Role of RAB5A in FSHR-mediated signal transduction in human granulosa cells

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Abstract

Polycystic ovary syndrome, a common condition characterized by endocrine dysfunction, menstrual irregularity, anovulation and polycystic ovaries, affects 5–7% of reproductive-age women. *RAB5B*, which is identified by a genome-wide association study as a risk locus for this syndrome, encodes a small GTPase involved in control of receptor internalization and early endosome fusion. We found that *RAB5A* mRNA levels in luteinized granulosa cells of obese patients with polycystic ovary syndrome were lower than in those of obese women without the syndrome. *RAB5A* regulated follicle-stimulating hormone (FSH)-mediated translocation of the FSH receptor (FSHR) from the membrane to the cytoplasm and the subsequent FSH–FSHR signaling pathway. We showed that *RAB5A* negatively regulated aromatase expression and estradiol synthesis in human granulosa cells in association with changes in FSHR levels by way of the cAMP/PKA/CREB pathway. The regulation of FSHR by *RAB5A* may have been associated with two transcription factors, USF1 and USF2. In conclusion, *RAB5A* gene was abnormally expressed in luteinized granulosa cells of obese patients with polycystic ovary syndrome, which may help explain high FSHR levels found in this syndrome.

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Introduction

Polycystic ovary syndrome (PCOS), a heterogeneous syndrome characterized by endocrine dysfunction, menstrual irregularity, anovulation and polycystic ovaries, is present in 7% of women of reproductive age (Ehrmann 2005, Mastorakos *et al.* 2006). The pathogenesis of PCOS is extremely complicated, and its precise etiology remains unclear. A previously conducted genome-wide association study in Han Chinese populations identified several loci associated with a risk for PCOS, including insulin receptor (*INSR*), follicle-stimulating hormone receptor (*FSHR*), luteinizing hormone/chorionic gonadotropin receptor (*LHCGR*), *YAP1*, *C9ORF3*, *DENND1A*, *RAB5B*, *HMGA2*, *TOX3*, *SUMO1P1/ZNF217* and *THADA*. These genes are primarily associated with insulin signaling, hormone function, type 2 diabetes, calcium signaling and endocytosis (Chen *et al.* 2011, Shi *et al.* 2012). We hypothesized that the receptor genes in particular may play key roles in the pathogenesis of PCOS.

In response to extracellular stimuli, a ligand–receptor complex is internalized into the cell by endocytosis via the arrestin- and clathrin-mediated pathways. The

receptors are subsequently either recycled back to the plasma membrane or transported to lysosomes for degradation (Gong *et al.* 2008, Jean-Alphonse & Hanyaloglu 2011). However, evidence increasingly indicates that receptor internalization functions not only in receptor degradation but also in receptor signal transduction (Di Fiore & De Camilli 2001, Sorkin & Von Zastrow 2002, Miaczynska *et al.* 2004, Kang *et al.* 2005).

Rab proteins are low-molecular-weight guanine nucleotide-binding proteins (G proteins) that are located on distinct intracellular membranes and regulate different stages of intracellular membrane trafficking (Pereira-Leal & Seabra 2001, Schwartz *et al.* 2007). Rab GTPases are members of the Ras superfamily, in which at least 60 Rabs have been identified. Among these, *RAB5A* plays a crucial role in clathrin-coated vesicle formation, endosome motility and early endosome fusion (Stenmark 2009). It mediates a cascade of intracellular signaling events by recruiting different effectors to the endosome membrane (Olchowik & Miaczynska 2009). *RAB5A* is required for the interaction of insulin receptor substrate 1 with the p85 subunit of PI3K in NIH3T3 cells, which may be associated with insulin resistance in diabetes

(Su *et al.* 2006). Inactive RAB5A (RAB5A: S34N) blocks the epidermal growth factor (EGF)-activated Raf-ERK1/2 kinase pathway and EGF-induced cyclin D1 transcription (Barbieri *et al.* 2004). In the nucleus, APPL1, an effector of RAB5A, interacts with the nucleosome remodeling and histone deacetylase multiprotein complex, an effect essential for transcriptional regulation of genes regulating cell proliferation (Miaczynska *et al.* 2004). In response to EGF, RAB5A and its effector APPL1 may translocate from the membrane to the nucleus via an endosomal compartment.

