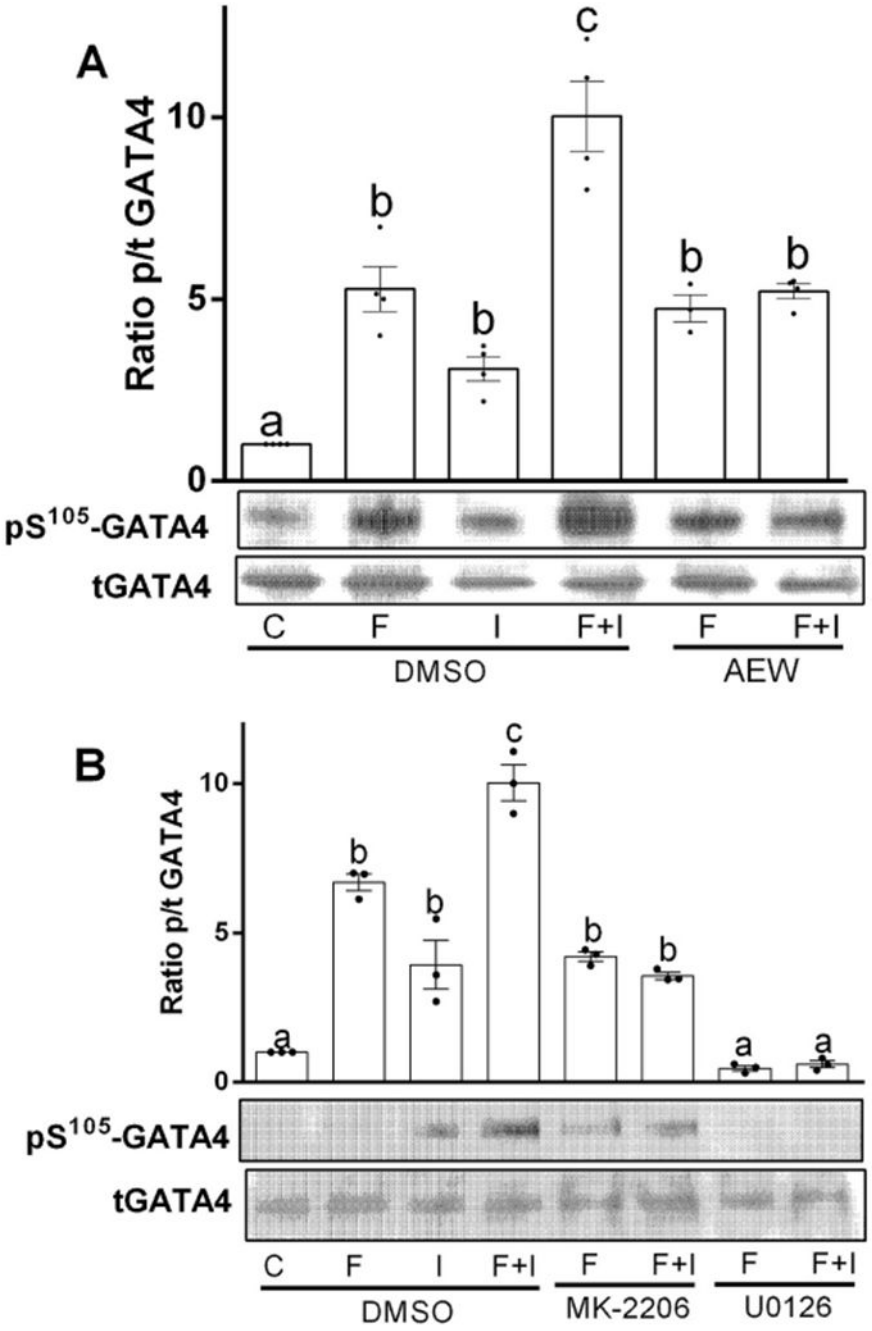


**Fig. 6. IGF1R and AKT activities are required for the synergistic effect of FSH and IGF1 on pS$^{105}$-GATA4.**

A- GCs were treated with vehicle (C, DMSO), FSH (F, 50 ng/mL), IGF1 (I, 50 ng/mL) or their combination (F + I) in the presence of absence of an IGF1R inhibitor (AEW). One hour later, total and pS$^{105}$-GATA4 were determine by Western blot in total cell lysates. The experiment was repeated 4 times, bands quantified, and the ratio of phosphorylated to total GATA4 calculated and plotted. Columns with different letters differ significantly a-b, b-c $p < 0.01$. B- GCs were treated with vehicle (C, DMSO), FSH (F, 50 ng/mL), IGF1 (I, 50 ng/mL) or their combination (F + I) in the presence of absence of an AKT inhibitor (MK-2206) or

an ERK1/2 inhibitor (U0126). One hour later, total and $pS^{105}$-GATA4 were determine by Western blot in total cell lysates. The experiment was repeated 3 times, bands quantified, and the ratio phosphorylated to total GATA4 calculated and plotted. Columns with different letters differ significantly a-b, b-c $p < 0.01$.