FSHR is a member of G-protein-coupled receptor family comprising a large extracellular domain and a transmembrane domain with seven α -helices (Jiang *et al.* 2012, Menon & Menon 2012). In testicular Sertoli cells, FSHR is involved in the initiation of spermatogenesis at puberty and maintenance of normal sperm production. In ovarian granulosa cells, it participates in follicular recruitment, growth and eventually maturation (Richards 1980, Simoni *et al.* 1997). *Fshr*-deficient male mice have small testes and partial failure of spermatogenesis (Dierich *et al.* 1998). *Fshr*-knockout female mice have thin uteri and small ovaries and are sterile because folliculogenesis is blocked before the formation of antral follicles (Balla *et al.* 2003).

In granulosa cells, FSH binding to FSHR leads to rapid activation of numerous signaling molecules with diverse effects. RAB5A mediates internalization of the FSH–FSHR complex (Krishnamurthy *et al.* 2003) but may also participate in FSHR-mediated signal transduction. We found differential *RAB5A* expression in ovarian granulosa cells from obese patients with PCOS compared with obese women without PCOS. Thus, we hypothesize that RAB5A could participate in the dynamic regulation of FSHR localization during folliculogenesis in human ovaries, which could imply involvement in the pathogenesis of PCOS. Further, RAB5A might negatively regulate aromatase expression and estradiol synthesis in association with changes in FSHR levels. This study focused on the function of RAB5A in human granulosa cells (termed hGCs when referring to cells obtained from study participants), attempting to elucidate the molecular mechanisms underlying PCOS.

Materials and methods

Subjects

A total of 55 women were recruited from the Center for Reproductive Medicine, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine. All women gave written informed consent to participate in this study. The study was approved by the assisted reproductive technology (ART) Ethics Committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. Twenty-six participants were women 20–35 years old undergoing *in vitro* fertilization (IVF) and embryo transfer who had been diagnosed with PCOS by meeting at least two or all three of the Rotterdam criteria. The

remaining 29 participants were healthy women undergoing IVF for tubal factor infertility or male factor infertility. These 29 women all had regular menstrual cycles (26–35 days) and normal ovarian morphology. Endocrine parameters were measured in this group to exclude hyperandrogenism. Both groups were divided on the basis of body mass index (BMI) into those with normal weight (BMI 18.5–24 kg/m²) and those who were obese (BMI $\geq$ 24 kg/m²). None of the participants had used hormonal therapy for at least 3 months before the study. All subjects were of Han ethnicity. Each of them underwent a gonadotropin-releasing hormone (GNRH) agonist long protocol consisting of decapeptyl 0.05 mg starting in the luteal phase (menstrual cycle day 21) of the previous cycle until human chorionic gonadotropin (hCG) administration and gonadotropins for 8–12 days after pituitary suppression (i.e. FSH $\leq$ 5 IU/L, LH $\leq$ 5 IU/L, estradiol (E₂) $\leq$ 55 pg/mL, and endometria $<$ 5 mm) with an hCG trigger. The patients' clinical information is presented in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article).

Follicular fluid samples were aspirated under ultrasound guidance transvaginally from individual follicles 36 h after hCG injection. To avoid post-aspiration cell death, the fluid was immediately centrifuged at 201 g for 10 min. The cell pellet was resuspended in 0.01% phosphate-buffered saline (PBS). The hGCs were isolated from the cell suspension with Ficoll gradient centrifugation and then digested with hyaluronidase (Sigma Chemical) for 10 min at 37°C. Red blood cells were removed with a 50% Percoll gradient. The remaining hGCs were purified by centrifugation and then used for extraction of RNA.

Cell culture

The human granulosa cell line KGN, kindly provided by Shandong University, was also used in our study. KGN cells were maintained in phenol red-free DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (Gibco) and were plated at 2×10^5 cells per well in six-well plates and then cultured at 37°C in humidified air with 5% CO₂. Cells at passages 5–15 were used in experiments. For FSH stimulation, the culture medium was replaced with medium supplemented with 25 ng/mL FSH (Merck Serono SA, Aubonne Branch, Switzerland) for 12 h. For the inhibition experiment, we added the protein kinase A (PKA) inhibitor H-89 10 μ M/L (Selleckchem, Houston, TX, USA) for 30 min.

Immunofluorescence

KGN cells (2×10^5) were seeded on glass coverslips in six-well cell culture plates overnight and grown to 60% confluence. Then, the cells were exposed to 400 ng/mL FSH for 30 min at 37°C. The cells were washed three times with PBS, fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.2% Triton X-100 for 20 min. After washing, the samples were blocked with 5% BSA in PBS for 1 h. To detect the location of FSHR and RAB5A, the cells were incubated with anti-RAB5A (Santa Cruz Biotechnology) at a 1:50 dilution overnight at 4°C and then incubated with anti-FSHR (Santa Cruz) at a 1:50 dilution at room temperature for

2 h. Cells were washed three times with PBS and incubated with AlexaFluor 488 goat anti-rabbit IgG (Proteintech, Wuhan, China) and AlexFluor Cy3 donkey anti-goat IgG (Proteintech) for 2 h at room temperature. After dyeing with DAPI (Thermo Fisher Scientific), the samples were scanned with a Carl Zeiss inverted fluorescence microscope using a $\times 40$ objective. Carl Zeiss Axio Vision software was used to collect data using a sequential scanning mode to minimize signal cross-over.

Extraction of RNA and analysis with real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from the subjects' hCGs and KGN cells using the Animal Total RNA Isolation Kit (Foregene, Chengdu, China) according to the manufacturer's instructions and reverse-transcribed to cDNA using a Prime Script RT Master Mix Perfect Real Time kit (TaKaRa). The mRNA levels of target genes were determined with RT-qPCR using the transcribed cDNA and Power SYBR Premix ExTaq (TaKaRa). The primers were obtained from the Primer Bank or designed using Primer3 software. Primer sequences and PCR efficiencies are shown in Table 1. The relative mRNA levels in each sample were calculated according to a standard curve using serial dilutions of known amounts of specific templates against corresponding cycle threshold values. The RNA levels of target genes were weighted to *ACTB* as an internal control.

Transfection of siRNA

siRNA sequences directed against *RAB5A*, *RAB5B* and *RAB5C* were synthesized by Gene Pharma Co., Ltd. (Shanghai, China). Transfection of siRNA (20 nmol/L) was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The siRNA sequences specific for *RAB5A* (5'-AACCAGGAATCAGTGTGTAG-3'), *RAB5B* (5'-AAGACAGCTATGAACGTGAAT-3'), and *RAB5C* (5'-ACCAACACAGATACATTTGCA-3') were selected on the basis of their potency to inhibit target gene expression.

Western blot analysis

KGN cells were washed with cold PBS (50 mM phosphate, pH 7.4, 100 mM NaCl, and 10 mM KCl) and lysed with ice-cold radioimmunoprecipitation assay lysis buffer (Active Motif, Carlsbad, CA, USA) containing a protease inhibitor cocktail (Roche). Membrane protein and cytoplasmic protein were extracted using a Membrane and Cytoplasmic Protein

Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. Equal amounts (40 μ g) of protein were separated on 8% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking with 5% BSA in Tris-buffered saline for 1 h, the membranes were incubated overnight at 4°C with primary antibodies diluted in 5% BSA/Tris-buffered saline. Antibodies used included anti-RAB5A (Proteintech) at a dilution of 1:500, anti-FSHR (Proteintech) at 1:500, anti-Aromatase (Abcam) at 1:1000, anti-ATP1A2 (Proteintech) at 1:500, anti-T-cAMP-regulatory element-binding protein (CREB) (Cell Signaling) at 1:1000 and anti-P-CREB (Cell Signaling) at 1:1000. The membrane was then incubated with an appropriate secondary antibody conjugated with horseradish peroxidase (Sigma) at room temperature for 1 h. An enhanced chemiluminescent detection system (Millipore) was used to detect bands with peroxidase activity. The bands were visualized using a G-Box iChemi Chemiluminescence image capture system (Syngene, Maryland, USA) and quantitated by Gel-Pro Analyzer image analysis software. Anti-GAPDH (Cell Signaling) and anti-ATP1A2 were used as internal loading controls for cytoplasmic and membrane proteins, respectively.

Transfection of recombinant EGFP-RAB5A: WT

A full-length human *RAB5A* cDNA was amplified by PCR and then digested and inserted into EGFP-N1 green fluorescent protein (GFP) to construct a recombinant EGFP-RAB5A: WT. The vectors were transfected with DNA Transfection Reagent (Biotool, Selleckchem, Houston, TX, USA). One day before transfection, 2×10^5 KGN cells were plated per well in six-well plates. DNA, 4 μ g, and transfection reagent, 10 μ L, were each diluted in 200 μ L and incubated for 5 min at room temperature. They were then combined and incubated for 20 min, and 400 μ L of the mixture was added to each well. The medium was changed 6 h after transfection. The cells were incubated at 37°C in humidified air with 5% CO₂ for 48 h prior to testing for *RAB5A* gene expression.