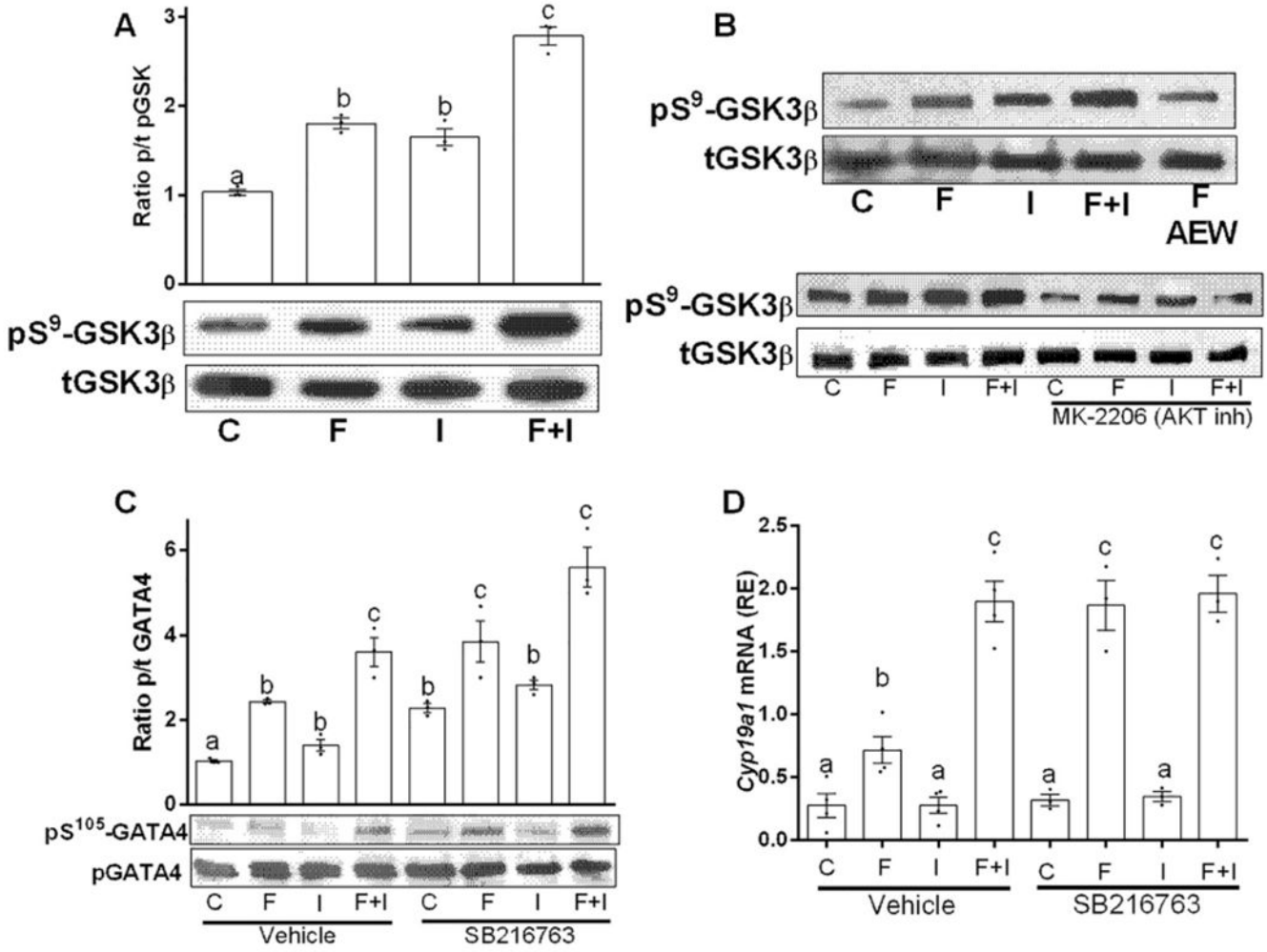


**Fig. 7. Inhibition of GSK3β activity increases FSH and IGF1 actions.**

A- GCs were treated with vehicle (C), FSH (F, 50 ng/mL), IGF1 (I, 50 ng/mL), or their combination (F + 1). One hour later, total and $pS^{9}$-GSK3γ were determined by Western blot. The experiment was repeated 4 times, bands quantified, and the ratio of phosphorylated to total GATA4 was calculated and plotted. Columns with different letters differ significantly a-b, b-c $p < 0.01$, $n = 3$. B- GCs were treated with vehicle (DMSO), FSH (F, 50 ng/mL), IGF1 (I, 50 ng/mL), or their combination (F + I) in the presence of absence of AEW or MK-2206. One hour later, total and $pS^{9}$-GSK3β were determined by Western blot. The experiment was repeated 3 times, one representative blot is shown. C- GCs were treated with vehicle (DMSO), FSH (F, 50 ng/mL), IGF1 (I, 50 ng/mL) or their combination (F + I) in the presence of absence of an GSK3β inhibitor, SB216763. One hour later, total and $pS^{105}$-GATA4 were determined by Western blot. The experiment was repeated 4 times, bands quantified, and the ratio of phospho to total GATA4 was calculated and plotted. Columns with different letters differ significantly a-b, b-c $p < 0.01$, $n = 3$. D- GCs were treated with vehicle (C), FSH (F, 50 ng/mL), IGF1 (I, 50 ng/mL) or their combination (F + I) in the presence of absence of SB216763. *Cyp19a1* mRNA levels were determine 48 h after

initiation of treatments. Columns with different letters differ significantly a-b, b-c $p < 0.01$, $n = 3$.

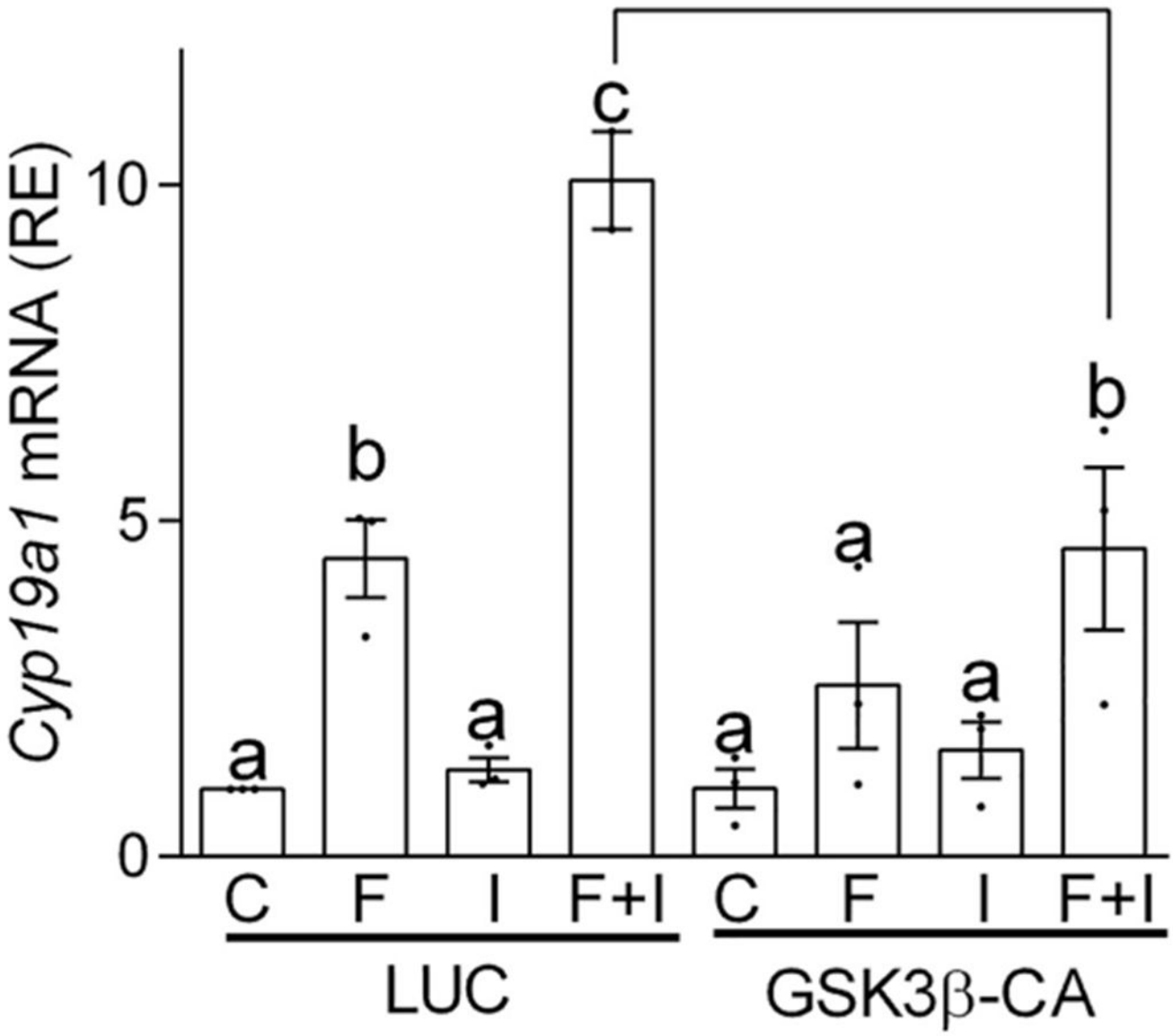


**Fig. 8.**

Constitutively active GSK3β decreases FSH and IGF1 actions. GCs were infected with lentivirus carrying Luciferase cDNA (LUC) or GSK3β-CA cDNA. 24 h after infection, cells were treated with vehicle (C), FSH (F, 50 ng/mL), IGF1 (I, 50 ng/mL), or their combination (F + I). *Cyp19a1* mRNA levels were quantified 48 h after initiation of treatments. Columns with different letters differ significantly a-b, b-c $p < 0.01$, n = 3.