Steroid assays

Testosterone (1×10^{-7} ng/mL) (Sigma) was added as the substrate for E₂ synthesis. After 4 h, the levels of E₂ in the spent media were measured using an electrochemiluminescence immunoassay (ECLIA) kit (Roche) according to the manufacturer's instructions. The levels of E₂ in the peripheral blood of recruited subjects were also detected by ECLIA.

Table 1 Primer sequences used for RT-qPCR and PCR efficiencies.

Gene	Primer sequence (5'–3')		PCR efficiency (%)
	Forward	Reverse	
<i>ACTB</i>	GGGAAATCGTGCGTGACATTAAG	TGTGTTGGCGTACAGGTCTTTG	99.2
<i>FSHR</i>	TCTGTCACTGCTCTAACAGGG	TGCACCTTTTGGATGACTCG	97.5
<i>CYP19A1</i>	TGGAAATGCTGAACCCGATAC	AATTCCCAGTAGCCAGG	99.3
<i>RAB5A</i>	AGACCCAACGGGCCAAATAC	GCCCCAATGGTACTCTCTTGAA	94.7
<i>RAB5B</i>	TGGTACCCAGTGAAAAAGG	CCATGGCGATGTACTTTCCT	93.6
<i>RAB5C</i>	CCGCTTTGTCAAGGGACAGTT	AGGCTGTGATACCGTCCT	93.0
<i>USF1</i>	CTGCTGTTGTACTACCCAGG	TCTGACTTCGGGGAATAAGGG	93.6
<i>USF2</i>	CTGTGATCCAAATCCCTTCAGC	GGTCTGTGGTCTGTACGGAC	94.8

Statistical analysis

After inspection for normal distribution of the data, differences between groups were determined using a one-way ANOVA test followed by the Newman–Keuls multiple comparison test. Multiple group comparisons were performed with a two-way ANOVA test followed by the Bonferroni post-test where appropriate to assess significant differences. Correlations between groups were analyzed by Pearson's correlation coefficient. Data analyses were conducted using SPSS 21.0 for Windows (IBM) and GraphPad Prism, version 5.01 (GraphPad). *P* values <0.05 were considered statistically significant.

Results

RAB5A is abnormally expressed in granulosa cells of patients with PCOS

To examine whether *RAB5A*, *RAB5B* and *RAB5C* were differentially expressed in PCOS and non-PCOS granulosa cells, we collected hGCs from 55 women undergoing IVF with a GnRHa long protocol, 26 with and 29 without PCOS. We also compared results of the normal group ($18.5 \text{ kg/m}^2 \leq \text{BMI} < 24 \text{ kg/m}^2$) vs those in the obese group ($\text{BMI} \geq 24 \text{ kg/m}^2$). *RAB5A* mRNA levels in hGCs from patients with PCOS were lower than in those without PCOS, but this difference was observed only among the women who were obese. There were no statistically significant differences in *RAB5B* and *RAB5C* expression (Fig. 1).

RAB5A mediated FSH-induced internalization of FSHR

We found both FSHR and *RAB5A* were localized on the KGN cell membrane (Fig. 2). With FSH stimulation, FSH ligand–receptor complexes were endocytosed, as indicated by reduced amounts of FSHR on the cell membrane and increased levels in the cytoplasm. *RAB5A* underwent a similar dramatic change. This simultaneous subcellular localization of FSHR and *RAB5A* implies in part that *RAB5A* was involved in the FSH-induced internalization of FSHR.

We examined FSHR levels in the cytoplasm and on the membrane by Western blotting (Fig. 3). In the absence of FSH treatment, FSHR was mainly located on the cell membrane. After FSH stimulation, FSHR underwent internalization, indicated by the finding that,

following interaction with its ligand, FSHR expression on the membrane decreased and that in the cytoplasm increased. That is, the ratio of FSHR on the membrane to that in the cytoplasm in treated cells was lower than the ratio in negative control cells. However, in the *RAB5A* siRNA-transfected cells, there was no significant reduction of FSHR on the membrane after FSH stimulation. These results suggest that downregulation of *RAB5A* prevents FSH-induced internalization of FSHR.

RAB5A expression affected the total FSHR levels in KGN cells

We tested FSHR levels in KGN cells after knockdown of *RAB5A*. We found that FSHR levels increased after FSH treatment both in the NC group and in the *RAB5A* siRNA-transfected group in a similar manner. Higher FSHR protein and mRNA levels were observed in KGN cells after *RAB5A* knockdown in the presence or absence of FSH (Fig. 4A and B). Treatment of cells with siRNA targeting *RAB5B* or *RAB5C* had no significant effect on *FSHR* mRNA levels (Supplementary Fig. 1A). To further study the role of *RAB5A* in FSHR expression, we overexpressed *RAB5A* in KGN cells (Fig. 4C). Addition of FSH increased FSHR protein levels in GFP controls but had no effect in cells overexpressing *RAB5A* (Fig. 4D). *FSHR* mRNA levels were reduced in *RAB5A*-transfected cells in the presence of FSH (Fig. 4E).

The basal helix-loop-helix transcription factors upstream stimulatory factor (USF) 1 and USF2, two transcription factors, which bind to the E-box of the *FSHR* promoter, play key roles in regulating *FSHR* transcriptional activity (Heckert *et al.* 1998, 2000). We found that downregulation of *RAB5A* increased *USF1* and *USF2* mRNA levels (Supplementary Fig. 1B), and *RAB5A* upregulation decreased *USF1* and *USF2* mRNA levels (Supplementary Fig. 1C). Therefore, the regulation of FSHR by *RAB5A* was probably related to USF1 and USF2.

RAB5A regulated aromatase expression and estradiol synthesis in KGN cells

In granulosa cells, the FSH signaling cascade has diverse effects that are crucial for human fertility. One primary

Figure 1 Decreased expression of *RAB5A* in ovarian granulosa cells of patients with polycystic ovary syndrome (PCOS). *RAB5A*, *RAB5B* and *RAB5C* mRNA levels detected by RT-qPCR in ovarian granulosa cells from women who were undergoing *in vitro* fertilization, 26 with and 29 without PCOS. Data are presented as mean $\pm$ s.e.m. **P* < 0.05.

Figure 2 Localization of FSHR and RAB5A in human granulosa cells. Expression and localization of FSHR (red signal) and RAB5A (green signal) in KGN cells detected by immunofluorescence assay. Cells were treated with medium alone (control group) or with FSH (400 ng/mL) (stimulation group).

and well-characterized effect is the secretion of E_2 , which is synthesized by an aromatase enzyme encoded by the *CYP19A1* gene. Our study showed that *CYP19A1* mRNA, aromatase protein and E_2 levels all increased in KGN cells stimulated by FSH (25 ng/mL) for 12 h. *RAB5A* knockdown increased both basal and FSH-stimulated *CYP19A1* and aromatase levels as well as E_2 levels in KGN cells. There was no difference in the extent of aromatase increase after FSH treatment (Fig. 5A, B and C). On the other hand, in cells transfected with either an empty vector or with a recombinant *RAB5A* vector, there was an increase in *CYP19A1* mRNA levels. However, aromatase levels increased only in the cells transfected with the empty vector. *RAB5A* overexpression was associated with decreases only in FSH-stimulated *CYP19A1* mRNA and aromatase protein levels (Fig. 5D and E). Compared with *RAB5A*, *RAB5B* and *RAB5C* had similar although smaller effects in regard to regulation of *CYP19A1* expression and E_2 synthesis (Supplementary Fig. 2B and C).

The role of *RAB5A* in the activation of cAMP/PKA/CREB pathway

Of the many signaling pathways regulating aromatase activity, the cAMP/PKA/CREB pathway is the primary one. Upon FSH stimulation, FSH combines with its receptor, activating adenyl cyclase, which results in increased cyclic AMP (cAMP) production (Richards *et al.* 1979). cAMP, in turn, activates AMP-dependent PKA, leading to

the phosphorylation of CREB (Walker *et al.* 1995). CREB then binds to the *CYP19A1* promoter region to facilitate the gene's transcription (Mendelson & Kamat 2007). To explore the molecular mechanism of *RAB5A* in the regulation of aromatase expression, Western blotting was used to assess activation of cAMP/PKA/CREB pathway in FSH-stimulated KGN cells with or without *RAB5A* knockdown. FSH induced phosphorylation of CREB at Ser 133, reaching a maximum when the cells were treated with FSH for 1 h. The degree of CREB phosphorylation in cells transfected with *RAB5A* siRNA was greater than that in cells transfected with NC siRNA. Maximum phosphorylation was also achieved after 1 h of FSH treatment in *RAB5A*-downregulated KGN cells. Therefore, *RAB5A* knockdown did not increase the speed of CREB phosphorylation (Fig. 6A). KGN cells were incubated with 10 µmol/L of the PKA inhibitor H-89 for 30 min before FSH treatment. H-89 decreased E_2 and aromatase protein levels in both the NC group and the *RAB5A* siRNA-transfected group. Similarly, the increased expression of E_2 and aromatase induced by *RAB5A* knockdown was also blocked by H-89 (Fig. 6B and C). Conversely, upregulation of *RAB5A* weakened FSH-induced CREB phosphorylation. CREB was phosphorylated after 1 h of FSH stimulation in KGN cells transfected with an empty vector but not in cells with high *RAB5A* expression (Fig. 6D). These results demonstrate that *RAB5A* participates in the regulation of aromatase expression in part by way of the cAMP/PKA/CREB pathway.

Figure 3 *RAB5A* mediated FSH-induced internalization of the FSHR. FSHR protein levels detected by Western blot in the cytoplasm or cell membrane after *RAB5A* knockdown in KGN cells. GAPDH was used as the reference gene for the cytoplasm and ATPase Na⁺/K⁺ transporting subunit alpha 2 (ATP1A2) as the reference gene for the cell membrane. The ratio of FSHR on the membrane to that in the cytoplasm was calculated by the numerical values normalized with their respective references. The blots are representative of assays performed in triplicate. ***P* < 0.01.

Figure 4 RAB5A affected the total level of FSHR in KGN cells. (A) FSHR protein levels detected by Western blot analysis after *RAB5A* knockdown in KGN cells with or without FSH (25 ng/mL) stimulation for 12 h. Above, a representative image is shown. Below, the immunoreactive bands are densitometrically quantified. (B) *FSHR* mRNA levels detected by RT-qPCR after *RAB5A* knockdown with or without FSH (25 ng/mL) stimulation for 12 h. (C) A representative image of an *RAB5A* construct after an *RAB5A* recombinant was transfected into KGN cells is shown. (D) FSHR protein levels after *RAB5A* overexpression detected by Western blot in KGN cells with or without FSH (25 ng/mL) stimulation for 12 h. Above, a representative image is shown. Below, the immunoreactive bands are densitometrically quantified. (E) *FSHR* mRNA levels detected by RT-qPCR after *RAB5A* overexpression with or without FSH (25 ng/mL) stimulation for 12 h. Data are means $\pm$ S.E.M. from four experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The negative correlation between *RAB5A* expression and E_2 levels in patients undergoing IVF

To further study the relationship between *RAB5A* mRNA expression and E_2 levels, we tested hGCs from patients with or without PCOS who were undergoing the same IVF protocol. We assessed the correlation between *RAB5A* mRNA levels in primary hGCs of patients with or without PCOS and peripheral blood E_2 levels on the third day of the menstrual cycle. *RAB5A* mRNA levels in hGCs were negatively correlated with E_2 levels in both groups (Fig. 7).

Discussion

RAB5A, a small GTPase protein, mediates vital signaling transduction by recruiting several RAB5A effectors, such as PI3Ks, rabaptin-5, EEA-1 and rabenosyn-5, on the endosome membrane (Stenmark *et al.* 1995, Christoforidis *et al.* 1999, Nielsen *et al.* 2000, Dou *et al.* 2013). Increasing evidence confirms that RAB5A

Figure 5 RAB5A regulated aromatase expression and E_2 synthesis in KGN cells. (A) Aromatase protein levels detected by Western blot after *RAB5A* knockdown in KGN cells with or without FSH (25 ng/mL) stimulation for 12 h. Above, a representative image is shown. Below, the immunoreactive bands are densitometrically quantified. (B) *CYP19A1* mRNA levels detected by RT-qPCR after *RAB5A* knockdown with or without FSH (25 ng/mL) stimulation for 12 h. (C) E_2 levels in the cell culture supernatant measured after *RAB5A* knockdown and further incubation with FSH. (D) Aromatase protein levels detected by Western blot after *RAB5A* overexpression in KGN cells with or without FSH (25 ng/mL) stimulation for 12 h. Above, a representative Western blot is shown. Below, the immunoreactive bands are densitometrically quantified. (E) *CYP19A1* mRNA levels detected by RT-qPCR after *RAB5A* overexpression with or without FSH stimulation for 12 h. Data are means $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

is essential for the maintenance of normal reproductive function. In mammals, the initiation and maintenance of gametogenesis depends not only on the rhythmic variation of sex hormones but also on the dynamic expression of hormone receptors on the germ cell membrane. RAB5A plays an indispensable role in the dynamic recycling of hormone receptors between the cell membrane and cytoplasm (Segretain *et al.* 2010). RAB5A-regulated transport of the LH receptor from the cell surface and its subsequent lysosomal degradation may be associated with dynamic regulation of LH receptor expression during the ovarian cycle by permitting the entry of newly synthesized receptors to the cell surface (Gulappa *et al.* 2011).

In this study, we tested the critical role of RAB5A in FSHR-mediated signal transduction in the human granulosa KGN cell line. The activated receptor complex is reportedly internalized into endosomes after FSH binds to FSHR (Krishnamurthy *et al.* 2003). We found that downregulation of *RAB5A* blocked internalization

Figure 6 RAB5A regulated aromatase and E_2 levels via the cAMP/PKA/CREB pathway. (A) Levels of phosphorylated CREB (P-CREB) and total CREB (T-CREB) after RAB5A knockdown in KGN cells detected at different time points by Western blot analysis. Above, a representative Western blot is shown. Below, the immunoreactive bands of CREB phosphorylation are densitometrically quantified. (B) E_2 levels in the cell culture supernatant measured after RAB5A knockdown and incubation with 10 $\mu\text{mol/L}$ H-89 (PKA inhibitor) for 30 min and subsequently with 25 ng/mL FSH for 12 h. (C) Aromatase and RAB5A protein levels detected by Western blot after RAB5A knockdown and incubation with 10 $\mu\text{mol/L}$ H-89 (PKA inhibitor) for 30 min and subsequently with 25 ng/mL FSH for 12 h. A representative Western blot image is shown. (D) P-CREB and T-CREB protein levels detected by Western blot after RAB5A overexpression in KGN cells. Above, a representative Western blot is shown. Below, the immunoreactive bands are densitometrically quantified. Data are means $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of the receptor, as indicated by an increase in FSHR on the membrane. Meanwhile, RAB5A downregulation also increased FSHR expression with or without FSH stimulation. Previous studies have shown a double effect of FSH on FSHR expression. In the absence of FSH or

Figure 7 Negative correlation between RAB5A expression and E_2 levels in patients undergoing *in vitro* fertilization. Relationship between RAB5A mRNA levels in primary human granulosa cells and peripheral blood E_2 levels on day 3 of the menstrual cycle of 26 patients with (left) and 29 without (right) polycystic ovary syndrome. The results are based on the calculated Pearson correlation r and P values according to distribution of the relevant variables.

a cAMP analog, granulosa cells lose FSHR expression, whereas the addition of FSH or cAMP analog prevents this loss (Galway *et al.* 1990). In preovulatory granulosa cells, FSH stimulation increases both FSHR mRNA levels and the receptors' binding ability. In contrast, the concentration of FSH during ovulation decreases FSHR mRNA levels and binding ability and then promotes granulosa cell luteinization. In an *in vitro* experiment, a certain concentration of FSH promoted FSHR expression in luteinized granulosa cells, but when the dose was high enough, FSHR no longer increased (Galway *et al.* 1990, LaPolt *et al.* 1992). In our study, we found that 25 ng/mL of FSH significantly increased FSHR expression in KGN cells. After RAB5A knockdown, FSH may also promote FSHR expression. Therefore, we postulated that the interaction between RAB5A and FSH affected the expression of FSHR. However, RAB5A downregulation did not enhance the effect of FSH on FSHR.

RAB5A downregulation weakens a number of receptor-mediated signaling pathways (Barbieri *et al.* 2004, Su *et al.* 2006). In our study, we found that RAB5A knockdown increased the phosphorylation of CREB, strengthened FSH-stimulated activation of the cAMP/PKA/CREB pathway in KGN cells and increased aromatase expression and E_2 synthesis. FSHR cannot be activated without ligand binding. However, we demonstrated that RAB5A knockdown increased aromatase levels in the absence of FSH stimulation. Previous evidence indicated a crucial role of RAB5A in the regulation of autophagy, with low RAB5A expression levels prevents autophagy (Su *et al.* 2011, Marlin & Li 2015). It has also been reported that autophagy affects aromatase expression by changing its distribution in microsomes and lysosomes (Zhang *et al.* 2010). Thus, the negative effect of RAB5A on aromatase expression might be related to the inhibition of autophagy. In order to further demonstrate the role of RAB5A in the regulation of E_2 synthesis, we assessed the correlation between RAB5A expression and E_2 levels. We found that the RAB5A mRNA levels in hGCs were negatively correlated with peripheral blood E_2 levels on the third day of the menstrual cycle. Although the pituitary was almost completely suppressed by the GnRH agonist on the third day of the cycle and E_2 production was mainly derived from the basal ovarian secretory function, E_2 blood levels are not totally representative of hGC E_2 synthesis because of hepatic E_2 degradation. We therefore hypothesized a relationship between RAB5A and E_2 synthesis.

RAB5A, RAB5B and RAB5C share more than 90% sequence identity, but the genes have different functions. Firstly, they are phosphorylated by different kinases *in vitro* (Chiariello *et al.* 1999, Chen *et al.* 2009). RAB5A, but not RAB5B or RAB5C, plays a critical role in early phagosome-endosome interactions and governs the maturation of the early phagosome, leading to phagosome-lysosome fusion (Alvarez-Dominguez

& Stahl 1999). Secondly, RAB5A, RAB5B and RAB5C function differently in EGF-induced cell motility. RAB5C, but not RAB5A or RAB5B, is selectively associated with growth factor activation of RAC1 and with enhanced cell motility (Chen *et al.* 2014). Moreover, RAB5A, RAB5B and RAB5C differentially regulate the trafficking and degradation of the EGF receptor (EGFR). Suppression of RAB5A or RAB5B hampers EGFR degradation, whereas RAB5C depletion has very little effect (Chen *et al.* 2009). We found that RAB5A, RAB5B and RAB5C might have different roles in FSHR-mediated signal transduction in KGN cells. We knocked down the three genes with their specific siRNA sequences. Their low expression increased aromatase expression, but RAB5A had the most obvious effect, whereas that of RAB5B and RAB5C was much lower.

In this study, we used KGN cells as a model for investigating FSHR signaling. Because the KGN cell line was established from a transformed granulosa cell tumor, it does not authentically replicate the physiological environment of the human body. However, it is more likely to be closer to normal physiological conditions than other human cell lines, which, it has been speculated, lost native FSHR upon cell transformation (Nishi *et al.* 2001).

Previous research showed an increased testosterone-to-estrogen ratio in the follicular fluid of patients with PCOS, implying decreased aromatase expression in granulosa cells in PCOS (Jakimiuk *et al.* 1998). E₂ blood levels are also slightly lower in those with PCOS than those without PCOS (Amato *et al.* 2003). We found that RAB5A expression was reduced in hGCs from obese women with PCOS and confirmed that the low RAB5A expression level promoted aromatase expression, which seemed somewhat contradictory. But other studies have suggested that decreased aromatase expression in PCOS is due not only to granulosa cell dysfunction but also to an FSH deficiency and decreased conversion of androgens to estrogen (Hillier 1994, la Marca *et al.* 2002). In fact, granulosa cells from patients with PCOS have been demonstrated to have increased aromatase activity and E₂ synthesis in response to FSH stimulation (Araki *et al.* 1996). Other studies have indicated that aromatase activity is related to FSH levels and the hormone's capacity to combine with its receptor (Franks *et al.* 2000, 2008). In order to offset an FSH deficiency, feedback may induce increased FSHR levels in granulosa cells from patients with PCOS. This would enhance the sensitivity of FSH–FSHR binding, thereby promoting the conversion of androgens to estrogens. In this study, we found that RAB5A not only regulated FSH-mediated translocation of FSHR from the membrane to the cytoplasm of granulosa cells but also mediated the FSHR expression level, which affected activation of the cAMP/PKA/CREB pathway. In conclusion, our study focused on the function of PCOS-associated genes

and might provide further insight into the molecular mechanisms involved in the pathogenesis of PCOS.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0015>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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